PDE4 inhibitors roflumilast and rolipram augment PGE2 inhibition of TGF-β1-stimulated fibroblasts

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Togo S, Liu X, Wang X, Sugura H, Kamio K, Kawasaki S, Kobayashi T, Ertl RF, Ahn Y, Holz O, Magnussen H, Fredriksson K, Skold CM, Rennard SI. PDE4 inhibitors roflumilast and rolipram augment PGE2 inhibition of TGF-β1-stimulated fibroblasts. Am J Physiol Lung Cell Mol Physiol 296: L959–L969, 2009. First published March 20, 2009; doi:10.1152/ajplung.00508.2007.—Fibrotic diseases are characterized by the accumulation of extracellular matrix together with distortion and disruption of tissue architecture. Phosphodiesterase (PDE)4 inhibitors, by preventing the breakdown of cAMP, can inhibit fibroblast functions and may be able to mitigate tissue remodeling. Transforming growth factor (TGF)-β1, a mediator of fibrosis, can potentially modulate cAMP by altering PGE2 metabolism. The present study assessed whether PDE4 inhibitors functionally antagonize the profibrotic activity of fibroblasts stimulated by TGF-β1. The PDE4 inhibitors roflumilast and rolipram both inhibited fibroblast-mediated contraction of three-dimensional collagen gels and fibroblast chemotaxis toward fibronectin in the widely studied human fetal lung fibroblast strain HFL-1 and several strains of fibroblasts from adult human lung. Roflumilast was ~10-fold more potent than rolipram. There was a trend for PDE4 inhibitors to inhibit more in the presence of TGF-β1 (0.05 < P < 0.08). The effect of the PDE4 inhibitors was mediated through cAMP-stimulated protein kinase A (PKA), although a PKA-independent effect on gel contraction was also observed. The effect of PDE4 inhibitors depended on fibroblast production of PGE2 and TGF-β1-induced PGE2 production. PDE4 inhibitors together with TGF-β1 resulted in augmented PGE2 production together with increased expression of COX mRNA and protein. The present study supports the concept that PDE4 inhibitors may attenuate fibroblast activities that can lead to fibrosis and that PDE4 inhibitors may be particularly effective in the presence of TGF-β1-induced fibroblast stimulation.

phosphodiesterase 4; chemotaxis; collagen gel contraction; transforming growth factor-β1; prostaglandin E2

MANY LUNG DISEASES ARE CHARACTERIZED by fibrotic alterations in tissue structure. The accumulation of interstitial mesenchymal cells, classically termed “fibroblasts,” and the extracellular matrix produced by these cells together with the contraction that characterizes fibrotic tissues can disrupt lung function. This process occurs not only in the pulmonary interstitium in interstitial lung diseases (46), but also in the airways in asthma and chronic obstructive pulmonary disease (COPD) (43). Inhibition of the fibrotic process, therefore, has the potential to alter the natural history of many important lung diseases.

The pluripotential cytokine transforming growth factor (TGF)-β1 is believed to be a key mediator in normal tissue repair (6) and in the development of fibrosis (15, 42). TGF-β1 directly stimulates fibroblasts to produce increased amounts of extracellular matrix, including fibronectin and collagen (25, 51). In addition, TGF-β1 stimulates fibroblast chemotactic activity (35) and augments fibroblast-mediated contraction of extracellular matrix (31), activities that may contribute to fibrosis.

In contrast to the profibrotic action of TGF-β1, prostaglandin (PG)E2 generally inhibits fibroblast activity (12, 21, 26). This inhibition is mediated through the EP2 and EP4 receptors, which, in turn, activate adenylly cyclase and increase cAMP (17, 44, 45, 49). Since fibroblasts can produce endogenous PGE2, PGE2 can function through this pathway as an autocrine/paracrine modulator of profibrotic responses. Consistent with this concept, previous studies have demonstrated that PDE4 inhibitors can inhibit fibroblast-mediated contraction of three-dimensional (3D) collagen gels and chemotaxis by a PGE2-dependent mechanism (22). TGF-β1 induces augmented PGE2 release (28, 29). By activating inhibitory pathways mediated by cAMP, this augmented PGE2 production could serve as a mechanism of negative feedback control of TGF-β1 activity.

The present study was designed to extend these earlier studies. PGE2 can signal through several receptors that can have varying effects (8). Since TGF-β1 can modulate many cell functions, it is by no means clear that the effect of a PDE inhibitor would be similar in the presence and absence of TGF-β. Thus one key goal of the present study was to determine whether PDE4 inhibitors would inhibit profibrotic activity in the presence of TGF-β1. Since TGF-β1 stimulates PGE2 release, it is very likely that PDE4 inhibitors are more effective at blocking TGF-β1-augmented profibrotic activity than they are at inhibiting those same activities under baseline conditions.

Using rolipram and roflumilast, the present study evaluated the potential for PDE4 inhibitors to block TGF-β1-
induced profibrotic activity. These PDE4 inhibitors were found to antagonize profibrotic activities of TGF-β1 through mechanisms that depended on PGE2 production and, in part, on cAMP signaling through protein kinase A (PKA). The PDE4 inhibitors were equally or more effective in inhibiting profibrotic activity in the presence of TGF-β1. Interestingly, an interaction between the PDE4 inhibitors and TGF-β1 leading to augmented production of PGE2 was demonstrated. The results of the present study therefore support the concept that PDE4 inhibitors may have utility as antifibrotic agents and that these may be particularly effective in situations in which TGF-β1 is driving the fibrotic activity.

**MATERIALS AND METHODS**

*Materials*

Cell culture medium and fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA). The PDE4 inhibitors roflumilast and rolipram were provided by Altana Pharmaceuticals (Konstanz, Germany) and were dissolved in 100% dimethyl sulfoxide (DMSO). The amounts of DMSO added have no effect on the bioassays assessed (16). PGE2 and indomethacin (Sigma, St. Louis, MO) were dissolved in 100% ethanol. Preliminary experiments with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) demonstrated that the concentrations of roflumilast and rolipram, as well as those of ethanol and DMSO used in this study, did not show any significant cytotoxicity for fibroblasts (data not shown).

**Materials and Methods**

*Fig. 1. Effect of roflumilast and rolipram on transforming growth factor (TGF)-β1-stimulated HFL-1 contraction of collagen gels. Cells were grown to subconfluence and cast into collagen gels with 100 × 10⁻¹² M TGF-β1 or without TGF-β1 in the presence of varying concentrations of roflumilast (Rof, A) or rolipram (Roli, B). Gel size is shown after 2 days of incubation and expressed as % of initial size. All values are means ± SE for at least 6 separate experiments, each performed in triplicate. *P < 0.05.*

*Fig. 2. Effect of roflumilast and rolipram on adult human lung fibroblast-mediated collagen gel contraction. Cells were grown to subconfluence, cast into collagen gels, and cultured with 100 × 10⁻¹² M TGF-β1 or without TGF-β1 in the presence of 10⁻⁶ M roflumilast or 10⁻³ M rolipram in serum-free medium. Gel sizes are shown after 2 days of incubation and expressed as % of original size. Each symbol represents a single strain of cells and is the mean ± SE for at least 2 separate experiments, each performed in triplicate. Each subject is indicated by a unique symbol that is the same in all panels, and the lines connect the values for a single strain with and without the PDE4 inhibitor. A: roflumilast in the presence or absence of TGF-β1. B: rolipram in the presence or absence of TGF-β1. *P < 0.05 assessed by paired Student’s t-test. C: inhibitory index in the presence or absence of TGF-β1. *P < 0.05 for a difference in the inhibitory index of roflumilast or rolipram for individual strains in the presence of TGF-β1. Trends for the groups are indicated.*
Human Fetal Lung Fibroblasts

Human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin, and 2.5 μg/ml Fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and passaged once a week at a 1-to-3 ratio. Fibroblasts were used between the 14th and 17th passages.

Adult Human Lung Fibroblasts

Seven adult human lung fibroblast cell lines were studied. Six lines were established from tissue samples from patients undergoing lung resection for localized tumor as approved by the Human Studies Committee of the Medical Board of the State of Schleswig-Holstein, Germany. One line was obtained by similar methods from a patient at the Karolinska University Hospital Solna under a protocol approved by the Ethics Committee of Karolinska Institutet. Tissue was taken from areas of macroscopically normal lung parenchyma distal to any tumor mass. Care was taken to avoid large airways, vessels, and the pleural surface. All subjects were current or former smokers, but all had normal lung function. These cells were cultured as described above.

Human Fibronectin

Human fibronectin was prepared from human plasma by gelatin-Sepharose affinity chromatography, as previously described (14). After elution with 4 M urea, the fibronectin was further purified by heparin-agarose affinity chromatography and eluted with 500 mM NaCl. Samples were frozen at −80°C until use.

Fig. 3. Effect of phosphodiesterase (PDE)4 inhibitors on TGF-β-stimulated HFL-1 chemotaxis. HFL-1 cells were grown to subconfluence, harvested with trypsin, and used for chemotaxis in the blindwell Boyden chamber assay. Fibronectin (20 μg/ml) was used as the chemoattractant. Varying concentrations of rolumilast or rolipram were added to the fibroblasts in the upper wells with 100 × 10^{-12} M TGF-β1 or without TGF-β1. Chemotaxis was measured as the number of migrated cells after 6-h incubation and expressed as cell number in 5 high-power fields (5HPF, ×400) under microscope. A: rolumilast. B: rolipram. All values are means ± SE for 4 separate experiments, each performed in triplicate. Fn−, random migration without chemoattractant fibronectin. *P < 0.05.

Fig. 4. Effect of rolumilast and rolipram on adult human lung fibroblast chemotaxis. Cells were grown to subconfluence, harvested with trypsin, and used for chemotaxis in the presence of rolumilast (1 × 10^{-6} M) or rolipram (10 × 10^{-6} M) with and without the addition of 100 × 10^{-12} M TGF-β1. Chemotaxis was measured as the number of migrated cells after a 12-h incubation and expressed as number of migrated cells per 5HPF. Each subject is indicated by a unique symbol that is the same in all panels, and lines connect the values for a single strain with and without the PDE4 inhibitor. A: rolumilast in presence or absence of TGF-β1. B: rolipram in presence or absence of TGF-β1. *P < 0.05 assessed by paired Student’s t-test. C: inhibitory index in presence or absence of TGF-β1. *P < 0.05 for difference in inhibitory index of rolumilast or rolipram for individual strains in presence of TGF-β1. Trends for the groups are indicated.
Fibroblast Chemotaxis

HFL-1 chemotaxis was assessed by the Boyden blindwell chamber technique (7). Further details of the chemotaxis assays are provided in the supplemental material for this article.1

Type I Collagen

Type I collagen (rat tail tendon collagen, RTTC) was extracted from rat tail tendons as previously described (13) and as detailed in the supplemental material for this article.

Collagen Gel Contraction Assay

The ability of PDE4 inhibitors to affect fibroblast-mediated gel contraction was measured in the presence or absence of TGF-β1, with a modification of the method developed by Bell et al. (4).

Quantification of TGF-β1, Fibronectin, and PGE2

TGF-β1 and fibronectin concentrations were measured by an enzyme-linked immunoabsorbent assay. Cultures were maintained for 24 h in monolayer and for 48 h for 3D culture to quantify TGF-β1 or PGE2. Cultures were maintained for 48 h in monolayer and for 72 h for 3D culture to quantify fibronectin. Media were then collected, frozen, and stored at −80°C until assay. To measure TGF-β1, samples were assayed both with and without acidification and neutralization to convert the latent form of TGF-β1 to active forms.

PGE2 production from cells was measured by enzyme immunoassay (ELIA; Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

Fig. 5. Effect of indomethacin on roflumilast and rolipram modulation of HFL-1-mediated collagen gel contraction in the presence of TGF-β1. Subconfluent HFL-1 cells were pretreated with or without indomethacin (Indo, 2 × 10−6 M) for 1 h. Cells were then cast into 3-dimensional (3D) collagen gels and allowed to contract in the presence or absence of indomethacin (2 × 10−6 M) or TGF-β1 (100 × 10−12 M) with and without roflumilast (1 × 10−6 M) or rolipram (10 × 10−6 M). Collagen gel contraction was determined by quantifying the area of the gels after 2 days; gel size is expressed as % of initial area. All values are means ± SE for 3 separate experiments, each performed in triplicate. *P < 0.05.

Immunoblotting Analysis

Cells were grown to 80% confluence in DMEM-10% FCS in 60-mm dishes, after which media were changed to DMEM without serum for 24 h. Cultures were then exposed to treatment for 8 or 24 h. Cell lysates were then collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting for cyclooxygenase (COX)-1 and COX-2. β-Actin staining was used as an internal control. Detailed methods are provided in the supplemental material for this article.

COX-1 and -2 mRNA Detection by Real-Time Polymerase Chain Reaction Assay

To investigate the effect of roflumilast and rolipram on COX-1 and -2 mRNA expression levels, monolayer-cultured HFL-1 cells were assessed with reverse transcription (RT) real-time quantitative polymerase chain reaction (PCR). Total RNA was extracted with the single-step method (3). The amount of RNA was quantified by spectrophotometric absorbance at 260/280 nm. Gene expression was assessed with the use of the ABI Prism 7500 Sequence Detection System as described previously (10). Primers and TaqMan probes were designed with Primer Express 1.0 (ABI) software to amplify <150 base pairs. Probes were labeled at the 5’ end with the reporter dye molecule 6-carboxy-fluorescein [FAM; emission (maximum absorbance) max = 518 nm] and at the 3’ end with the quencher dye molecule 6-carboxy-tetramethylrhodamine ( TAMRA; emission max = 582 nm). Human rRNA (VIC-labeled probe, Applied Biosystems, Foster City, CA) was used as an internal control. Values are expressed as 105 rRNA units. Real-time PCRs of cDNA specimens were conducted in a total volume of 50 μl with 1× TaqMan Master Mix (Perkin-Elmer), primers at 300 nM, and probes at 200 nM. Sequences used were as follows: COX-1 (forward) 5’-CAA TCA

Fig. 6. Effect of indomethacin on roflumilast and rolipram modulation of HFL-1 chemotaxis in the presence of TGF-β1. Subconfluent HFL-1 cells were pretreated with or without indomethacin (2 × 10−6 M) for 1 h. Cells were then harvested and used for assay of chemotaxis toward fibronectin in the presence or absence of indomethacin (2 × 10−6 M), with and without TGF-β1 (100 × 10−12 M) and with and without either roflumilast (1 × 10−7 M) or rolipram (10 × 10−9 M). Chemotaxis is expressed as number of cells/5HPF. All values are means ± SE for 3 separate experiments, each performed in triplicate. *P < 0.05.

1 The online version of this article contains supplemental material.
Absence of TGF-β1, but in the presence of TGF-β1 the effect of rolflumilast was nearly maximal over the entire range tested (10⁻⁶–10⁻⁷ M). The inhibitory effect of both rolipram and roflumilast was consistently observed on collagen gel contraction in normal adult lung fibroblasts. While there was strain-to-strain variability, of seven strains tested, six showed inhibition in the absence and all in the presence of TGF-β1 (Fig. 2, A and B). Similar results were observed over other time points up to 5 days (Supplemental Fig. S1). When expressed as inhibitory index, both roflumilast and rolipram inhibited six of seven strains significantly more in the presence of TGF-β1, while one strain was inhibited more in the absence of TGF-β1 (Fig. 2C). For all strains together, there was a trend toward more inhibition in the presence of TGF-β1 for both roflumilast (P = 0.053) and rolipram (P = 0.072).

**Effect of PDE Inhibitors on TGF-β1-Augmented Fibroblast Chemotaxis**

Both roflumilast and rolipram inhibited HFL-1 fibroblast chemotaxis toward fibronectin in a concentration-dependent manner (Fig. 3). In the presence of TGF-β1, which augmented chemotaxis, the inhibition caused by rolipram was clearly concentration dependent while that of roflumilast was similar for the concentrations tested. Rolipram was more potent. Roflumilast at a concentration of 1 μM had approximately the same effect as rolipram at a concentration of 10 μM both in the presence and in the absence of TGF-β1. The inhibitory effect of both rolipram and roflumilast was also consistently observed on chemotaxis in several strains of normal adult lung fibroblasts. Of the strains tested, all showed inhibition both in the

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**RESULTS**

**Effect of PDE4 Inhibitors on TGF-β1-Stimulated Fibroblast-Mediated Gel Contraction**

The PDE4 inhibitors roflumilast and rolipram inhibited both baseline and TGF-β1-augmented HFL-1 fibroblast-mediated gel contraction (Fig. 1). Roflumilast at a concentration of 1 μM had an effect similar to rolipram at a concentration of 10 μM. The effect of rolipram was clearly concentration dependent. Roflumilast showed clear concentration dependence in the

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**Statistical Analysis**

The results are expressed as means ± SE. To compare the effect of the PDE4 inhibitors among various cell strains, an inhibitory index was calculated, which was [(gel size with PDE inhibitor) − (control gel size)]/100 for contraction and [(control chemotaxis) − (PDE inhibitor chemotaxis)]/100 for chemotaxis. Grouped data were evaluated by one-way analysis of variance (ANOVA), corrected by the Tukey test. Samples that appeared different within a series were further assessed by Student’s t-test. Comparisons of paired data from individual subjects with and without treatment were made by the Wilcoxon test. Comparisons were considered statistically significant if P < 0.05.
absence and in the presence of TGF-β1 (Fig. 4). Expressed as percent inhibition, both roflumilast and rolipram inhibited four of five strains tested significantly more in the presence of TGF-β1, while one strain responded equally to roflumilast in the presence and absence of TGF-β1, and this same strain responded slightly more to rolipram in the absence of TGF-β1 (Fig. 4C). For the group as a whole, there was a trend toward greater inhibition in the presence of TGF-β1 for both roflumilast (P = 0.054) and rolipram (P = 0.087).

Role of Endogenous PGE2 as Source of cAMP in Actions of Roflumilast and Rolipram

Elevation of cAMP is known to inhibit fibroblast-mediated collagen gel contraction and chemotaxis. Both forskolin, a direct activator of adenylyl cyclase, and dibutyryl cAMP, a cAMP analog, were found to inhibit both baseline and TGF-β1-stimulated collagen gel contraction (Supplemental Table S1). Inhibition of chemotaxis by these agents has been previously reported (21). To determine whether endogenous cAMP production played a role in the effect of roflumilast and rolipram, two experimental approaches were used. First, since the production of PGE2 is believed to be a major means of autocrine/paracrine stimulation of adenylyl cyclase, the ability to elevate cAMP-activated PKA, while PKA plays a more important role in the effect of PGE2 in modulating chemotaxis.

Effect of PDE4 Inhibitors on Fibroblast Mediator Release

Because fibroblasts can release mediators including fibronectin and TGF-β1 and PGE2 that can modulate contraction and chemotaxis in an autocrine or paracrine manner, the ability of PDE4 inhibitors to modulate release of these mediators was assessed in the presence or absence of exogenous TGF-β1.

**TGF-β1.** Neither roflumilast nor rolipram had any effect on TGF-β1 release by HFL-1 fibroblasts in either monolayer or 3D gel culture; nor did either PDE4 inhibitor alter Smad2 or Smad3 phosphorylation in the presence of exogenous TGF-β1 (Supplemental Fig. S2). Similarly, neither roflumilast nor rolipram had an effect on fibronectin release by fibroblasts (Table 1).

**Fibronectin.** In contrast, in the presence of exogenous TGF-β1, which stimulated fibronectin release, both roflumilast and rolipram significantly inhibited fibronectin release. Both indomethacin and KT-5720 completely blocked the ability of the PDE4 inhibitors to inhibit TGF-β1-stimulated fibronectin release (Fig. 9), suggesting that the effect of the PDE4 inhibitors was completely dependent on endogenous PGE2 production and mediated through PKA.

**PGE2 release.** Similar to their effect on fibronectin release, neither roflumilast nor rolipram affected PGE2 release when added to fibroblasts in either monolayer or 3D gel culture in the absence of TGF-β1 (Fig. 10). In contrast, in monolayer culture in the presence of TGF-β1, which resulted in stimulation of PGE2 release, both PDE4 inhibitors further augmented PGE2 release significantly. In monolayer culture, the PDE inhibitors, which had no effect when added alone, approximately doubled the stimulation of PGE2 release by TGF-β1. In 3D gel culture, neither TGF-β1 nor the PDE4 inhibitors added alone resulted in a measurable increase in PGE2 release (Fig. 10). In contrast, when TGF-β1 and a PDE4 inhibitor were added together, PGE2 release was augmented, suggesting a synergistic interaction increasing PGE2 production.

The ability of the PDE4 inhibitors to modulate PGE2 release was further assessed by evaluating the expression of COX-1 and COX-2 in HFL-1 cells in collagen gel contraction (Supplemental Table S3). TGF-β1 resulted in a 158% increase in COX-1 expression (P = 0.069) and a 238% increase in COX-2 expression (P = 0.06; Fig. 11). Both roflumilast and rolipram, when added alone to fibroblasts, resulted in a marginal increase in both COX-1 and COX-2 expression. When they were added together with TGF-β1, however, the expression of both COX-1 and COX-2 was greatly amplified (Fig. 11). The increase in COX-2 protein expression was paralleled by an increase in mRNA levels. TGF-β1, added alone, resulted in an 8.4-fold increase in

### Table 1. Effect of PDE4 inhibitors on HFL-1 fibroblast fibronectin release in presence of TGF-β1 in both monolayer and collagen gel culture

<table>
<thead>
<tr>
<th>TGF(−)</th>
<th>Control</th>
<th>Roflumilast</th>
<th>Rolipram</th>
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<tbody>
<tr>
<td></td>
<td>988±97</td>
<td>1,048±144</td>
<td>1,063±190</td>
</tr>
<tr>
<td>TGF(+)</td>
<td>3,952±1,040*</td>
<td>1,851±190†</td>
<td>1,869±196†</td>
</tr>
</tbody>
</table>

All values are means ± SD for at least 3 separate experiments, each performed in triplicate. Fibroblasts were cultured in monolayer or in 3-dimensional (3D) collagen gels in the presence or absence of 100 × 10⁻¹² M transforming growth factor (TGF)-β1 with the addition of phosphodiesterase (PDE4) inhibitors roflumilast (10⁻⁶ M) or rolipram (10⁻⁵ M). Monolayer culture media were harvested after 24 h, and 3D collagen gel media were harvested after 48 h. *P < 0.05 compared with control with no TGF-β1; †P < 0.05 compared with TGF-β1 exposed without PDE4 inhibitor.
Both roflumilast and rolipram had modest effects that did not achieve statistical significance on COX-2 when they were added alone. When they were added together with TGF-β1, however, roflumilast and rolipram resulted in 19.9- and 21.0-fold increase, respectively, in COX-2 mRNA expression, although these differences did not achieve statistical significance.

DISCUSSION

The present study demonstrates that the PDE4 inhibitors roflumilast and rolipram inhibit fibroblast chemotaxis and contraction of collagen gels in TGF-β1-stimulated fibroblasts. The inhibitory effect of the PDE4 inhibitors depends on endogenous PGE2 production that leads to a subsequent increase in cAMP, which signals, at least in part, through PKA. There was a strong trend for both roflumilast (0.053 and 0.054) and rolipram (0.072 and 0.087) to be more effective in TGF-β1-stimulated cells than in control cells for inhibiting both chemotaxis and contraction.

PGE is the major prostanoid produced by cultured fibroblasts, and it can function in an autocrine or paracrine manner to regulate fibroblast behavior. In this context, PGE can inhibit many profibrotic fibroblast functions including chemotaxis (21), proliferation (5, 17), matrix production (17, 40), differentiation (24, 47), and matrix contraction (30). In general, these inhibitory effects are mediated by the EP2 and EP4 receptors that signal primarily through cAMP. Consistent with the importance of these inhibitory pathways, we previously demonstrated (23) that PDE inhibitors can attenuate fibroblast chemotaxis and contraction of 3D collagen gels and that this effect depends on potentiation of endogenous PGE2 signaling.

There is, however, considerable opportunity for complexity in PGE signaling as PGE interacts with four receptors that can signal through several pathways. TGF-β1 is a pluripotent...
cytokine believed to play an important role in tissue repair and remodeling (6, 36). TGF-β1 induces profibrotic responses through direct actions on mesenchymal cells (28, 29). TGF-β1 also induces several “feedback” pathways that can serve as endogenous downregulators of the profibrotic effects of TGF-β1. These include the induced cytoplasmic localization of the inhibitory protein Smad7 that can block TGF-β signaling (32) and the production of PGE2 (48). The present study extends our earlier observations by demonstrating that PDE4 inhibitors also attenuate fibroblast responses in the face of TGF-β1 stimulation and that the inhibitory effect demonstrates a trend toward potentiation. This suggests that the complex effects that TGF-β1 induces in fibroblasts do not attenuate the cAMP-mediated inhibition. The observation by Dunkern and colleagues (11) that PDE4 inhibitors can also block TGF-β1 induction of fibroblast α-smooth muscle actin expression is consistent with our findings and with the concept that PDE4 inhibitors have the potential to function as therapeutic blockers of TGF-β1 effects.

The present study also demonstrated that the PDE4 inhibitors potentiated TGF-β1 induction of PGE2 production that was mediated by augmenting COX expression. A similar effect of PDE4 inhibition inducing COX-2 expression has been reported in osteoblastic precursor cells for some, but not all, PDE4 inhibitors (34, 41). Similarly, Barry and colleagues (2) noted that PDE4 inhibition resulted in increased PGE2 release from bovine airway smooth muscle, although the mechanism for PGE2 induction was not defined. In contrast, in lympho-

![Fig. 11. Effect of PDE4 inhibitors on cyclooxygenase (COX)-1 and -2 protein expression in the presence of TGF-β1. Subconfluent HFL-1 cells were treated with or without 100 × 10^{-12} M TGF-β1 with the addition of either roflumilast (10^{-6} M) or rolipram (10^{-5} M) 8 h (for detection of COX-2) or 24 h (for detection of COX-1), cells were harvested for the detection of COX-1 or COX-2 by immunoblot. A: COX-1. B: COX-2. Representative Western blots are shown at top with experimental conditions indicated. All values are means ± SE for at least 6 separate experiments. *P < 0.05.]

![Fig. 12. PDE4 inhibitors induce COX-1 and -2 mRNA synthesis in the presence of TGF-β1. Subconfluent HFL-1 cells were treated with or without 100 × 10^{-12} M TGF-β1 with the addition of either roflumilast (10^{-6} M) or rolipram (10^{-5} M). After 6 h, total RNA was extracted and used for real-time RT-PCR. A: COX-1 mRNA levels normalized to ribosomal RNA. B: COX-2 mRNA levels normalized to ribosomal RNA. All values are means ± SE for at least 6 separate experiments. *P < 0.05.]

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cytes PDE4 inhibitors are reported to inhibit COX-2 expression and block PGE2 production (19). The effect of PDE4 inhibitors on PGE2 production, therefore, appears to be cell type specific.

In the present study, a number of strains of adult lung fibroblasts were assessed, and there was some strain-to-strain variation in sensitivity to PDE4 inhibition. The basis for these strain differences remains undefined. However, in the majority of strains, the inhibitory effect of PDE4 inhibition was significantly greater in the presence of TGF-β1, consistent with a mechanistic interaction between TGF-β1 and cAMP-mediated inhibition that is likely to be present in most lung fibroblasts.

The present study, by demonstrating augmentation of PGE2 production in fibroblasts, establishes a potential synergistic mechanism for PDE4 inhibitors to block TGF-β1-mediated effects. Interestingly, PDE4 inhibition did not affect PGE2 production in the absence of TGF-β1. This suggests the potential for selective synergies between PDE4 and TGF-β1. Although the mechanisms for such remain to be defined, the observation that COX mRNA levels are increased suggests a role for mRNA transcription, degradation, or both. The complexity of PGE signaling suggests a number of steps at which TGF-β could act, including PGE production, expression of the four EP receptors, several of which can undergo differential splicing and alternate G protein coupling, modulation of downstream signaling pathways, and regulation of phosphatase pathways. With regard to the latter, evidence is accumulating that specific PDE can be localized to specific receptors by anchoring proteins, creating increased specificity for cAMP-dependent pathways (37, 39). Which isoforms and receptors are relevant for the interactions between TGF-β and PGE signaling remains to be defined.

Potentiation of PGE2-mediated effects by PDE4 inhibition has been demonstrated in several in vitro cell systems. PDE4 inhibition, for example, can potentiate PGE2 inhibition of monocyte proliferation (1), stimulate PGE2-driven formation of osteoblasts (41), and can potentiate PGE2-mediated inhibition of osteoblast formation (33, 50). In the present study, the nonspecific cyclooxygenase inhibitor indomethacin largely blocked the PDE4 effect, consistent with dependence on endogenous PGE2 production. However, indomethacin alone potentiated chemotaxis in the absence of TGF-β1, but not in the presence of TGF-β1. While indomethacin blocks cyclooxygenase, it can also have other effects including activation of nuclear receptors (18). This suggests that in the presence of TGF-β1 indomethacin may be affecting multiple pathways that modulate chemotaxis.

PGE2 inhibition of fibroblast chemotaxis and contraction is believed to be mediated by signaling through the EP2 and EP4 receptors, which activate adenyl cyclase and increase intracellular cAMP levels (17, 44, 45, 49). Consistent with this, the PKA inhibitor KT-5720 blocked the ability of PDE4 inhibitors to antagonize TGF-β1-activated chemotaxis. Interestingly, KT-5720 only partially blocked PDE4 inhibition of TGF-β1-augmented contraction of 3D collagen gels, consistent with the possibility of an action through signaling pathways in addition to PKA.

Augmented fibronectin production has also been suggested to play a role in the development of fibrosis. In the present study, PDE4 inhibitors were demonstrated to antagonize TGF-β1-induced production of fibronectin. This was also blocked by PKA inhibition. A similar PKA-dependent inhibition of fibronectin release due to activation of the IP receptor has been described by Kamio et al. (20). Similarly, Burgess et al. (9) reported that roflumilast inhibits TGF-β1-induced fibronectin production from both normal and asthmatic airway smooth muscle cells.

The two PDE4 inhibitors differed in their dose response. Roflumilast was more potent than rolipram. While both demonstrated concentration dependence in the absence of TGF-β1, in the presence of TGF-β1 all concentrations of roflumilast that were tested demonstrated near-maximal activity, consistent with increased potency of the PDE4 inhibitors in the presence of TGF-β1.

The effects of PDE4 inhibition observed on TGF-β-mediated signaling are consistent with previous studies showing that cAMP inhibits TGF-β-induced profibrotic effects. However, given the complexity of PGE signaling and the multiplicity of TGF-β effects, there is considerable potential for unexpected interactions. In this context, the present study suggests the potential for synergistic interactions between the PDE4 inhibitors and TGF-β1, which, in turn, suggests possible advantages for therapeutic use. In regions where there are high levels of TGF-β1, the effect of PDE4 inhibition to reduce fibroblast activity may be selectively greater. In contrast, at tissue sites that are normal and without active TGF-β1, the effect of the PDE4 inhibitors will be less, with less potential for adverse effect. This may be particularly important in a disease such as COPD where fibrotic narrowing of the small airways and inadequate maintenance of alveolar wall in the face of injury both contribute to disease (38). On the other hand, fibroblasts from fibrotic tissues have been reported to have reduced sensitivity to cAMP-mediated inhibition, and increased cAMP levels may be less effective (27). Nevertheless, the potential of an agent to selectively inhibit the airway fibrosis, while having less of an effect on alveolar repair, could have an important therapeutic advantage.

In summary, the present study demonstrates that the PDE4 inhibitors roflumilast and rolipram inhibit several profibrotic activities of fibroblasts and that these effects are more pronounced in the presence of TGF-β1. The inhibitory effect of the PDE4 inhibitors is mediated by potentiating endogenous PGE2 signaling, which in turn acts, at least in part, by stimulating cAMP and PKA. In addition to inhibiting the breakdown of cAMP, PDE4 inhibitors potentiate TGF-β1-induced PGE2 production. By augmenting an endogenous feedback control mechanism, PDE4 inhibitors have the potential, as therapeutic agents, to limit TGF-β1-driven fibrosis.

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REFERENCES

INHIBITION OF TGF-β1-STIMULATED FIBROBLASTS BY PDE4 INHIBITORS


