ORIGINAL RESEARCH

Inhibitors of Phosphodiesterase 4, but Not Phosphodiesterase 3, Increase β_2-Agonist–Induced Expression of Antiinflammatory Mitogen-Activated Protein Kinase Phosphatase 1 in Airway Smooth Muscle Cells

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Abstract

β_2-agonists are principally used in asthma to provide bronchodilation; however, they also have antiinflammatory properties, due, in part, to their ability to up-regulate mitogen-activated protein kinase phosphatase (MKP) 1 in a cAMP-dependent manner. Phosphodiesterases (PDEs) are attractive targets for potentiating the antiinflammatory response. There are 11 subfamilies of PDE enzymes; among these, inhibition of PDE3 and PDE4 are the main targets for airway smooth muscle (ASM). PDE enzymes are important intracellular regulators that catalyze the breakdown of cyclic adenosine monophosphate (cAMP) and/or 3′,5′-cyclic guanosine monophosphate to their inactive forms. Given that MKP-1 is cAMP dependent, and inhibition of PDE acts to increase β_2-agonist–induced cAMP, it is possible that the presence of PDE inhibitors may enhance β_2-adrenoceptor–mediated responses. We address this herein by comparing the ability of a panel of inhibitors against PDE3 (cilostamide, cilostazol, milrinone) or PDE4 (cilomilast, piclamilast, rolipram) to increase cAMP, MKP-1 mRNA expression, and protein up-regulation in ASM cells induced in response to the β_2-agonist formoterol. Our data show that inhibitors of PDE4, but not PDE3, increase β_2-agonist–induced cAMP and induce MKP-1 mRNA expression and protein up-regulation. When cAMP was increased, there was a concomitant increase in MKP-1 levels and significant inhibition of TNF-α–induced CXCL8 (IL-8). This result was consistent with all PDE4 inhibitors examined but not for the PDE3 inhibitors. These findings reinforce cAMP-dependent control of MKP-1 expression, and suggest that PDE4 is the predominant PDE isoform responsible for formoterol-induced cAMP breakdown in ASM cells. Our study is the first to demonstrate that PDE4 inhibitors augment antiinflammatory effects of β_2-agonists via increased MKP-1 expression in ASM cells.

Keywords: cAMP; β_2-adrenoceptor agonist; mitogen-activated protein kinase phosphatase 1; phosphodiesterase 3 inhibitor; phosphodiesterase 4 inhibitor

Clinical Relevance

By blocking cAMP degradation, phosphodiesterase (PDE) inhibitors can enhance the antiinflammatory action of β_2-agonists. PDE3 and PDE4 are the main isoforms found in airway smooth muscle (ASM), and isoform-specific inhibitors exist. Our study is the first to demonstrate that inhibitors of PDE4, but not PDE3, augment antiinflammatory effects of β_2-agonists by increasing expression of the antiinflammatory protein mitogen-activated protein kinase phosphatase 1 in human ASM cells.

(Received in original form September 7, 2014; accepted in final form September 24, 2014)

This work was supported by National Health and Medical Research Council (NHMRC) grant APP1025637 (A.J.A.) and philanthropic funding from Mr. Maurice Renshaw (President, Faculty of Pharmacy Foundation), by an Australian Postgraduate Award (B.S.P.), by scholarship support from Mr. Renshaw (P.P.), and by NHMRC Career Development Fellowship APP1026880 (B.G.O.).

Author Contributions: B.S.P., P.P., B.G.O., and A.J.A. conceived, designed, and performed the experiments; B.S.P., P.P., and A.J.A. analyzed and interpreted the data; B.G.O. and A.J.A. edited the manuscript for important intellectual content; B.S.P. and A.J.A. wrote the manuscript.

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Am J Respir Cell Mol Biol Vol 52, Iss 5, pp 634–640, May 2015

Originally Published in Press as DOI: 10.1165/rcmb.2014-0344OC on October 8, 2014
Internet address: www.atsjournals.org

Chronic respiratory disease is typified by airway inflammation, and the development of efficacious pharmacotherapeutic strategies is the subject of intense interest worldwide. The antiinflammatory actions of β_2-agonists are increasingly recognized, and their combination with phosphodiesterase (PDE) inhibitor(s) is increasingly recognized as a novel antiinflammatory approach.

Cyclic nucleotide PDEs function as negative regulators of cAMP and 3′,5′-cyclic guanosine monophosphate (cGMP)–activated signaling pathways. PDEs hydrolyze the phosphodiester bond of purine cyclic nucleotides, cAMP and cGMP, to the inactive metabolites 5′-AMP and 5′-GMP, respectively. At least 11 PDE families have now been distinguished on the basis of substrate specificity and inhibition by selective inhibitors (1–3). PDE1–5 have been found in human airway smooth muscle (ASM) cells, with PDE3 and PDE4 serving as the two major cAMP hydrolyzing enzymes (4). PDE4 is composed of four different genes (PDE4A, -B, -C, and -D), but mainly the PDE4D isoform appears to play a pivotal role in cAMP degradation in ASM cells (4). PDE3 enzyme occurs in two major isoforms, PDE3A and -B, where PDE3A is the predominant form found in ASM cells (1). The selective inhibitors of PDE3 and PDE4 cause bronchodilator and/or antiinflammatory actions, and hence there is potential for their use in the treatment of respiratory disease (5–7).

It is well established that a rapid increase in cAMP levels in ASM is critical for effective relaxation of bronchospasm by β_2-agonists. More recently, the cAMP-driven up-regulation of antiinflammatory proteins, including mitogen-activated protein kinase phosphatase (MKP) 1, has been recognized as an important mechanism responsible for β_2-agonist–induced antiinflammatory action (8–11). In this study, we compare the ability of PDE3 inhibitors (cilostamide, cilostazol, and milrinone) or PDE4 (cilomilast, piclamilast, and rolipram) to increase cAMP, MKP-1 mRNA expression, and protein up-regulation in ASM cells induced in response to the β_2-agonist, formoterol. Our data show that inhibitors of PDE4, but not PDE3, increase β_2-agonist–induced cAMP and induced MKP-1 mRNA expression and protein up-regulation, and repress synthetic function of ASM cells.

Material and Methods

ASM Cell Culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or from lung transplant donors, in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney (Sydney, NSW, Australia). ASM cells were dissected, purified, and cultured as previously described by Johnson and colleagues (12). A minimum of three different ASM primary cell lines were used for each experiment.

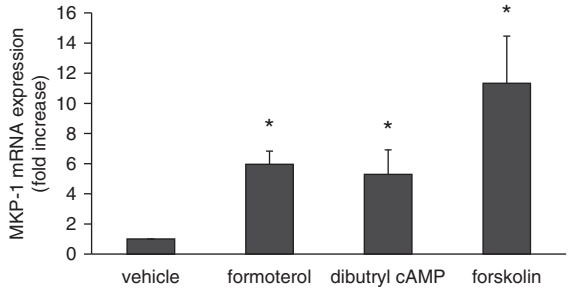

Figure 1. Mitogen-activated protein kinase phosphatase (MKP) 1 mRNA expression is mediated by the cyclic adenosine monophosphate (cAMP)–adenylate cyclase pathway. Growth-arrested airway smooth muscle (ASM) cells were treated with vehicle, formoterol (10 nM), dibutryl cAMP (1 mM), or forskolin (10 μM) for 1 hour. MKP-1 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared with vehicle-treated cells). Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *Significant increase ($P < 0.05$). Data are mean ± SEM values from $n = 6$ primary ASM cell cultures.

Chemicals

TNF-α was purchased from R&D Systems (Minneapolis, MN). Cilomilast (CB-207499) was from Selleck Chemicals (Houston, TX). Unless otherwise specified, all other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

cAMP Assay

cAMP was measured by enzyme immunoassay (cAMP EIA 58100; Cayman, Ann Arbor, MI).

ELISAs

Cell supernatants were collected and stored at −20°C for later analysis by ELISA. IL-8 ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

Real-Time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Doncaster, VIC, Australia) and reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7,500 with MKP-1 (DUSP1: Hs00610256_g1) TaqMan Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems, Foster City, CA) subjected to the following cycle parameters: 50°C for 2 minutes, 1 cycle; 95°C for 10 minutes, 1 cycle; 95°C for 15 seconds, 60°C for 1 minutes, 40 cycles. mRNA expression (fold increase) was quantified by ΔΔ Ct calculations.

Western Blotting

MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) compared with α-tubulin, which was used as the loading control (mouse monoclonal IgG_1, DM1A: Santa Cruz Biotechnology). Primary antibodies were detected with goat anti-rabbit or anti-mouse horseradish peroxiase–conjugated secondary antibodies (Cell Signaling Technology, Foster City, CA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA and then Fisher's *post hoc* multiple comparison test. *P* values less than

0.05 were sufficient to reject the null hypothesis for all analyses.

Results

MKP-1 mRNA Expression Is Mediated by the cAMP/Adenylate Cyclase Pathway

It is well established that β_2-agonists act on ASM cells to increase cAMP via activation of G_s-coupled adenylate cyclase activation. MKP-1 is a cAMP-dependent gene (13, 14); thus, to confirm that the β_2-agonist, formoterol, increases MKP-1 mRNA expression by this pathway, ASM cells were treated for 1 hour with formoterol (10 nM), permeable cAMP analog–dibutryl cAMP (1 mM), and the adenylate cyclase activator, forskolin (10 μM), and compared with vehicle. As shown in Figure 1, formoterol, dibutryl cAMP, and forskolin all significantly up-regulate MKP-1 mRNA in ASM cells by 6.0 (± 0.9)-, 5.3 (± 1.6)-, and 11.3 (± 3.1)-fold respectively, compared with vehicle-treated cells ($P < 0.05$).

PDE4 Inhibitors Increase Formoterol-Induced cAMP and MKP-1 mRNA Expression

Because PDE enzymes act to degrade cAMP, we were interested in examining the effect of PDE inhibition on formoterol-induced cAMP and subsequent MKP-1 expression. The major PDE isoforms present in ASM cells are the PDE4 and PDE3, with PDE4 considered predominant (15). Accordingly, we first examined the ability of increasing concentrations (0.1–10 μM) of three PDE4 inhibitors (cilomilast, piclamilast, and rolipram) to enhance formoterol-induced cAMP and MKP-1 mRNA expression. As shown in Figures 2A, 2C, and 2E, all PDE4 inhibitors significantly up-regulated formoterol-induced cAMP ($P < 0.05$). There appeared to be a close concordance between the ability of the PDE4 inhibitors to increase cAMP and the resulting MKP-1 expression (Figure 2). As shown in Figures 2B, 2D, and 2F, activation of cAMP-mediated pathway significantly induced MKP-1 mRNA expression compared with formoterol treatment alone ($P < 0.05$).

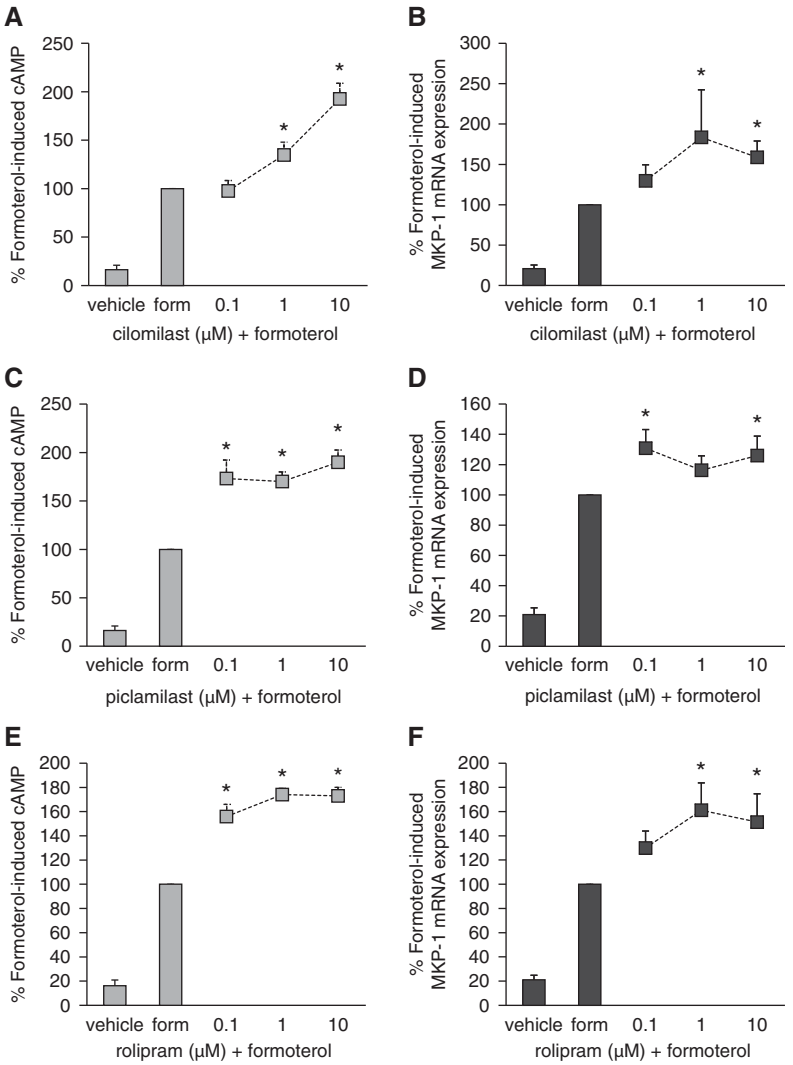

Figure 2. Phosphodiesterase (PDE) 4 inhibitors increase formoterol-induced cAMP and MKP-1 mRNA expression. Growth-arrested ASM cells were pretreated for 30 minutes with the PDE4 inhibitors cilomilast (*A* and *B*), piclamilast (*C* and *D*), or rolipram (*E* and *F*) (0.1–10 μM). The impact of PDE4 inhibition was assessed by: (*A*, *C*, and *E*) measuring cAMP production in response to stimulation with 10 nM formoterol for 15 minutes in comparison to vehicle (results expressed as percentage of formoterol-induced cAMP); and (*B*, *D*, and *F*) measuring MKP-1 mRNA expression by RT-PCR in response to stimulation with 10 nM formoterol for 1 hour in comparison to vehicle (results expressed as percentage of formoterol-induced MKP-1). Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *Significant increase ($P < 0.05$). Data are mean ± SEM values from $n = 7$ primary ASM cell cultures.

PDE3 Inhibitors Do Not Increase Formoterol-Induced cAMP and MKP-1 mRNA Expression

We then examined whether PDE3 inhibitors had a similar potentiating effect to PDE4 inhibitors. Growth-arrested ASM cells were treated with three PDE3 inhibitors (cilostazole, cilostamide, and milrinone) and experiments performed in a manner identical to that described above with the PDE4 inhibitors. As shown in Figures 3A, 3C, and 3E, in contrast to the PDE4 inhibitor data, all PDE3 inhibitors tested were unable to significantly increase formoterol-induced cAMP levels. The lack of augmented cAMP-inducibility suggests that formoterol-induced MKP-1 expression would also not be significantly enhanced. This was indeed the case, with results shown in Figures 3B, 3D, and 3F, confirming that PDE3 inhibitors did not significantly enhance formoterol-induced MKP-1 mRNA level in ASM cells.

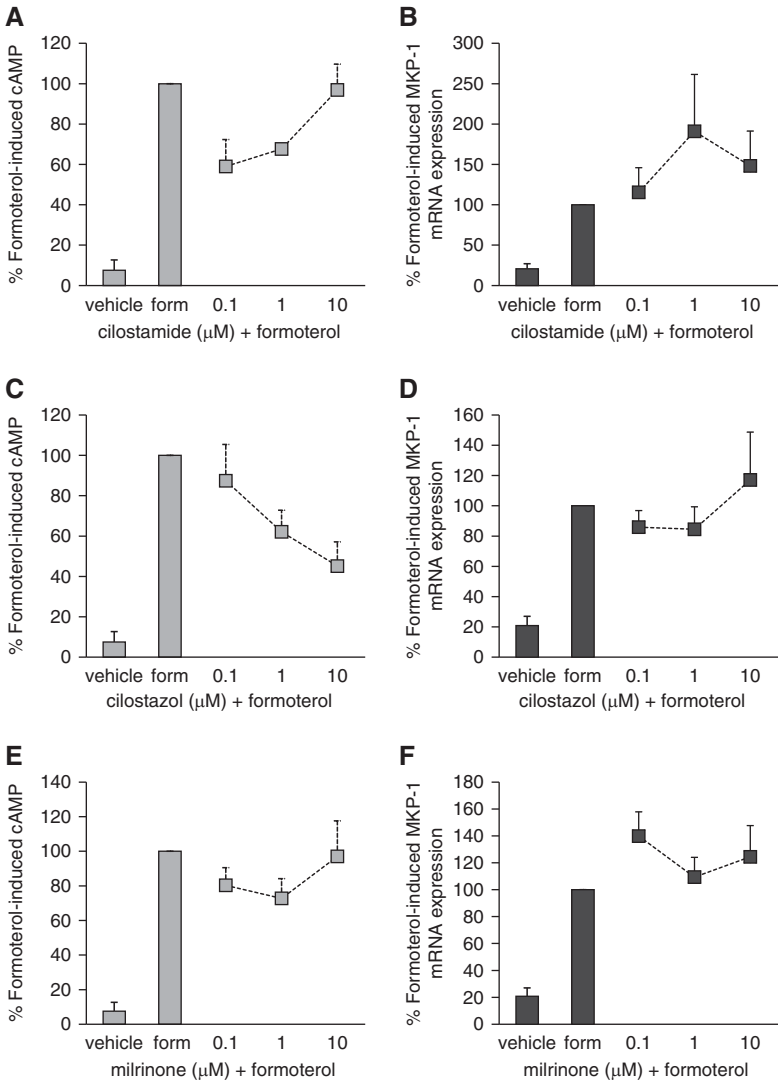

Figure 3. PDE3 inhibitors do not increase formoterol-induced cAMP and MKP-1 mRNA expression. Growth-arrested ASM cells were pretreated for 30 min with the PDE3 inhibitors, cilostamide (*A* and *B*), cilostazol (*C* and *D*), or milrinone (*E* and *F*) (0.1–10 μM). The lack of effect of PDE3 inhibition was assessed by: (*A*, *C*, and *E*) measuring cAMP production in response to stimulation with 10 nM formoterol for 15 minutes in comparison to vehicle (results expressed as percentage of formoterol-induced cAMP); and (*B*, *D*, and *F*) measuring MKP-1 mRNA expression by RT-PCR in response to stimulation with 10 nM formoterol for 1 hour in comparison to vehicle (results expressed as percentage of formoterol-induced MKP-1). Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. Data are mean ± SEM values from $n = 5$ primary ASM cell cultures.

PDE4 Inhibitors, but Not PDE3 Inhibitors, Augment Formoterol-Induced MKP-1 Protein Up-regulation

Taken together, these results indicate that inhibitors of PDE4, but not PDE3, increase β_2-agonist–induced cAMP and induced MKP-1 mRNA expression. To confirm that gene expression results translate into effects on protein up-regulation, growth-arrested ASM cells were pretreated for 30 minutes with PDE4 or PDE3 inhibitors (all at 10 μM) or vehicle. Cells were stimulated with 10 nM formoterol for 1 hour in comparison to vehicle, and MKP-1 protein quantified by Western blotting using α-tubulin as the loading control. The protein results confirm the mRNA expression data, with PDE4 inhibitors, but not PDE3, significantly increasing formoterol-induced MKP-1 protein regulation (Figure 4).

The Combination of Formoterol with PDE4 Inhibitors, but Not PDE3 Inhibitors, Represses TNF-α–Induced IL-8 Secretion

To demonstrate the functional antiinflammatory effects on the synthetic function of ASM cells, formoterol was used, alone or in combination with PDE4 (Figure 5A) or PDE3 inhibitors (Figure 5B), with the resultant repression of IL-8 secretion induced by TNF-α examined. As shown in Figure 5A, we found that the PDE4 inhibitors, cilomilast, piclamilast, and rolipram, significantly repressed TNF-α–induced IL-8 secretion to 61.1 (± 5.8)%, 78.4 (± 2.6)%, and 80.0 (± 10.2)%, respectively ($P < 0.05$). PDE3 inhibitors, in contrast, did not up-regulate MKP-1 level, and thus, no repression of IL-8 was found in ASM cells (Figure 5B). Taken together, our results indicate that the combination of a long-acting β_2-agonist with PDE4 inhibitors enhanced cAMP-mediated MKP-1 to repress cytokine secretion in ASM cells.

Discussion

In addition to serving as effective bronchodilators, β_2-agonists are increasingly recognized as having antiinflammatory effects in respiratory disease. Up-regulation of cAMP-mediated antiinflammatory proteins plays a role, and we and others have demonstrated the impact of the mitogen-activated protein kinase deactivator, MKP-1 (8–11). Given the importance of cAMP in mediating this antiinflammatory effect, in this study, we examined the impact of various PDE inhibitors on β_2-agonist–induced effects. As PDE enzymes catalyze the hydrolysis of cAMP to form inactive 5′ monophosphate products, inhibition of PDE is a useful way to elevate cAMP within cells.

The clinical use of PDE inhibitors in respiratory disease is the subject of intense investigation worldwide (5). PDE enzymes are important intracellular regulators that catalyze the breakdown of cAMP and/or cGMP to their inactive forms. There are 11 subfamilies of PDE enzymes, and, among these, PDE3 and PDE4 are considered the major isoforms responsible for cAMP degradation in ASM cells (16). To test this, we investigated the ability of various PDE3 and PDE4 inhibitors to enhance β_2-agonist–induced cAMP in ASM cells. We found that selective PDE4 inhibitors

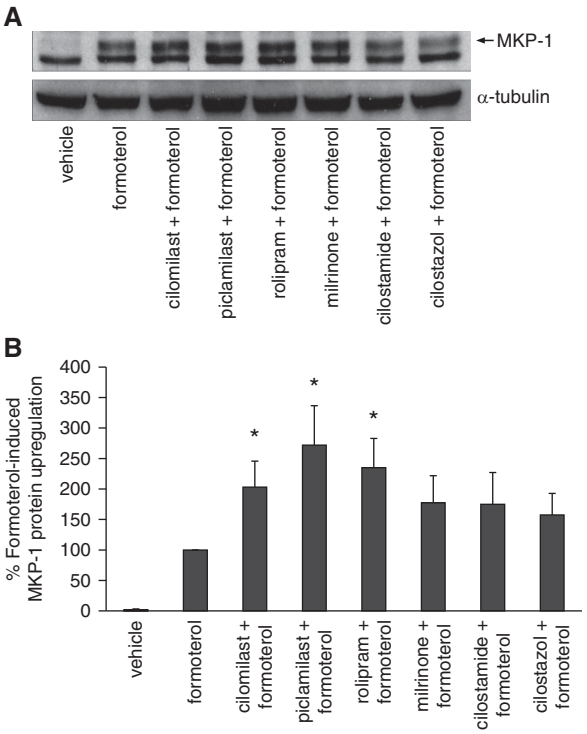

Figure 4. PDE4 inhibitors, but not PDE3 inhibitors, augment formoterol-induced MKP-1 protein up-regulation. Growth-arrested ASM cells were pretreated for 30 minutes with PDE4 or PDE3 inhibitors (all at 10 μM), or vehicle. Cells were stimulated with 10 nM formoterol for 1 hour in comparison to vehicle, and MKP-1 protein quantified by Western blotting, using α-tubulin as the loading control. Results are shown as (*A*) representative Western blots and (*B*) densitometric analysis (expressed as fold increase over vehicle-treated cells). Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *Significant increase ($P < 0.05$). Data are mean ± SEM values from $n = 5$ primary ASM cell cultures.

(cilomilast, piclamilast, and rolipram) significantly increased cAMP levels in ASM cells. In contrast, PDE3 inhibitors (cilostamide, cilostazole, and milrinone) did not significantly increase cAMP. The ability of PDE inhibitors to increase formoterol-induced cAMP was directly related to their ability to increase MKP-1 gene expression and protein up-regulation. Taken together, our study shows that inhibitors of PDE4, but not PDE3, increase β_2-agonist–induced expression of antiinflammatory MKP-1 to repress ASM synthetic function. These results are in agreement with those of Korhonen and colleagues (17), who reported that the PDE4 inhibitor, rolipram, increased the expression of MKP-1 and attenuated experimentally induced inflammation in a MKP-1–dependent manner, as confirmed by experiments with macrophages from MKP-$1^{-/-}$ mice (17).

Many studies have reported the antiinflammatory properties of PDE4 inhibitors on immune cells and airway cell function (5, 7, 18). In addition, it has been suggested that PDE4 inhibition can exert antiinflammatory action on other cell types, including respiratory epithelial cells and submucosal glands (19). PDE4 inhibitors, in combination with β_2-agonists, act additively to suppress a variety of proinflammatory mediators (20). As highlighted by Korhonen and colleagues (17) in mouse macrophages, PDE4B knockout suppressed TNF-α production. To support the above assertion that increased expression of MKP-1 may explain the beneficial effects of β_2-agonist/PDE4 combination therapies in the repression of inflammatory gene expression, we performed *in vitro* studies measuring secretion of IL-8 from ASM cells after stimulation with TNF-α. We were able to confirm that long-acting β_2-agonists, in combination with all selective PDE4 inhibitors, demonstrated an enhanced repressive effect. These results are consistent with previous reports that suggest that rolipram and cilomilast inhibit TNF-α–induced cytokines and chemokines in ASM cells (3, 21).

Interest in PDE3 as a therapeutic target in respiratory disease was derived from the finding that PDE3 inhibitors promote bronchodilation (22, 23); however, it is clear that PDE3 has a high affinity for both cyclic nucleotides (cAMP and cGMP) and remains membrane bound, whereas PDE4 has a high affinity for cAMP hydrolysis and has a wide distribution. These results suggest that PDE3 enzymes are less distributed and have lower affinities for cAMP in ASM cells compared with PDE4. Our results are in accord with earlier studies that emphasize the greater role played by PDE4, compared with PDE3, in the regulation of cAMP in bronchial smooth muscle cells (1, 15, 24), and that PDE4 plays a greater role in the activation of the cAMP signaling cascade (25).

It is possible that targeting one PDE type may not fully inhibit airway inflammation, and there is an increasing realization that simultaneous targeting of several PDE isoforms may be the more effective strategy. Moreover, the fact that PDE4 is not the only isoform present in immune and inflammatory cells further suggests that targeting both PDE3 and PDE4 can have additive or synergistic effects (1, 7). In recent developments, inhibition of PDE3 and PDE4, or mixed PDE3/PDE4 inhibition, has shown greater bronchodilator and antiinflammatory action. In addition, novel approaches are required to use newer drugs, such as roflumilast-n-oxide to target specific PDE4 isoforms.

Taken together, these findings reinforce cAMP-dependent control of MKP-1 expression, and show that, in combination with β_2-agonists, PDE4 inhibitors, but not PDE3 inhibitors, exert significant repressive effects on cytokine secretion from ASM cells. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank their colleagues in the Respiratory Research Group and acknowledge the collaborative effort of the cardiopulmonary transplant team and the pathologists at St. Vincent's Hospital, Sydney, and the thoracic physicians and pathologists at Royal Prince Alfred Hospital, Concord Repatriation Hospital, Strathfield Private Hospital, and Healthscope Pathology, Sydney.

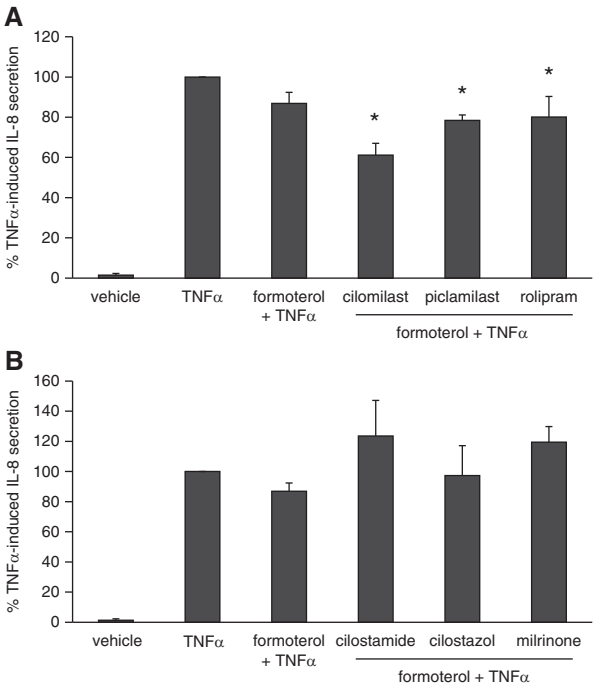

Figure 5. The combination of formoterol with PDE4 inhibitors, but not PDE3 inhibitors, represses TNF-α–induced IL-8 secretion. Growth-arrested ASM cells were pretreated for 30 minutes with (*A*) PDE4 or (*B*) PDE3 inhibitors (all at 10 μM), or vehicle. Cells were treated with 10 nM formoterol or vehicle for 1 hour before stimulation for 24 hours with TNF-α (10 ng/ml). IL-8 secretion was measured by ELISA and results expressed as percentage of TNF-α–induced IL-8 secretion. Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *Significant decrease ($P < 0.05$). Data are mean ± SEM values from $n = 4$ primary ASM cell cultures.

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