Novel Phosphodiesterase 4 Inhibitor T-440 Reverses and Prevents Human Bronchial Contraction Induced by Allergen

KAZUHIKO FUJII, HIROTSUGU KOHROGI, HAJIME IWAGOE, JUNJI HAMAMOTO, NAOMI HIRATA, EISUKE GOTO, OSAMU KAWANO, KAZUTERU WADA, SHINSUKE YAMAGATA and MASAYUKI ANDO

First Department of Internal Medicine and the Department of Laboratory Medicine, Kumamoto University School of Medicine and Lead Optimization Research Laboratory, Tanabe Seiyaku Co., Ltd., Kumamoto, Japan

Accepted for publication September 16, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

To study the roles of phosphodiesterase (PDE) 4 in the human airways, we examined the effect of the novel PDE4 inhibitor T-440 in the isolated human bronchus. T-440 inhibited PDE4 extracted from human bronchial smooth muscle. IC50 values for the effect of T-440, rolipram (a PDE4 inhibitor) and theophylline on PDE4 activity of the bronchial tissues were 0.08 μM, 2 μM and >100 μM, respectively. T-440 (10⁻⁶ M to 10⁻⁵ M) and aminophylline (3.3 × 10⁻⁶ M) significantly reversed the 10⁻⁶ M histamine-induced contraction, the efficacy of 10⁻⁶ M T-440 being almost the same as that of 3.3 × 10⁻⁵ M aminophylline. T-440 (10⁻⁶ M to 10⁻⁵ M) and aminophylline (3.3 × 10⁻⁵ M) significantly reversed the 10⁻⁴ M ACh-induced contraction. But their reversal effects on the ACh-induced contraction were weaker than those on the histamine-induced contraction. T-440 (10⁻⁵ M) significantly reversed the contraction induced by allergen in passively sensitized bronchi. The efficacy of the reversal effect of T-440 (10⁻⁵ M) was significantly higher than that of aminophylline (10⁻⁵ M). T-440 and aminophylline significantly relaxed the basal tension, but pretreatment with T-440 or aminophylline did not significantly prevent histamine- or ACh-induced contraction. In contrast, both T-440 (10⁻⁵ M) and aminophylline (3.3 × 10⁻⁵ M) prevented the contraction induced by allergen, which suggests that PDE4 inhibitor inhibits the release of chemical mediators probably from bronchial mast cells in the allergic response. T-440 (10⁻⁶ M to 10⁻⁵ M) caused the accumulation of cAMP at the concentration that relaxed histamine-induced contraction. Thus selective PDE4 inhibitor is a candidate for the treatment of asthma.

Xanthines such as theophylline and aminophylline are widely used in the treatment of asthma. Although xanthines operate through multiple mechanisms in the airways and lungs, the most likely mechanism of the bronchodilating effect has been thought to be the accumulation of cAMP in bronchial smooth muscle by inhibition of cyclic nucleotide PDE. Furthermore, because eosinophilic inflammation plays a crucial role in the pathogenesis of asthma, much attention has been paid to the anti-inflammatory and immunomodulatory effects of xanthines (Howell, 1990; Ward et al., 1993; Kidney et al., 1995). However, the complex multiple actions of xanthines include side effects on the cardiovascular system, GI system and/or CNS, and these side effects limit their use.

PDEs comprise at least seven isozyme families, PDE1 through PDE7. Individual PDE isozymes are encoded by a distinct cDNA sequence and differ with respect to their tissue distributions, physical and kinetic activities and substrate preferences (cAMP and/or cGMP) (Beavo, 1995). The inhibitions of PDE activities by xanthines are weak and are not isozyme-selective. Thus, when one isozyme of PDE is inhibited selectively and strongly, it may result in more useful effects and fewer undesirable effects in the treatment of asthma.

The inhibition of PDE3 or PDE4, both of which hydrolyze cAMP, is of special interest because either inhibition induces bronchodilation by the accumulation of cAMP (Nicholson et al., 1991; Hall, 1993). Although some investigators reported that PDE3 inhibition relaxed airway smooth muscle more than PDE4 inhibition did (Rabe et al., 1993; Torphy et al., 1993), PDE3 inhibition causes a positive inotropic action, and it may result in arrhythmogenic potency of xanthines (Nicholson et al., 1991; Hall, 1993). In contrast, PDE4 inhibition has only mild effects on the cardiovascular system. In addition, PDE4 inhibition induces anti-inflammatory effects such as the reduction of chemical mediator release from mast cells, basophils, eosinophils, neutrophils and macrophages; it also inhibits eosinophil infiltration and airway vascular leakage in the airways in guinea pigs (Dent et al., 1991; Souness et al., 1991). However, the complex multiple actions of xanthines include side effects on the cardiovascular system, GI system and/or CNS, and these side effects limit their use.

Received for publication March 6, 1997. 1Supported by Scientific Grants-in-Aid for Scientific Research (C) 02670343 and 06670622 from the Ministry of Education, Science and Culture of Japan. Presented in part at the 1996 American Lung Association/American Thoracic Society International Conference.

ABBREVIATIONS: PDE, phosphodiesterase; FEV₁,0, ratio of FEV₁ to FVC (forced expiratory volume in one second) to FVC (forced vital capacity); VC, vital capacity; cGMP, cyclic guanosine 3',5'-monophosphate.
et al., 1991; Ortiz et al., 1992; Raeburn and Karlsson, 1993; Lagente et al., 1994; Souness et al., 1994; Underwood et al., 1994). These facts suggest that the selective inhibition of PDE4 may be useful in treating asthma. There are, however, only a few studies in the human on the relaxing effect of the selective inhibition of PDE4 on precontracted bronchial smooth muscle (Cortijo et al., 1993; Rabe et al., 1993; Torphy et al., 1993), and there is no study on the preventive effect of the selective inhibition of PDE4 on bronchial smooth muscle contraction induced by histamine, ACh and allergen.

T-440, 6,7-dietoxy-2,3-bis[(hydroxymethyl)-1-{1-(2-methoxyethyl)-2-oxo-pyrid-4-yl}napthalene, is a novel PDE4 inhibitor (fig. 1; table 1 and Iwasaki et al., 1996). T-440 inhibits antigen- and chemical mediator-induced bronchoconstrictions in guinea pigs (Kaminuma et al., 1996). However, the effect on human bronchial tissues is not yet known. The goal of this study is to explore the reversal and preventive effects of the selective inhibition of PDE4 by T-440 on the human bronchial contraction induced by histamine, ACh and allergen. We also studied the effect of T-440 on cAMP accumulation in human bronchial tissue when the tissue was contracted by histamine, comparing it with the effect of aminophylline, one of the clinically used isoenzyme-nonselective PDE inhibitors.

Materials and Methods

Human bronchial tissues were obtained from 41 patients (32 males, 9 females) undergoing surgery for lung cancer. They had no history of atopic diseases and bronchial asthma, and their pulmonary function was within normal limits (FEV1 0.1sec. > 70%, %VC > 80%). They were 31 to 82 years old (mean age 64.0 ± 1.8). Immediately after resection of a lung or a lobe, the tissues were placed in Krebs-Henseleit solution of the following millimolar composition: NaCl, 118.3; KCl, 4.7; MgSO4, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25.0; glucose, 11.1. Segmental and subsegmental bronchi were dissected free from apparently normal lung tissue and cut into ring preparations. The rings were cut open through the cartilage. All tissues were prepared within 2 h after the resection.

Effect of T-440 on Bronchial Contraction

The tissues were placed in 10-ml organ baths containing Krebs-Henseleit solution. The solution was maintained at 37°C and continuously aerated with a mixture of 95% oxygen and 5% carbon dioxide. A glass hook at the bottom of the organ bath held one end of the tissue, and the other end was fixed by a silk thread to an isometric force displacement transducer (TB-611T; Nihonkohden Kogyo Co., Tokyo, Japan). The bronchial tissues were set up for the tension force displacement transducer (TB-611T; Nihonkohden Kogyo Co., Tokyo, Japan). The bronchial tissues were set up for the tension

![Fig. 1. Chemical structure of T-440.](image)
specificity for a substrate and by influences of cGMP and rolipram (a specific inhibitor of PDE4). The PDE4 fractions were collected and used for inhibition assays.

PDE activity was measured by a modification of the two-step radioisotope method (Thompson et al., 1979). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, and 4 mM 2-mercaptoethanol. All assays were performed at 30°C for 30 min, and the concentration of substrate was 1 μM for [³H]-cAMP or [³H]-cGMP. Inhibition assays for T-440, rolipram and theophylline were performed in triplicate. For each drug, 3 to 5 different concentrations were tested. IC₅₀ values were obtained by fitting concentration-response curves to the four-parameter logistic equation.

### Measurement of cAMP Content

We prepared 40 mg of human bronchial tissues (diameter 1 to 2 mm), denuding the epithelium by gentle rubbing. To examine whether T-440 causes the accumulation of cAMP in the tissue, we measured cAMP content in the presence and absence of histamine. In this study, we used smaller bronchi because they are easily homogenized. The tissues were incubated free-floating in Krebs-Henseleit solution aerated with a mixture of 95% oxygen and 5% carbon dioxide at 37°C. The tissues were equilibrated for more than 60 min in the solution, during which time the solution was replaced every 15 min. At 20 min after the addition of histamine (10⁻⁶ M) or its solvent, when the histamine-induced bronchial contraction reached a plateau in the study described above, we added T-440 (10⁻⁶ or 10⁻⁵ M), aminophylline (3.3 × 10⁻⁵ M) or their solvent to the tissues. They were then incubated for 60 min, because the reversal effect of the drugs reached plateau at around 60 min. Next they were rapidly removed, blotted on dry absorbent paper and snap-frozen by immersion in liquid nitrogen. The frozen tissues were stored at −80°C until assay for cAMP content.

We added 1 ml of cold 10% trichloroacetic acid (0°C) to a cold glass homogenizing tube containing approximately 40 mg of frozen tissue. The tissue was homogenized for 6 × 10-sec bursts at setting 6 (Polytron PCU-2, Kinematica GmbH, Luzern, Switzerland) and transferred to a 1.5-ml microtube. The homogenate was centrifuged at 600 × g for 15 min at 4°C (MR-150, Tomy, Tokyo, Japan) to form soluble extract and particulate fractions. Trichloroacetic acid in soluble fraction was removed by four successive extractions with water-saturated ether. After being heated at 70°C for 5 min to remove ether, the soluble fraction was stored at −20°C until assay for cAMP content. The residual precipitation was used for the measurement of protein content. cAMP was acetylated, and its amount was subsequently estimated by enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI). cAMP content was expressed as picomoles of cAMP per milligram of protein. Protein content was estimated by the method of Lowry et al. (Lowry et al., 1951).

### Data Analysis and Statistics

Data were expressed as the mean ± S.E. In the study of reversal effect, contraction was expressed as the percent of contraction induced by each spasmogen. In the study of preventive effect, the contractile response was expressed as follows:

\[
\% \text{ Contraction} = \frac{\text{Contraction at each concentration to spasmogen pretreated with drug}}{\text{Contraction to } 10^{-5} \text{ M acetylcholine}} \times 100
\]

The EC₅₀ value of the histamine- and ACh-induced concentration-response curve was calculated by linear regression analysis and expressed as a negative logarithm.

Unless otherwise mentioned, data were analyzed by one-factor analysis of variance (ANOVA), and the P values were corrected by Fisher’s protected least significant difference (Fisher’s PLSD) for multiple comparisons. A P value smaller than .05 was considered significant.

### Drugs

T-440 and rolipram were synthesized by Tanabe Seiyaku Co. Ltd. (Osaka, Japan). Aminophylline, ACh and histamine were purchased from Sigma Chemical Co. (St. Louis, MO), theophylline was from Nacalai Tesque (Kyoto, Japan) and house dust antigen was from Torii Co. (Tokyo, Japan). T-440, rolipram, aminophylline and theophylline were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in the vehicle solution or reaction mixture. The final concentration of DMSO was 0.01%. ACh, histamine, and house dust antigen were dissolved in Krebs-Henseleit solution just before each experiment. The concentration given is the final bath concentration.

### Results

#### Effect of T-440 on Bronchial Contraction

**Reversal effects of T-440 on bronchial contraction induced by histamine, ACh and allergen.** The magnitude of the bronchial contraction induced by 10⁻⁵ M histamine was similar between groups (control, 0.87 ± 0.14 g; 10⁻⁷ M T-440, 0.59 ± 0.14 g; 10⁻⁶ M T-440, 0.51 ± 0.14 g; 10⁻⁵ M T-440, 0.82 ± 0.11 g; 10⁻⁵ M aminophylline, 0.65 ± 0.18 g; 3.3 × 10⁻⁵ M aminophylline, 0.91 ± 0.11 g; P > .05). T-440 and aminophylline reversed the contraction in a concentration-dependent fashion (fig. 2A). 10⁻⁶ and 10⁻⁵ M T-440 and 3.3 × 10⁻⁵ M aminophylline significantly reversed the contraction compared with the control, but 10⁻⁵ M aminophylline did not (ANOVA with repeated measures). The reversal effect of 10⁻⁶ M T-440 was significantly greater than that of 10⁻⁵ and 3.3 × 10⁻⁵ M aminophylline.

### Table 1

**Effects of PDE inhibitors on the activity of PDE isozymes in guinea pigs and dogs**

<table>
<thead>
<tr>
<th>Guinea Pigs</th>
<th>T-440</th>
<th>Rolipram</th>
<th>CI-930</th>
<th>Theophylline</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1 (heart)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PDE2 (adrenal gland)</td>
<td>23</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PDE3 (heart)</td>
<td>49</td>
<td>100</td>
<td>0.88</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PDE4 (lung)</td>
<td>0.071</td>
<td>0.71</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PDE5 (lung)</td>
<td>67</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Dogs</td>
<td>T-440</td>
<td>Rolipram</td>
<td>CI-930</td>
<td>Theophylline</td>
</tr>
<tr>
<td>PDE3 (heart)</td>
<td>28</td>
<td>&gt;100</td>
<td>0.44</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PDE4 (trachea)</td>
<td>0.13</td>
<td>2.1</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

The magnitude of the bronchial contraction induced by 10^{-4} M ACh was also similar between groups (control, 0.79 ± 0.15 g; 10^{-7} M T-440, 0.88 ± 0.34 g; 10^{-6} M T-440, 0.74 ± 0.17 g; 10^{-5} M T-440, 1.03 ± 0.10 g; 10^{-5} M aminophylline, 0.71 ± 0.16 g; 3.3 × 10^{-5} M aminophylline, 0.72 ± 0.08 g; P > .05). 10^{-5} M T-440 and 3.3 × 10^{-5} M aminophylline significantly reversed the contraction (fig. 2B).

The magnitude of the bronchial contraction induced by allergen was similar between groups (control, 0.88 ± 0.06 g; 10^{-6} M T-440, 0.77 ± 0.08 g; 10^{-5} M T-440, 0.77 ± 0.10 g; 10^{-5} M aminophylline, 0.79 ± 0.10 g; 3.3 × 10^{-5} M aminophylline, 0.81 ± 0.12 g; P > .05). T-440 reversed the contraction induced by allergen in a concentration-dependent fashion, and 10^{-5} M T-440 significantly relaxed the tissues compared with the control (ANOVA with repeated measures) (fig. 2C). 10^{-5} and 3.3 × 10^{-5} M aminophylline reversed the contraction, but this effect was not significant. The reversal effect of 10^{-5} M T-440 was significantly greater than that of 10^{-5} M aminophylline. The reversal potency of T-440 in the contraction induced by allergen was similar to that induced by histamine. In contrast, the effects of T-440 on the ACh-induced contraction were obviously weaker than those on histamine- and allergen-induced contractions.

**Preventive effects of T-440 on bronchial contraction induced by histamine, ACh and allergen.** T-440 and aminophylline reversed the contraction induced by 10^{-5} M histamine, 10^{-5} M ACh and 10^{-5} M allergen. The magnitude of the bronchial contraction induced by 10^{-5} M aminophylline, 0.81 ± 0.06 M T-440, 0.77 ± 0.10 g; 10^{-5} M T-440 and 3.3 × 10^{-5} M aminophylline caused significant relaxation compared with the control. Moreover, 10^{-5} M T-440 caused significant relaxation compared with 10^{-5} M aminophylline. After adjusting the tension to 1 g again, we cumulatively added histamine, ACh or allergen to the tissues.

T-440 and aminophylline did not inhibit the contraction induced by histamine (fig. 4A). The magnitude of the bronchial contraction induced by 10^{-5} M acetylcholine was similar between groups (control, 1.33 ± 0.15 g; 10^{-7} M T-440, 1.54 ± 0.27 g; 10^{-6} M T-440, 1.44 ± 0.21 g; 10^{-5} M T-440, 1.28 ± 0.17 g; 10^{-5} M aminophylline, 1.38 ± 0.25 g; 3.3 × 10^{-5} M aminophylline, 1.20 ± 0.19 g; P > .05). T-440 and aminophylline slightly prevented the histamine-induced contraction but did not significantly change the concentration-response curve to histamine (EC_{50}, and maximal contraction).

T-440 and aminophylline did not inhibit the contraction induced by ACh (fig. 4B). The magnitude of the bronchial contraction induced by 10^{-5} M ACh was similar between groups (control, 0.81 ± 0.20 g; 10^{-7} M T-440, 0.81 ± 0.26 g; 10^{-6} M T-440, 0.82 ± 0.28 g; 10^{-5} M T-440, 0.80 ± 0.15 g; 10^{-5} M aminophylline, 0.82 ± 0.21 g; 3.3 × 10^{-5} M aminophylline, 0.83 ± 0.12 g; P > .05). T-440 and aminophylline did not significantly change the concentration-response curve to ACh (EC_{50}, and maximal contraction).

In contrast, T-440 significantly inhibited the maximal contraction induced by allergen (fig. 4C). The magnitude of the bronchial contraction induced by 10^{-5} M ACh was similar between groups (control, 0.82 ± 0.21 g; 10^{-7} M T-440, 0.77 ± 0.08 g; 10^{-6} M T-440, 0.77 ± 0.10 g; 10^{-5} M T-440, 0.77 ± 0.10 g; 10^{-5} M aminophylline, 0.79 ± 0.10 g; 3.3 × 10^{-5} M aminophylline, 0.81 ± 0.12 g; P > .05). T-440 reversed the contraction induced by allergen in a concentration-dependent fashion, and 10^{-5} M T-440 significantly relaxed the tissues compared with the control (ANOVA with repeated measures) (fig. 2C). 10^{-5} and 3.3 × 10^{-5} M aminophylline reversed the contraction, but this effect was not significant. The reversal effect of 10^{-5} M T-440 was significantly greater than that of 10^{-5} M aminophylline. The reversal potency of T-440 in the contraction induced by allergen was similar to that induced by histamine. In contrast, the effects of T-440 on the ACh-induced contraction were obviously weaker than those on histamine- and allergen-induced contractions.

**Preventive effects of T-440 on bronchial contraction induced by histamine, ACh and allergen.** T-440 and aminophylline relaxed the basal tension in a concentration-dependent fashion, and 10^{-5} M T-440 and aminophylline did not inhibit the contraction induced by histamine, ACh and allergen.
between groups (control, $1.05 \pm 0.22$ g; $10^{-6}$ M T-440, $1.02 \pm 0.13$ g; $10^{-5}$ M T-440, $1.02 \pm 0.18$ g; $10^{-5}$ M aminophylline, $1.12 \pm 0.16$ g; $3.3 \times 10^{-5}$ M aminophylline, $1.11 \pm 0.21$ g; $P > .05$). The threshold ($1$–$100 \text{ mg} \text{ ml}^{-1}$) and concentration-response curve of the contraction induced by allergen in control tissues varied among the subjects, and some tissues reached maximal contraction at their threshold concentration of allergen. Thus we could not calculate their EC$_{50}$ value and evaluated only their maximal contraction. T-440 inhibited the maximal response in a concentration-dependent manner, and $10^{-5}$ M T-440 showed a significant inhibition compared with the control (65% of the control). The maximal response was also significantly inhibited (79% of the control) by $3.3 \times 10^{-5}$ M aminophylline.

**Extraction, Separation and Characterization of PDE Isozymes from Human Bronchus**

Cytosolic cAMP-PDE activities of human bronchial tissue were separated by Mono-Q column chromatography into three peaks (fig. 5A). In accordance with the nomenclature proposed by Beavo and Reifsnyder (Beavo and Reifsnyder, 1990), the first peak (fraction 14) and the second peak (fraction 22) of cAMP-PDE activity were characterized as PDE1 and PDE2, respectively. But because the first peak overlapped another peak of cGMP-PDE activity (fig. 5A), it was suggested to be a mixture of PDE1 and PDE5. The third peak (fraction 26) was the major peak of cAMP-PDE activity (45% of total cAMP-PDE activity). It did not hydrolyze cGMP, and its cAMP-PDE activity was unaffected by the addition of cold cGMP. Furthermore, it was potently inhibited by rolipram.

**Fig. 4.** Preventive effect of T-440 on histamine-, ACh- and allergen-induced bronchial contraction. Here % contraction was defined as described in “Materials and Methods.” Each value is mean in histamine-induced contraction ($n = 8$, panel A) and ACh-induced contraction ($n = 4$, panel B) and mean ± S.E. in allergen-induced contraction ($n = 8$, panel C). The inset shows the preventive effect of T-440 on allergen-induced maximal contraction. □ control, ● $10^{-7}$ M T-440, ▲ $10^{-6}$ M T-440, ■ $10^{-5}$ M T-440, □ $10^{-5}$ M aminophylline, ◆ $3.3 \times 10^{-5}$ M aminophylline. * $P < .05$, ** $P < .01$ compared with control.

**Fig. 5.** Elution profile of PDE activities from human bronchus after separation on a Mono-Q anion exchange column. A) PDE activity when 1 $\mu$M cAMP (●) or 1 $\mu$M cGMP (○) was used as a substrate. B) PDE activity when cAMP was used as a substrate in the absence (●) and presence of 1 $\mu$M cold cGMP (○) or in the presence of 10 $\mu$M rolipram (○).
an inhibitor of PDE4 (fig. 5B). Therefore, it was characterized as PDE4. The PDE4 fractions (Kleine et al., 1992; Columbo et al., 1993; Griswold et al., 1993) were mixed, and the preventive effects of T-440, rolipram and theophylline were evaluated. The IC_{50} values of these inhibitors were 0.08 μM, 2 μM and >100 μM, respectively (fig. 6). PDE3 activity could not be detected.

**Measurement of cAMP Content**

T-440 (10^{-5} M) significantly increased cAMP content in the absence and presence of histamine (fig. 7). Interestingly, 10^{-5} M T-440 evoked a greater accumulation of cAMP in the presence of histamine than in its absence. On the other hand, 3.3 \times 10^{-5} M aminophylline evoked a slight accumulation of cAMP, but this effect was not significant. The accumulation induced by 10^{-5} M T-440 was significantly greater than that induced by 3.3 \times 10^{-5} M aminophylline.

**Discussion**

We examined the effects of the selective inhibition of PDE4 by the novel PDE4 inhibitor T-440 on human bronchial contraction induced by histamine, ACh and allergen. T-440 reversed in a concentration-dependent fashion the contraction induced by histamine, ACh and allergen. Pretreatment with T-440 also inhibited the maximal contraction induced by allergen, but not by histamine or ACh. The potency of T-440 was greater than that of isozyme-nonspecific PDE inhibitor aminophylline. We also confirmed that T-440 inhibits human bronchial PDE4 activity and that inhibition of PDE4 by T-440 causes the accumulation of cAMP in human bronchial tissues at the concentration that reverses histamine-induced contraction.

In the human bronchial tissues, we could not detect PDE3 activity in the cytosolic fraction, but PDE3 is detected in the human airway tissues, including smooth muscle, and PDE3 inhibitors have reversal effects on precontracted human bronchial tissues (Torphy et al., 1992; Rabe et al., 1993). We speculate that the cytosolic fraction in the present study did not contain enough PDE3 isozyme. Therefore, we examined the potency of T-440 against PDE isozymes in guinea pigs and dogs (table 1 and Iwasaki et al., 1996). T-440 is a PDE4 inhibitor with preferential potency against PDE4 isozyme (Iwasaki et al., 1996). As table 1 shows, T-440 potently inhibits PDE4 activity in the guinea pig lung (IC_{50} = 0.071 μM), and is approximately 10-fold more potent than rolipram (IC_{50} = 0.71 μM). and it displayed a 690-fold selectivity for PDE4 compared with PDE3 in the guinea pig heart (IC_{50} against PDE3, 49 μM) (Yamagata et al., 1997). In the present study, the preventive potency of T-440 against PDE4 in the human bronchus (IC_{50} = 0.08 μM) was similar to that against PDE4 in the guinea pig lung, and T-440 was approximately 25-fold more potent than rolipram (IC_{50} = 2 μM). We used T-440 at a concentration lower than 10 μM because the inhibition of PDE4 is selective at that concentration.

T-440 reversed in a concentration-dependent fashion the contraction induced by histamine and allergen. The effects of 10^{-6} M T-440 on the contraction induced by histamine and allergen were similar to those of 3.3 \times 10^{-5} M aminophylline. The concentration 3.3 \times 10^{-5} M aminophylline is equivalent to 13.9 μg/ml, which is the adequate serum concentration clinically used. These data suggest that PDE4 inhibition is potent enough to relax human bronchial contraction. This result supports previous reports (Cortijo et al., 1993).

Reversal effects of T-440 and aminophylline on the contraction induced by ACh were remarkably smaller (fig. 2, A and B). This result, which is consistent with reports of previous studies in animals (Andersson et al., 1978; Souness et al., 1994; Kaminuma et al., 1996), may be due to the cholinergic inhibition of adenylate cyclase in the airway smooth muscle (Andersson et al., 1978; Jones et al., 1987). Harris and colleagues reported that the bronchorelaxant activity of a series of PDE4 inhibitors in guinea pigs in vivo and in vitro correlates with the ability of these agents to interact with high-affinity rolipram-binding site, not with their ability to inhibit PDE4 catalytic activity (Harris et al., 1989). We studied the potency of T-440 and rolipram against [3H]-rolipram binding site in the guinea pig brain, and ability of T-440 and rolipram to inhibit PDE4 in the guinea pig lung. We found that T-440 has lower potency against rolipram binding site and higher PDE4-inhibiting ability than rolipram (unpublished data).

Although we did not study the reversal effect of rolipram, we speculate that T-440 may have lower reversal effect than rolipram.

T-440 and aminophylline relaxed bronchial basal tone in a concentration-dependent manner. We do not know which mediator regulates the basal tone, but cAMP may be one of

![Fig. 6. Inhibition of PDE4 activity by T-440, rolipram and theophylline in human bronchial tissue. IC_{50} values for the effect of T-440 (●), rolipram (□) and theophylline (△) on PDE4 activity were 0.08 mM, 2 mM and >100 mM, respectively.](image-url)
the important regulators of airway tone. The effect of $10^{-6}$ M T-440 on basal tension was similar to that of $3.3 \times 10^{-5}$ M aminophylline. These data suggest that T-440 is about 33-fold more potent than aminophylline in relaxing human bronchial basal tone.

Pretreatment with T-440 and aminophylline significantly prevented the contraction induced by allergen. They slightly prevented the histamine-induced contraction, but this effect was not significant. These results are also obtained in guinea pigs (Gustafsson and Persson, 1991). Thus there are differences between the reversal and the preventive effects of T-440 and aminophylline on the histamine- and ACh-induced constrictions. Because T-440 and aminophylline relaxed the basal tension, we adjusted it before adding histamine and ACh. Therefore, when the preventive effects of T-440 and aminophylline were examined, the bronchial tissues did not contain the factors that contributed to the basal tone associated with T-440 and aminophylline. By contrast, when the reversal effects of T-440 and aminophylline were examined, the bronchial tissues did contain the factors that contributed to the basal tone associated with T-440 and aminophylline. Therefore, we speculate that the reversal effects were greater than the preventive ones. In spite of these facts, T-440 and aminophylline prevented the human bronchial contraction induced by allergen, which suggests that inhibition of PDE4 suppresses the release of chemical mediator from mast cells, thus inhibiting the contraction induced by allergen. Another possible mechanism in the inhibition of allergen-induced bronchial contraction is that T-440 inhibits the effects of other contractile mediators, especially leukotrienes, which are important mediators in asthma. But this possibility is remote, because T-440 did not have leukotriene B$_4$ and D$_4$ receptor antagonist activity in the guinea pig lung membrane (data not shown) and because PDE4 inhibition by rolipram did not inhibit LTD$_4$-induced bronchial contraction and constriction in guinea pigs (Howell et al., 1993; Underwood et al., 1994). In animal studies, rolipram inhibited histamine-induced bronchoconstriction significantly less than antigen-induced bronchoconstriction in the sensitized guinea pig in vivo (Howell et al., 1993; Underwood et al., 1993; Underwood et al., 1994). It also reduced the antigen-induced release of prostaglandin D$_2$ in the guinea pig trachea and that of histamine and leukotriene C$_4$ in murine mast cells (Griswold et al., 1993; Underwood et al., 1993). Thus our human bronchial ex vivo study is consistent with the animal studies. We conclude that 1) there are difference between the reversal and the preventive effects of T-440 and aminophylline on histamine- and ACh-induced bronchial smooth muscle contraction, and 2) T-440 and aminophylline prevent allergen-induced bronchial smooth muscle contraction. These findings are new insights in the pharmacology of PDE inhibitors in the human bronchus.

In human basophils, the PAF- or IgE-mediated release of histamine and leukotriene C$_4$ is reduced by the inhibition of PDE4, which appears to be the major PDE isozyme in mast cells and basophils (Kleine et al., 1992; Peachell et al., 1992; Torphy et al., 1992; Columbo et al., 1993). Inhibition of PDE4 reduces the functions of other inflammatory cells, including eosinophils (Dent et al., 1994; Souness et al., 1994), and of monocytes (Griswold et al., 1993; Molnar et al., 1993). Furthermore, T-440 inhibits interleukin-5 production in the peripheral mononuclear cells of asthmatic patients (Kaminuma et al., 1995). Inhibition of PDE4 also potentiates nonadrenergic, noncholinergic relaxation (Fernandes et al., 1994). In the present study, because the bronchial tissues are normal, these types of inflammatory cells may be not contributed.

T-440 accumulated cAMP content in bronchial smooth muscle in the presence or absence of histamine. This suggests that the inhibition of PDE4 causes the accumulation of cAMP, resulting in the relaxation of bronchial smooth muscle (Cortijo et al., 1993; Howell et al., 1993; Underwood et al., 1993). On the other hand, $3.3 \times 10^{-5}$ M aminophylline did not induce a significant accumulation of cAMP, although this concentration significantly reversed histamine-induced contraction as much as $10^{-6}$ M T-440 did. These results suggest that aminophylline relaxed the bronchial contraction through mechanisms other than the inhibition of PDEs that hydrolyze cAMP.

The accumulation of cAMP induced by T-440 was significantly greater in the presence of histamine than in its absence. Histamine stimulates cAMP synthesis via H$_2$ receptors that are coupled to adenylyl cyclase in the airway tissue, including human airway smooth muscle, though its precise role remains in dispute whether this activation of H$_2$ receptors results in physiological changes (Dunlop et al., 1977; Chandler and Eyre, 1978; Duncan et al., 1980; Florio et al., 1992). Therefore, our result may be due to the effect of T-440 on the accumulation of cAMP synthesized by histamine and on the accumulation of basal cAMP. In the present study, we could not show that histamine itself significantly induced cAMP synthesis. This may be due to the long incubation with histamine (60 min), because histamine-induced cAMP synthesis was rapid and took less than 6 min (Murad and Kimura, 1974; Duncan et al., 1980).

The bronchodilatory and anti-inflammatory effects of PDE4 inhibition may be useful in treating asthma. On the other hand, the inhibition of PDE3, which may relax the bronchus more than the inhibition of PDE4, has little or no effect on anti-inflammatory action (Rabe et al., 1993; Torphy et al., 1993). Additionally, the inhibition of PDE3 causes positive inotropic and arrhythmogenic actions (Nicholson et al., 1991; Hall, 1993), and long-term inhibition of PDE3 is associated with increased morbidity and mortality for patients with severe chronic heart failure (Packer et al., 1991). To treat asthmatic patients subject to chronic heart failure and arrhythmia, it is reasonable to try to use a selective PDE4 inhibitor.

The drawback of PDE4 inhibitors is emesis. Because the dose of T-440 that induced emesis is about 10-fold higher than that of rolipram in suncus (unpublished data), and because the PDE4-inhibiting activity of T-440 is 10-fold more potent than that of rolipram, the potency for emesis of T-440 may be 100-fold less than that of rolipram.

In summary, the inhibitor of PDE4, T-440, reversed histamine-, ACh- and allergen-induced bronchial contraction in the human bronchus. Pretreatment with T-440 also prevented allergen-induced contraction, but not histamine- and ACh-induced contractions, which suggests that T-440 inhibits the release of chemical mediators, probably from mast cells. The effects of T-440 were more potent than those of aminophylline. In addition, T-440 caused the accumulation of cAMP at the concentration that relaxed histamine-induced contraction. Thus the present study suggests that the selec-
tive inhibition of PDE4 is a candidate for the treatment of asthma.

**References**


Send reprint requests to: Hirotsugu Kohrogi, M.D., First Department of Internal Medicine, Kumamoto University School of Medicine, 1-1-1, Honjo, Kumamoto 860, Japan.