Modulation of human airway smooth muscle proliferation by type 3 phosphodiesterase inhibition

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Modulation of human airway smooth muscle proliferation by type 3 phosphodiesterase inhibition. Am. J. Physiol. Lung Cell. Mol. Physiol. 20: L412–L419, 1999.—Elevation in cell cAMP content can inhibit mitogenic signaling in cultured human airway smooth muscle (HASM) cells. We studied the effects of the type 3-selective phosphodiesterase inhibitor siguazodan, the type 4-selective phosphodiesterase inhibitor rolipram, and the nonselective inhibitor 3-isobutyl-1-methylxanthine (IBMX) on proliferation of cultured HASM cells. At concentrations selective for the type 3 phosphodiesterase isoform, siguazodan inhibited both [3H]thymidine incorporation (IC50 2 µM) and the increase in cell number (10 µM; 64% reduction) induced by platelet-derived growth factor-BB (20 ng/ml). These effects were mimicked by IBMX. At concentrations selective for type 4 phosphodiesterase inhibition, rolipram was without effect. A 20-min exposure to siguazodan and rolipram did not increase whole cell cAMP levels. However, in HASM cells transfected with a cAMP-responsive luciferase reporter (p6CRE/Luc), increases in cAMP-driven luciferase expression were seen with siguazodan (3.9-fold) and IBMX (16.5-fold). These data suggest that inhibition of the type 3 phosphodiesterase isoform present in airway smooth muscle results in inhibition of mitogenic signaling, possibly through an increase in cAMP-driven gene expression.

mitogenesis; adenosine 3′,5′-cyclic monophosphate; transfection; luciferase

PRIMARY CULTURES of human airway smooth muscle cells provide a useful system for studying the regulation of mitogenic responses in human airway smooth muscle (13). These cells have been shown to respond to a number of mitogens, including both the AB and BB forms of platelet-derived growth factor (PDGF), thrombin, histamine, mast cell tryptase, endothelin, and epidermal growth factor (3, 11, 15, 21, 23). An increase in airway smooth muscle mass is observed in the airways of patients with chronic asthma (9) and is believed to contribute to the irreversible component of airway obstruction seen in chronic asthma when airway remodeling has occurred. Hence it is important to gain an understanding of the mechanisms underlying control of airway smooth muscle cell number.

Although there are extensive data in the literature regarding the effects of potential mitogens on the proliferative responses of these cells (3, 11, 15, 21, 23), less is known about the regulation of mitogenic signaling in airway smooth muscle. A previous study (29) suggested that agents that elevate cAMP are able to inhibit mitogenic signaling; for example, salbutamol, isoproterenol, and the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) are able to inhibit tritiated thymidine incorporation in response to mitogens such as PDGF in human airway smooth muscle cells in culture. In preliminary studies, it has been demonstrated that this effect is likely to be cAMP mediated because microinjection of the catalytic subunit of protein kinase A into individual airway smooth muscle cells inhibits 5-bromo-2′-deoxyuridine incorporation in response to mitogenic stimulation (20).

Breakdown of cAMP within airway smooth muscle cells is dependent on the activity of phosphodiesterase isoenzymes (2). Airway smooth muscle cells have been shown to express a number of isoforms of phosphodiesterase (7, 28, 30). The predominant forms responsible for the physiological control of cAMP levels in this tissue appear to be members of the type 3 and type 4 isomorph families (30, 31). The aim of the present study was, therefore, to investigate the ability of selective inhibitors of these phosphodiesterase isoenzymes to modulate mitogenic signaling in cultured human airway smooth muscle cells. A preliminary account of these data has been presented (16).

MATERIALS AND METHODS

Culture of human airway smooth muscle cells. Primary cultures of human airway smooth muscle cells were prepared from explants of trachealis muscle obtained from individuals without respiratory disease within 12 h of death as previously described (6). Hall and Kotlikoff (13) and others (10, 22) have extensively characterized the phenotype of these cells, which retain many properties of acutely isolated airway smooth muscle cells. A segment of trachea was removed from immediately above the carina. A strip of the trachealis ~2 × 1 cm was then dissected clear from the surrounding tissue and transported to the laboratory in DMEM containing penicillin G (200 U/ml), streptomycin (200 µg/l), and amphotericin B (0.5 µg/l). The tissue was washed several times in 10 ml of DMEM containing antibiotics and antifungal agents at double the above concentrations. The overlying mucosa was dissected free from the airway smooth muscle under sterile conditions. Small (0.2 × 0.2-cm) explants of the airway muscle were then excised, and ~15 explants were placed in each of several 60-mm Petri dishes. After the explants adhered, DMEM containing antibiotics, amphotericin B, 10% fetal calf serum (FCS), and glutamine (2 mM) was added to just cover the explants. The medium was changed each day for the first 3 days to reduce the incidence of fungal infection. Smooth muscle cell growth usually occurred ~7–10 days after the explants were placed in culture. When growth commenced, the cultures were supplemented with fresh DMEM contain-
ing 10% FCS and 2 mM glutamine approximately every 3 days. When the cells were approaching confluence in some parts of the vessel, the explants were removed, and 24 h later, the cells were harvested by trypsinization. Cells from an individual dish or flask were then plated in one 75-cm² flask and grown to confluence. When confluent, each flask was split into four new flasks. Antibiotics and amphotericin B were not added to the medium used for all subsequent passages after this stage (passage 2). Cells for experiments were seeded in 24-well (for cAMP, thymidine incorporation, and cell counts) or 6-well (for transfection) plates unless otherwise stated. All primary cell cultures from each donor were examined with standard immunocytochemical techniques with anti-smooth muscle actin antibody (1:100 dilution; Sigma) to confirm the presence of smooth muscle type cells. Primary cell cultures used for the experiments described in this study showed >95% of cells staining for smooth muscle actin. Cells from preparations from four individuals were used.

Determination of cAMP responses. Accumulation of [3H]cAMP was measured by a modification of a previously described method (26). In brief, confluent monolayers of cells plated in 24-well plates were labeled with [3H]adenine (2 µCi/well) for 2 h in DMEM at 37°C. In an incubator constantly gassed with air-5% CO₂. At the end of this period, the cells were washed three times with 1 ml of Hanks-HEPES buffer and allowed to rewarm to 37°C for 20 min in the presence or absence of phosphodiesterase inhibitors. At the end of this period, the agonists were added for 20 min before the reactions were terminated by the addition of 50 µl of concentrated HCl. The cells were then stored at −20°C. [3H]cAMP was determined by column chromatography after the cells were rewarmed as previously described (8, 12). Aliquots of [3H]cAMP were added to each sample, and the counts obtained from this recovery marker were used to correct for variations in recovery from each column. In addition, a 100-µl aliquot was taken from each well of the plate after the reactions were stopped and counted for tritium to correct for variations in the number of cells per well.

[3H]thymidine incorporation. Subconfluent cultures (70–90%) of human airway smooth muscle cells in 24-well plates were washed and then incubated in 1 ml of DMEM containing 0.1% FCS and 2 mM glutamine for 48 h to induce partial growth arrest. Phosphodiesterase inhibitors and/or growth factors were added in 5- or 10-µl aliquots (time 0), and the cells were washed twice with PBS before being fixed with methanol-glacial acetic acid (3:1) for at least 1 h at room temperature. Two further washes with methanol-water (4:1) were performed before the cells were lysed with 1 ml of 1 M NaOH (adapted from Ref. 5). Nine hundred microliters of the supernatant were transferred to a scintillation vial along with 10 ml of scintillation fluid (Packard, Meriden, CT) and counted on an LKB scintillation counter (efficiency ~30%), the results being expressed as disintegrations per minute or as a multiple of stimulation over the control value.

Determination of cell number. Subconfluent cultures (30–40%) of human airway smooth muscle cells grown in 24-well plates were washed twice and then incubated in 1 ml of DMEM containing 0.1% FCS and 2 mM glutamine for 48 h to induce partial growth arrest. Phosphodiesterase inhibitors and/or growth factors were added in 5- or 10-µl aliquots (time 0), and the cells were washed twice with PBS before being fixed with methanol-glacial acetic acid (3:1) for at least 1 h at room temperature. Two further washes with methanol-water (4:1) were performed before the cells were lysed with 1 ml of 1 M NaOH (adapted from Ref. 5). Nine hundred microliters of the supernatant were transferred to a scintillation vial along with 10 ml of scintillation fluid (Packard, Meriden, CT) and counted on an LKB scintillation counter (efficiency ~30%), the results being expressed as disintegrations per minute or as a multiple of stimulation over the control value.

Determination of cell number. Subconfluent cultures (30–40%) of human airway smooth muscle cells grown in 24-well plates were washed twice and then incubated in 1 ml of DMEM containing 0.1% FCS and 2 mM glutamine for 48 h to induce partial growth arrest. Phosphodiesterase inhibitors and/or growth factors were added in 5- or 10-µl aliquots (time 0), and the cells were washed twice with PBS before being fixed with methanol-glacial acetic acid (3:1) for at least 1 h at room temperature. Two further washes with methanol-water (4:1) were performed before the cells were lysed with 1 ml of 1 M NaOH (adapted from Ref. 5). Nine hundred microliters of the supernatant were transferred to a scintillation vial along with 10 ml of scintillation fluid (Packard, Meriden, CT) and counted on an LKB scintillation counter (efficiency ~30%), the results being expressed as disintegrations per minute or as a multiple of stimulation over the control value.

Measurement of luciferase activity. Luciferase activity was measured in the whole lysate of cultured smooth muscle cells with a commercially available kit (Promega) as described in the product information but with minor modifications. Medium was aspirated from the wells, and the cells were washed twice with 1 ml of phosphate-buffered saline solution. Three hundred microliters of lysis buffer were then added to each well, and the cells were incubated for 10–15 min at room temperature. Any cell debris together with the lysis buffer was then removed from the individual wells of each six-well plate. The lysates were spun at 13,000 rpm for 30 s to pellet large cell debris. Twenty microliters of the supernatant were then assayed for luciferase activity in a Turner luminometer; measurement was made for 30 s after an initial delay of 10 s after addition of the substrate. Protein concentrations in the cell lysate supernatant were determined with a miniaturized Bradford assay with 96-well plates read in a plate reader. All luciferase activities were then corrected for protein content to normalize for variation in cell number and lysis efficiency between experiments.

Materials. [2,8-3H]adenine (26 µCi/mmol) and [3H]cAMP (42.4 µCi/mmol) were purchased from Amersham (Little Chalfont, UK). The firefly luciferase vector pGL3 (used as a control), Transfectam reagent, and luciferase assay kits were obtained from Promega UK. p6CRE/Luc was a gift from S. Rees (Glaxo Wellcome). Siguazodan was a gift from Dr. T. Toprhy (SmithKline Beecham, King of Prussia, PA). Rolipram...
was obtained from Calbiochem (Nottingham, UK). All other chemicals were obtained from Sigma (Poole, UK). Relevant vehicle controls were included in all experiments involving rolipram and siguazodan, for which stock solutions were made as follows: 10 mM rolipram in 10% DMSO and 20 mM siguazodan in 100% DMSO. The antibodies used for immunocytochemistry were anti-smooth muscle α-actin (Sigma) and mouse IgG whole molecule (host goat; Sigma). Plastic ware was obtained from Costar (High Wycombe, UK).

Data analysis and statistics. EC_{50} values for PDGF-BB and IC_{50} values for the phosphodiesterase inhibitors were defined in each individual experiment and used to calculate mean values. Each data point in individual experiments was calculated from the mean of triplicate determinations.

Statistical analysis of the data was performed with paired or unpaired t-tests, Dunnett’s test, Tukey’s honestly significant difference test, or analysis of variance as appropriate. Data were log transformed where appropriate. All values are means ± SE of n separate experiments.

RESULTS

Induction of [3H]thymidine incorporation by PDGF-BB. Initial experiments were performed to confirm that PDGF-BB is able to induce [3H]thymidine incorporation into cultured human airway smooth muscle cells. Figure 1 shows the concentration-response relationship for PDGF-BB-induced thymidine incorporation in these cells. The maximum increase in stimulation was observed with 100 ng/ml of PDGF-BB, and the response was increased 6.3 ± 1.4-fold compared with the basal value (P < 0.05; n = 4). The EC_{50} for this response was 8.3 ± 1.5 ng/ml (n = 4).

Inhibition of PDGF-BB-stimulated [3H]thymidine incorporation by phosphodiesterase inhibitors. We next studied the potential of phosphodiesterase inhibitors to inhibit PDGF-BB-stimulated thymidine incorporation in cultured human airway smooth muscle cells. The agents chosen were the type 3 (siguazodan)- and type 4 (rolipram)-selective phosphodiesterase inhibitors together with the nonselective phosphodiesterase inhibitor IBMX, all of which have been shown to be effective relaxant agents in this tissue and also to inhibit the type 3 and type 4 phosphodiesterase activities present in trachealis homogenates in previous studies (24, 25, 30). Vehicle (DMSO, highest concentration 0.6%) was included at the same concentration in all relevant incubations, although no significant effect of vehicle alone was observed on thymidine incorporation. Siguazodan, rolipram, and IBMX produced a concentration-related inhibition of PDGF-BB-induced [3H]thymidine incorporation (Fig. 2). The maximum inhibition seen and the apparent IC_{50} for these responses are shown in Table 1. Interestingly, IBMX was only effective at inhibiting thymidine incorporation at concentrations of 100 µM and above, and the effect observed with IBMX was less than that with the isoenzyme-selective phosphodiesterase inhibitors. We attempted to study higher concentrations of IBMX but found that prolonged incubation with these higher concentrations of IBMX produced apparent cytotoxicity with marked cell detachment. Rolipram produced significant inhibition of thymidine incorporation only at concentrations > 10 µM. The combination of siguazodan and rolipram was not significantly more effective than the additive effects of either compound alone (Table 1). In addition, we studied the response to rolipram and siguazodan in combination with the β_{2}-adrenoceptor agonist isoproterenol (1 µM; Fig. 3). Isoproterenol alone induced a 63 ± 12% inhibition of the response to PDGF-BB (P < 0.05; n = 4). Siguazodan (10 µM) in addition to isoproterenol gave a 66 ± 25% reduction in the thymidine response compared with the effect of isoproterenol alone on the PDGF-BB response (P < 0.05; n = 4). Rolipram (10 µM) did not significantly increase the inhibitory effects of isoproterenol (30 ± 19% reduction; P > 0.05; n = 4). We also examined the effect of rolipram and siguazodan (both 10 µM) on the thymidine response to thrombin (100 U/ml; response 5.2 ± 0.4-fold over basal value; n = 3) to define whether the effects of siguazodan were mitogen specific. Siguazodan produced a 46 ± 5% inhibition of the thrombin response (P < 0.05; n = 3). No significant inhibition was seen with rolipram at this concentration.

Inhibition of PDGF-BB-stimulated cell proliferation by phosphodiesterase inhibitors. The effect of these agents on cell number was studied by incubating cells with the relevant agents and then performing Coulter counting (Fig. 4). Cells were initially placed in 1% FCS for 24 h before the agents were added, and cell counts were performed at that stage and 48, 72, or 120 h after the addition. One percent FCS (rather than 0.1% FCS) was used because with the longer incubation required to study changes in cell number (compared with thymidine incorporation), significant cell death was observed with lower levels of FCS. In the presence of 1% FCS but in the absence of other added mitogens, the cell number slowly increased (1.8 ± 0.2-fold compared with the starting cell number; P < 0.05; n = 12). The addition of PDGF-BB (20 ng/ml) produced an additional 1.8 ± 0.1-fold increase in cell number (P < 0.05; n = 12) compared with 1% FCS alone. In keeping with the data
obtained from the [3H]thymidine incorporation assay, no significant effect of vehicle alone on the changes in cell number was observed (data not shown). After incubation for 3–5 days, significant inhibition of PDGF-BB-driven increases in cell number was seen with siguazodan and IBMX but not with rolipram (10 µM siguazodan: 48 ± 7% inhibition, P < 0.05, n = 3; 50 µM IBMX: 81 ± 26% inhibition, P < 0.05, n = 4; 10 µM rolipram: 15 ± 5% inhibition, not significant, n = 3; Fig. 4).

Siguazodan and IBMX induce an increase in cAMP-driven luciferase activity in human airway smooth muscle cells. To determine the effect of these agents on cell cAMP content, we used two approaches. First, we measured cAMP levels at an early time point after the addition of phosphodiesterase inhibitors. As a positive control, we used isoproterenol, which Hall et al. (14) previously showed induces cAMP formation via β2-adrenoceptor stimulation in these cells. The overall change in cAMP levels with phosphodiesterase inhibitors at a single time point (20 min) is small in these cells; as previously reported (14), we observed very small responses to rolipram, siguazodan, and IBMX in the absence of other agonists (Table 2), with only the response to IBMX being significant. PDGF-BB (20 ng/ml) produced no significant change in cAMP levels itself. The apparent lack of effect of phosphodiesterase inhibitors was not due to the inability of the assay to detect change because the response to isoproterenol (1 µM; 6.6-fold) seen in these experiments was similar to that previously reported by Hall et al. (14). In addition,
we also measured cAMP with a radioimmunoassay and were again unable to demonstrate significant cAMP responses to the isoform-selective phosphodiesterase inhibitors used in this study (data not shown) despite the control levels of cAMP recorded in these cells with this assay being above the lower limit of detection of the assay determined with cAMP standards. One possible explanation for this apparent discrepancy is that it is the integrated cAMP response over time that is relevant for antimitogenic signaling. In the proliferation experiments, phosphodiesterase inhibitors were present throughout the experiment and prolonged phosphodiesterase inhibition would be expected to have occurred. Therefore, we studied the ability of these agents to modulate cAMP over longer time periods by utilizing a luciferase reporter construct under the control of six CREs (27) to provide an integrated readout for cAMP changes during the time course of the experiment. The luciferase response in cell lysates transfected with a range of agents capable of elevating cell cAMP content is shown in Fig. 5. The response of primary cultures of human airway smooth muscle cells transfected with this construct and stimulated with phosphodiesterase inhibitors is shown in Table 3. It can be seen that when measured with an assay that assesses the integrated change in cAMP levels over a longer time period, larger effects were observed, which reached significance with siguazodan and IBMX (P < 0.05; n = 11). None of these agents appeared to alter transfection efficiency per se because in a parallel series of experiments, the level of expression of a control (i.e., nonresponsive to cAMP) luciferase vector (pGL3) was not altered by exposure to any of the agents studied (data not shown).

**DISCUSSION**

These results demonstrate that nonselective and isoform-selective phosphodiesterase inhibitors are able to modulate proliferative responses of human airway smooth muscle cells in culture. These cells provide a useful model for studying proliferative responses to mitogens likely to be important in airway remodeling (10, 22). We were able to demonstrate effects by looking at a measure of DNA synthesis (thymidine incorporation) and also at total cell number, suggesting that the inhibition of PDGF-BB-driven DNA synthesis directly results in a reduction in cell number. In general, the effects on DNA synthesis of the agents studied reflected the effects seen on cell number, although it is interesting that IBMX appeared to have a greater effect on cell number than on thymidine incorporation. Cell number is dependent on the balance between cell proliferation, cell survival, and cell death; in the present study, we did not examine the ability of phosphodiesterase inhibi-

**Table 2. Effects of PDE inhibitors and isoproterenol on cAMP levels**

<table>
<thead>
<tr>
<th>PDE Inhibitor or Agonist</th>
<th>n</th>
<th>Multiple of Increase</th>
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</thead>
<tbody>
<tr>
<td>Siguazodan (50 µM)</td>
<td>14</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Rolipram (50 µM)</td>
<td>14</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>IBMX (50 µM)</td>
<td>14</td>
<td>3.7 ± 0.7*</td>
</tr>
<tr>
<td>Isoproterenol (1 µM)</td>
<td>14</td>
<td>6.6 ± 1.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of experiments. All were in presence of PDGF-BB, which alone had no effect. *Significant increase compared with basal value, P < 0.05.

**Table 3. Effects of PDE inhibitors on cAMP-driven increases in luciferase activity over 24 h in HASM cells transfected with p6CRE/Luc**

<table>
<thead>
<tr>
<th>PDE Inhibitor or Agonist</th>
<th>n</th>
<th>Multiple of Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siguazodan (50 µM)</td>
<td>11</td>
<td>3.9 ± 1.7*</td>
</tr>
<tr>
<td>Rolipram (50 µM)</td>
<td>11</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>IBMX (50 µM)</td>
<td>11</td>
<td>16.5 ± 6.4*</td>
</tr>
<tr>
<td>Rolipram + Siguazodan (50 µM)</td>
<td>11</td>
<td>5.4 ± 2.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of experiments. HASM, human airway smooth muscle. *Significant increase compared with basal value, P < 0.05.
tors to modulate cell death, and it is possible that the quantitative differences observed were due to effects on survival. However, we had to employ slightly different assay conditions (i.e., 1% FCS) for the cell number studies because over the longer time period of these experiments, we found that cells seeded in 0.1% FCS declined in number, presumably due to low background rates of apoptosis; this may also account for the quantitative differences seen in the two sets of experiments. At the concentrations of agents used, no cytotoxicity was observed, although at very high concentrations of IBMX (>100 µM), cytotoxicity was seen after prolonged (>24-h) incubation (data not shown). We also observed qualitatively similar effects of these agents when thrombin was used to induce DNA synthesis in these cells and when epidermal growth factor was used to increase cell number (data not shown), suggesting that the effects of siguazodan are not due to antagonism of the PDGF-BB receptor.

The effects of the type 3 phosphodiesterase inhibitor siguazodan on both DNA synthesis and proliferation occurred at concentrations likely to be selective for this isoform; the IC50 for siguazodan inhibition of PDGF-BB-driven increases in thymidine incorporation was 1.8 µM, and inhibition of PDGF-BB-driven increases in cell number was observed with 10 µM siguazodan (Fig. 4). In contrast, although rolipram produced inhibition of PDGF-BB-driven [3H]thymidine incorporation, this effect was only observed at concentrations unlikely to be selective for binding to either the high- or low-affinity sites on the type 4 phosphodiesterase isoform.

In keeping with the data on DNA synthesis, no effect of rolipram was observed on PDGF-BB-induced increases in cell number at the highest concentration likely to be selective for the type 4 isoform (Fig. 4). Hence it seems likely that the type 3 isoform is the more important target for inhibiting airway smooth muscle proliferation.

At least seven families of phosphodiesterase isoenzymes exist, each containing multiple isoforms and splice variants (1, 2). Airway smooth muscle contains a range of phosphodiesterase isoenzymes. In previous studies, Hall et al. (14) and others (7, 28, 30) have shown that inhibitors of the type 4 (cAMP-selective) and, to a lesser extent, the type 3 (cGMP-inhibited, cAMP-selective) phosphodiesterase families appear to have the greatest effect on cell cAMP content and tissue tone. Another study (30) has shown that although other phosphodiesterase isoforms are present in airway smooth muscle, the type 3 and type 4 isoforms are physiologically the most important in controlling cAMP breakdown in these cells. However, there is controversy regarding the mechanism of action of phosphodiesterase inhibitors in airway cells. Given the relatively small change in total cell cAMP content seen with these agents in the absence of other activators of adenyl cyclase, it has been suggested that the physiological effects of these agents are due to effects other than those directly related to changes in whole cell cAMP levels. In the present study, we were unable to demonstrate significant changes in whole cell cAMP levels assayed at a single time point after the addition of a type 3 phosphodiesterase inhibitor alone and only observed a small change after the addition of the nonselective inhibitor IBMX despite using two different, sensitive assays for cAMP formation. These data suggest that the physiological effects of these agents are unlikely to be due to changes in whole cell cAMP levels at early time points. However, two explanations could account for this anomaly. The first is that a small change in cAMP levels that is sustained may be important for the action of these drugs. The second is that there may be a larger change in cAMP levels in a cell compartment that will not be detected by whole cell lysate cAMP assays but that may be functionally relevant.

To investigate this apparent disparity between cAMP levels and the physiological effects of phosphodiesterase inhibitors further, we used a different approach that provides an integrated readout for changes in cell cAMP content over longer time periods. This approach involves transfection of primary cultures of human airway smooth muscle cells with a reporter construct that contains the gene for firefly luciferase under the control of six CREs (27). First, we demonstrated that when transiently expressed in cultured human airway smooth muscle cells, this construct enables changes in cAMP-driven luciferase expression to be studied after the elevation of cell cAMP content with a range of agonists acting through different mechanisms (Fig. 5). By using this approach, we believed that we should be able to detect effects mediated through either of the two mechanisms described above. When we assessed cAMP-driven luciferase expression as an integrated readout for cAMP elevation in these cells, significant responses to siguazodan and IBMX were observed. These data suggest that integrated or subcellular changes in cAMP levels in the absence of a detectable change in whole cell cAMP levels may be important for the effects of cAMP on mitogenic signaling.

It is also clear that cAMP inhibits mitogenic signaling in these cells when other mechanisms (e.g., direct receptor stimulation) are used to elevate cAMP levels. For example, inhibition of DNA synthesis has been observed when the catalytic subunit of protein kinase A was microinjected directly into airway smooth muscle cells but not when inactive (boiled) catalytic subunit was microinjected (20). In addition, inhibition of thymidine incorporation into cultured airway smooth muscle cells has previously been observed when cell CAMP content has been elevated by other agents including β2-adrenoceptor agonists such as salbutamol and isoproterenol and also vasoactive intestinal peptide (20, 29). We observed similar effects to those previously described with isoproterenol in the present study and, in addition, were able to show that the effects of phosphodiesterase inhibitors on thymidine incorporation were additive to the effects of isoproterenol. There are still, however, some quantitative differences in the magnitude of responses observed and the changes in whole cell cAMP seen with different agonists. Disparity between direct changes in cell cAMP content and physi-
ologial effects has been previously noted in airway smooth muscle (e.g., Ref. 33). One possible explanation might be that under some conditions, increases in cell cAMP content may actually enhance proliferative signaling, for example, when β-hexosaminidase is used as an agonist (17). We attempted to study the role of cAMP in more detail by using the protein kinase A inhibitor H-89 (4) to reverse the effects of siguazodan on PDGF-BB-induced thymidine incorporation. Interestingly, in these experiments, H-89 (100 nM) itself produced a small inhibition of PDGF-BB-induced thymidine incorporation (7.8 ± 4.2%), which made interpretation of the effects of H-89 on the response to siguazodan difficult.

Assuming that cAMP is important in the effect of phosphodiesterase inhibition leaves the question of the site of action of protein kinase A. This remains to be determined, but phosphorylation of Raf-1 may be one potential mechanism inhibiting mitogenic signaling driven through, e.g., MAP kinase pathways. In addition, cAMP may directly alter the expression of genes important in mitogenic signaling by the activation of CRE binding proteins binding to CREs in target genes. Our data with the pCRELuc construct demonstrate that this effect occurs with inhibition of the type 3 phosphodiesterase isozyme present in airway smooth muscle. Elevation in cell cAMP content has also been observed to inhibit mitogenic signaling in a range of other myofibroblast cell types including vascular smooth muscle cells and renal mesangial cells in primary culture; in both these cell types, inhibition of type 3 and type 4 phosphodiesterase isoforms was effective in inhibiting mitogenesis (18, 19, 32).

In conclusion, therefore, this study demonstrates that siguazodan, a selective inhibitor of the type 3 phosphodiesterase isoenzyme, is able to inhibit both DNA synthesis and increases in cell number in response to the mitogen PDGF-BB in cultured human airway smooth muscle cells. In contrast, the type 4-selective phosphodiesterase inhibitor rolipram only produced inhibition of DNA synthesis at concentrations of drug unlikely to be selective for type 4 phosphodiesterase inhibition. Administration of type 3 phosphodiesterase inhibitors to patients with airway diseases such as asthma may therefore potentially prevent some of the airway remodeling that leads, in part, to the irreversible component of the airflow obstruction observed in some patients with chronic asthma.

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