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Original Article

Inhibition of tracheal smooth muscle cell proliferation by phosphodiesterase inhibitors

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Abstract

Agents that increase intracellular cyclic 3′,5′-adenosine monophosphate (cAMP), such as forskolin, prostaglandin (PG)E_2, salbutamol and 8-bromo-cAMP, have been shownto inhibit the proliferation of airway smooth-muscle (ASM) cells *in vitro*. However, it has not yet been determined whether selective inhibitors of phosphodiesterase (PDE) isoenzymes III and IV that catalyze cAMPto 5′-adenosine monophosphate have the ability to inhibit ASM cell proliferation. To evaluate the effectsof PDE inhibitors on ASM cell proliferation, ASM cells isolated from bovine tracheae were cultured in the presence of fetal bovine serum (FBS), with or without a non-selective PDE inhibitor (theophylline), a selective PDE III inhibitor (cilostazol), and a selective PDE IV inhibitor (rolipram). The number of ASM cells cultured with 5% FBS was significantly reduced by the presence of theophylline at 10^{-3} and 3×10^{-4} mol/L, cilostazol at 10^{-5}, 10^{-6} and 10^{-7} mol/L, and rolipram at 10^{-4} and 10^{-5} mol/L. The release of lactic dehydrogenase from ASM cells cultured with any concentration of these agents was not significantly different from that with medium alone. Inhibitors of PDE III and IV were demonstrated to have an inhibitory effect on ASM cell proliferation induced by FBS. Our results suggest the value of the further development of PDE inhibitors for the treatment of hyperplasia of ASM cells characteristic of airway remodeling, in addition to bronchospasm and airway inflammation, in bronchial asthma.

Key words: phosphodiesterase inhibitors, proliferation, tracheal smooth muscle cells.

Introduction

One of the histologic features in the airways of patients with bronchial asthma is hypertrophy/hyperplasia of airway smooth muscle (ASM) cells.[1] The structural change of ASM, together with subepithelial fibrosis and hypertrophy/hyperplasia of submucosal glands, leads to thickening of the airway walls, resulting in the aggravation of the chronicity and severity of bronchial asthma by amplifying airway narrowing.[2] Several factors present in the airways of asthmatics, which include platelet-derived growth factor, transforming growth factor-β1, tumor necrosis factor-α, substance P, histamine, leukotriene D4 and tryptase, have been reported to promote the proliferation of ASM cells *in vitro*.[3–6] The activation of phospholipase C, tyrosine kinase and mitogen-activated protein kinase has been reported to be involved in the growth responses of ASM cells.[7,8] On the other hand, salbutamol, prostaglandin E2, forskolin, vasoactive intestinal peptide (VIP) and 8-bromo-cyclic 3′,5′-adenosine monophosphate (8-bromo-cAMP) have been demonstrated to inhibit the proliferation of ASM cells via a rise of intracellular cyclic 3′,5′-adenosine monophosphate.[9,10]

Cyclic 3′,5′-adenosine monophosphate and cyclic 3′,5′-guanosine monophosphate (cGMP) are well known as second messengers mediating intracellular signal transduction evoked by the binding of agonists to

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their cell surface receptors. The concentration of intracellular cAMP/cGMP is dependent on the catalysis rate of adenosine/guanosine 5′-triphosphate to cAMP/cGMP by adenylate/guanylate cyclase and cAMP/cGMP to 5′-adenosine/guanosine monophosphate by phosphodiesterase (PDE). The PDE comprise at least seven families of isoenzymes (types I–VII), the differentiation of which is based on the primary protein and cDNA sequence, substrate specificity, regulation of enzymatic activity and calcium/calmodulin dependence. Of the seven PDE isoenzymes, types III and IV hydrolyze cAMP, and type V cGMP.[11] Biochemical analysis has identified five distinct PDE isoenzymes (I, II, III, IV and V) in human and bovine tracheal smooth muscle. Phosphodiesterases III, IV and V are abundant in bronchus and trachea, while PDE I and II are sparse. Pharmacological investigations using selective and non-selective inhibitors of PDE isoenzymes have shown that PDE type III and IV inhibitors are more effective than PDE type I, II and V inhibitors in bronchorelaxation.[12] With regard to the effects of PDE inhibitors on the growth of ASM cells, a non-selective PDE inhibitor (3-isobutyl-1-methylxanthine; IBMX) has been shown to inhibit the proliferation of human ASM cells via a rise of cAMP.[9] However, the effects of selective PDE inhibitors have not yet been determined, although selective inhibitors of PDE III and IV, as well as IBMX, have been demonstrated to inhibit mitogenesis of vascular smooth muscle cells.[13]

Therefore, in this study we investigated the effects on fetal bovine serum-induced bovine ASM cell proliferation of selective inhibitors of PDE III (cilostazol) and PDE IV (rolipram), as well as a non-selective PDE inhibitor (theophylline), which has been widely used for the treatment of bronchial asthma.

METHODS

Preparation of ASM cells

Airway smooth muscle cells were obtained from bovine tracheae as described previously, with some modifications.[14] Fresh bovine tracheae were obtained from a slaughterhouse. Small strips of the tracheal muscle were dissected from epithelium and connective tissue under sterile conditions, and minced into approximately 1 mm^3 pieces. After washing with modified Krebs'-Ringer-Henseleit (KRH) solution ((in mmol/L) NaCl 115, KCl 5, $CaCl_2$ 2, KH_2PO_4 1, $MgSO_4$ 1, glucose 15, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 25 and 10 μg/mL phenol red, pH 7.4) containing 100 U/mL penicilline G (Meiji Seika Co., Tokyo, Japan), 100 μg/mL streptomycin (Meiji) and 0.5 μg/mL amphotericin B (Bristol-Myers Squibb Co., Tokyo, Japan) (PSA) several times at 4°C, the minced muscle pieces were resuspended in 10 mL KRH solution containing PSA, not 2 but 0.2 mmol/L $CaCl_2$, 6400 U collagenase (Sigma, St Louis, MO, USA), 10 mg soybean trypsin inhibitor (Sigma) and 100 U elastase (Sigma), and then stirred gently for 90 min at 37°C. The cell suspension was filtered through 100 μm nylon meshes (Cell Strainer; Becton Dickinson Labware, Franklin, NJ, USA), and centrifuged at 150 *g* for 10 min. Cells were resuspended in Ham's F-12 (GIBCO BRL, Grand Island, NY, USA) containing 10% FBS (Sanko Junyaku Co. Ltd, Tokyo, Japan) and PSA, seeded at a density of $1 \times 10^4/cm^2$ in 25 cm^2 culture flasks (Becton Dickinson Labware) and cultured in a humidified atmosphere at 37°C in 95% air/5% CO_2 (v/v). The medium was replaced every 2 days. After 5 to 7 days, when the cells grew to confluence, they were subcultured by removal from flasks with 0.25% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.01% ethylenediamine tetraacetic acid (EDTA) (Dojindo, Kumamoto, Japan). These cell preparations were identified as ASM cells by a characteristic 'hill and valley' appearance and by immunocytochemical staining with anti-α-smooth muscle actin monoclonal antibodies (Sigma) detected by the alkaline phosphatase antialkaline phosphatase method, as described previously.[15] Cells at passage numbers 5 to 10 were used for the following experiments.

Culture of ASM cells with theophylline or PDE inhibitors

Airway smooth muscle cells in Ham's F-12 containing 10% FBS and PSA, were seeded at a density of 2×10^4/mL in the wells of 24-well microplates (Becton Dickinson Labware) in duplicate. After postincubation for 24 h, the culture medium was replaced with a fresh one containing 2.5, 5 or 10% FBS without or supplemented with theophylline (Wako Pure Chemical Industries, Ltd, Osaka, Japan) (10^{-3} mol/L). The culture medium was changed and the cell numbers were counted every 2 days. The cells cultured in the presence of theophylline were counted on the fourth day. Cell numbers were determined by counting the released nuclei, as previously described.[16] For each well, counting was performed three times.

To examine the effects of theophylline and PDE inhibitors on the proliferation of ASM cells, after the cells were seeded and postincubated as mentioned above, the culture medium was replaced with Ham's

F-12 containing 5% FBS and PSA without or supplemented with theophylline (10^{-5}, 10^{-4}, 3×10^{-4} and 10^{-3} mol/L), cilostazol (provided by Ohtsuka Pharmaceutical Co. Ltd, Tokyo, Japan;[17] 10^{-7}, 10^{-6} and 10^{-5} mol/L), rolipram (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} mol/L) or zaprinast (10^{-6}, 10^{-5} and 10^{-4} mol/L).[18] Changing of the culture medium was performed on the second day and counting on the fourth day, as mentioned above.

Lactate dehydrogenase assay

Airway smooth muscle cells were cultured in Ham's F-12 containing 5% FBS and PSA with or without theophylline (10^{-4}, 3×10^{-4} and 10^{-3} mol/L), rolipram (10^{-5} and 10^{-4} mol/L) and cilostazol (10^{-7}, 10^{-6} and 10^{-5} mol/L), as described above. On the fourth day, the culture medium was collected followed by the addition of 1 mL 5% Triton X-100 (Sigma) in Ham's F-12 to each well. The content of lactic dehydrogenase (LDH) in the culture medium and the Triton X-100 solution was measured by an automatic analyzer (7350; Hitachi Kohki Co., Hitachinaka, Japan) as extra- and intracellular LDH (LDHe and LDHi, respectively). The LDH content in Ham's F-12 containing 5% FBS and PSA (LDHm) was also measured.

Lactic dehydrogenase release was calculated by the following formula:

$$\text{LDH release} = \frac{LDH_e - LDH_m}{LDH_i + (LDH_e - LDH_m)} \times 100$$

Data analysis

Data are shown as mean ± SEM or mean ± SD and were compared using Mann–Whitney *U*-test. Differences were considered to be significant when $P < 0.05$.

Results

The effect of theophylline on the proliferation of ASM cells

As shown in Fig. 1, the number of cells was increased in an FBS concentration-dependent manner. Because the number reached the maximum on day 6 at all concentrations of FBS, the counting in the following experiments was performed on day 4 when ASM cells grew subconfluently.

The effect of theophylline (10^{-3} mol/L) on ASM cell proliferation was examined at 2.5, 5 or 10% of FBS. The number of cells cultured in the presence of theophylline was expressed as a percentage of that in the absence of theophylline in each concentration of FBS. Theophylline

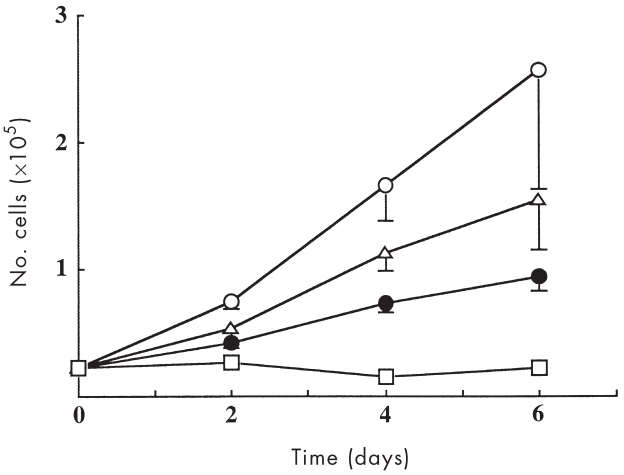

Fig. 1 Growth curve of airway smooth muscle cells in the presence of various concentrations of fetal bovine serum (FBS). Cells seeded at 2×10^4/well were cultured in the presence of 0% (□), 2.5% (●), 5% (△) and 10% (○) FBS and counted at days 2, 4 and 6. Each point represents mean ± SEM of four experiments.

significantly inhibited the proliferation of ASM cells at all concentrations of FBS (63.5% ± 10.6 (mean ± SD) at 2.5%; $P < 0.01$, 53.2% ± 12.4 at 5%; $P < 0.01$, and 60.9% ± 18.3 at 10% FBS; $P < 0.01$; Fig. 2a). However, there was no significant difference in the inhibitory effect of theophylline between these concentrations of FBS.

The effect of various concentrations of theophylline was also examined at the culture condition of 5% FBS. The number of cells cultured in the presence of theophylline was expressed as a percentage of that in the absence of theophylline. The number of ASM cells was significantly decreased by theophylline at concentrations of 10^{-3} (59.7 ± 15.9%, $P < 0.05$) and 3×10^{-4} (59.7 ± 16.0%, $P < 0.05$) mol/L, but not at 10^{-4} (99.1 ± 23.5%) and 10^{-5} (100.6 ± 10.4%) mol/L (Fig. 2b).

The effect of selective PDE inhibitors on the proliferation of ASM cells

The number of cells cultured with a selective PDE III inhibitor (cilostazol), a selective PDE IV inhibitor (rolipram), or a cGMP-specific PDE inhibitor (zaprinast), was expressed as a percentage of that without the inhibitors. Cilostazol significantly decreased the number in a dose-dependent manner (21.7% ± 7.0 at 10^{-5} mol/L; $P < 0.01$, 58.0% ± 29.0 at 10^{-6} mol/L; $P < 0.01$ and 77.8% ± 16.5 at 10^{-7} mol/L; $P < 0.03$; Fig. 3). Rolipram significantly inhibited the proliferation at concentrations of 10^{-4} (50.9% ± 7.4, $P < 0.01$) and 10^{-5}

(76.6% ± 10.7, $P < 0.05$) mol/L, but not at 10^{-6} (102.2% ± 23.5) and 10^{-7} (98.5 ± 17.8%) mol/L (Fig. 4). In contrast, zaprinast did not inhibit the number of ASM cells (Fig. 5).

Release of LDH from ASM cells cultured with agents

To examine whether or not the inhibitory effects of theophylline, rolipram and cilostazol on ASM cell proliferation were cytotoxic, LDH release was evaluated in each culture condition. Lactic dehydrogenase release from cells cultured with theophylline (10^{-3} mol/L, 2.4 ± 3.5% ($n = 3$) and 3×10^{-4} mol/L, 10.4 ± 6.3% ($n = 3$)), cilostazol (10^{-5} mol/L, 0.0 ± 0.0% ($n = 3$), 10^{-6} mol/L, 0.5 ± 0.5%($n = 3$) and 10^{-7} mol/L, 2.1 ± 1.2% ($n = 3$)) and rolipram (10^{-4} mol/L, 0.0 ± 0.0% ($n = 3$) and 10^{-5} mol/L, 0.0 ± 0.0% ($n = 3$)) was not significantly different from that with medium alone (6.4 ± 7.5% ($n = 5$)).

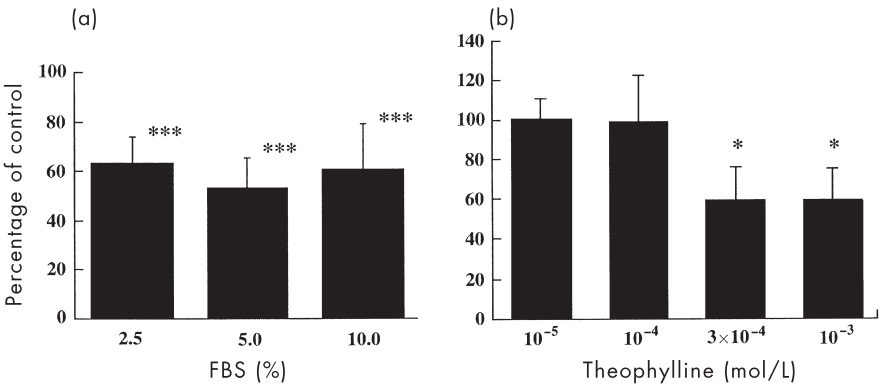

Fig. 2 Effect of theophylline on airway smooth muscle cell proliferation. (a) Cells seeded at 2×10^4/well were cultured with or without theophylline (10^{-3} mol/ L) in the presence of 2.5%, 5% and 10% fetal bovine serum (FBS), and counted at day 4. (b) Cells seeded at 2×10^4/well were cultured with or without theophylline (10^{-5}, 10^{-4}, 3×10^{-4} or 10^{-3} mol/L) at 5% FBS, and counted at day 4. Data are expressed as a percentage of counted cells cultured without theophylline. Each bar represents mean ± SD of six experiments. $^*P < 0.05$, $^{***}P < 0.01$.

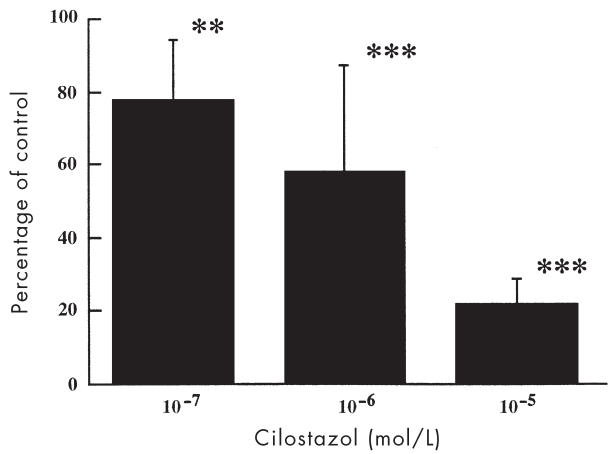

Fig. 3 Effect of cilostazol on airway smooth muscle cell proliferation. Cells seeded at 2×10^4/well were cultured with cilostazol (10^{-7}, 10^{-6} or 10^{-5} mol/L) at 5% fetal bovine serum, and counted at day 4. Data are expressed as a percentage of counted cells cultured without cilostazol. Each bar represents mean ± SD of six experiments. $^{**}P < 0.03$, $^{***}P < 0.01$.

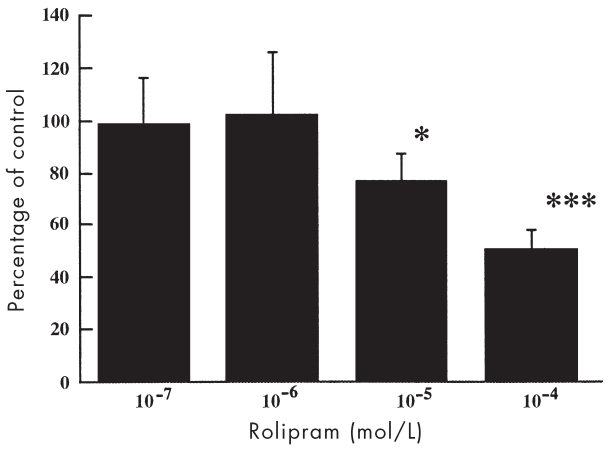

Fig. 4 Effect of rolipram on airway smooth muscle cell proliferation. Cells seeded at 2×10^4/well were cultured with rolipram (10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} mol/L) at 5% fetal bovine serum, and counted at day 4. Data are expressed as a percentage of counted cells cultured without rolipram. Each bar represents mean ± SD of four experiments. $^*P < 0.05$, $^{***}P < 0.01$.

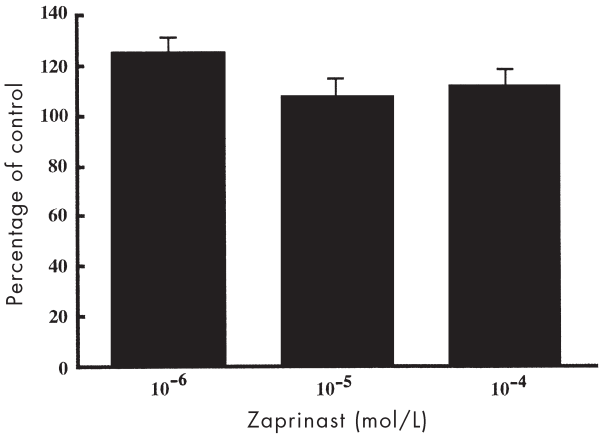

Fig. 5 Effect of zaprinast on airway smooth muscle cell proliferation. Cells seeded at 2×10^4/well were cultured with zaprinast (10^{-6}, 10^{-5} or 10^{-4} mol/L) at 5% fetal bovine serum and counted at day 4. Data are expressed as a percentage of counted cells cultured without zaprinast. Each bar represents mean ± SD of three experiments.

Discussion

In this study, we demonstrated for the first time that selective inhibitors of PDE isoenzyme type III (cilostazol) and type IV (rolipram) inhibited the FBS-induced proliferation of bovine ASM cells. The cAMP and cGMP content in ASM cells was not measured, but it has been reported that the inhibition of ASM cell proliferation, such as by corticosteroids, was not associated with an elevation of intracellular cAMP.[19] However, it has been shown that PDE III hydrolyzes mainly cAMP and PDE IV specifically hydrolyzes cAMP,[11] and that the inhibition of ASM cell proliferation by agents that elevate the cAMP content, including IBMX, (a non-selective PDE inhibitor), was correlated with cAMP levels,[9] and, furthermore, that this inhibitory effect was abolished by the addition of a selective inhibitor of cAMP-dependent protein kinase A (PKA).[10] In contrast, a cGMP-specific PDE inhibitor (zaprinast), showed no effect on the proliferation of ASM cells. Therefore, it is very likely that the inhibitory effects of theophylline, cilostazol and rolipram were mediated by the increased content of cAMP. The mechanisms by which cAMP inhibits the proliferation remains unclear. However, PDGF, which is a powerful mitogen of ASM cells in serum,[20] induces the proliferation of ASM cells through the activation of the tyrosine kinase system and phospholipase C.[7] The abolition of the inhibitory effect by a PKA inhibitor suggests the involvement of cAMP response element binding protein, which binds to the cAMP response element on the promoter region of target genes following phosphorylation by PKA, resulting in subsequent gene transcription.[21] Cyclic 3′,5′-adenosine monophosphate might inhibit Ras-dependent Raf-1 activation resulting in MAP kinase activation that is required for cell proliferation.[22,23] Therefore, the inhibitory effect of cAMP observed in this study might have been mediated by the inhibition of tyrosine kinase pathway.

Consistent with the previous results using IBMX,[9] theophylline inhibited ASM cell proliferation. The inhibitory activity of theophylline on ASM cell proliferation was lower than that of IBMX, which can be explained by the fact that the IC50 of theophylline on PDE activity is higher by 10–60-fold.[24] The lowest concentration examined at which the inhibitory effect of theophylline could be achieved (3×10^{-4} mol/L) was still higher than the therapeutic concentration in serum ($0.5–1 \times 10^{-4}$ mol/L),[25] although the inhibitory effect was not cytotoxic to ASM cells at the concentration used in this study, as indicated by LDH release. A combination of submaximally effective concentrations of sulbutamol and IBMX was reported to have a greater inhibitory effect on thrombin-induced mitogenesis in human ASM cells than that of either agent alone.[9] These results suggest that an inhibitory effect of theophylline, even at therapeutic concentrations, could be achieved by the combination with β-stimulants.

In addition to bronchospasm and airway inflammation, airway remodeling, including hyperplasia/hypertrophy of ASM cells leading to the amplification of airway narrowing, has been targeted in the treatment of bronchial asthma.[26] However, the pathogenesis of airway remodeling remains unclear, although there have been a few studies investigating the contribution of growth factors to the pathogenesis.[15,27,28] Furthermore, once it has begun, airway remodeling is difficult to resolve, even by the administration of corticosteroids.[29,30] Therapeutic strategies for airway remodeling have not yet been reported, whereas effective treatments for bronchoconstriction and airway inflammation, such as by theophylline, β-agonists and corticosteroids, have been developed. Our results indicating the inhibitory effects of PDE inhibitors on ASM-cell proliferation suggest the usefulness of these agents for the prevention of airway remodeling. Further investigation that also takes into account the bronchodilatory and anti-inflammatory effects[31,32] of PDE inhibitors will be needed for their development as anti-asthma drugs.

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