Studies on Mechanisms of Low Emetogenicity of YM976, a Novel Phosphodiesterase Type 4 Inhibitor

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ABSTRACT

YM976 is a novel and selective inhibitor of phosphodiesterase type 4 (PDE4) with a different chemical structure from rolipram. Orally administered YM976 showed anti-inflammatory activity (ED50 = 2.8 mg/kg) similar to rolipram (3.5 mg/kg). On the other hand, the emetogenicity of YM976, one of the main adverse effects of PDE4 inhibitors, was lower (maximal non-emetic dose = 10 mg/kg) than that of rolipram (1 mg/kg). The reasons for this low emetogenicity of YM976 remain unclear, and the present study endeavored to elucidate the mechanisms. Candidates for the possible mechanisms included 1) PDE4 subtype selectivity, 2) binding affinity for HAR-conformation, and 3) brain penetration. YM976 exhibited affinity for high affinity for rolipram-conformation (HAR-conformation) (IC50 = 2.6 nM) identical to that of rolipram (1.2 nM), and failed to show significant selectivity for the individual PDE4 subtype. These results suggested that neither subtype selectivity nor the affinity for HAR-conformation may be related to the low emetogenicity of YM976. YM976 showed a minor effect on reserpine-induced hyperthermia, in contrast to rolipram. To estimate brain penetration, we then measured cAMP contents in peripheral tissues (peritoneal macrophages) and in the brain. YM976 increased the cAMP content of peritoneal macrophages, but caused no significant increase in brain cAMP levels, while rolipram elevated the cAMP content of both tissues at the same dose. In conclusion, YM976 shows an apparent dissociation between its anti-inflammatory effects and emetogenicity, perhaps because of the poor brain penetration.

Cyclic AMP-specific phosphodiesterase type 4 (PDE4) is predominantly contained in immune and inflammatory cells and plays important roles in regulating intracellular levels of cAMP (Teixeira et al., 1997). PDE4 is encoded by four gene families (A, B, C, and D), each of which generates multiple isoforms. These appear to have very similar catalytic sites and are inhibited similarly by a variety of PDE4 inhibitors (Muller et al., 1996; Houslay et al., 1998).

PDE4 inhibitors have attracted considerable attention, primarily because of their wide suppressive effects on the functions of several inflammatory cells. Most interest to date has focused on the potential therapeutic effects on various diseases, including bronchial asthma (Dal and Giovannoni, 2000) chronic obstructive pulmonary disease (Torphy et al., 1988), arthritis (Sekut et al., 1995), and CNS disorders (Fleischhacker et al., 1992). Although PDE4 inhibitors have potential therapeutic activities, nausea and vomiting have been observed in the clinic following administration of PDE4 inhibitors, including rolipram (Bertolino et al., 1988), tibenelast (Israel et al., 1988), and CP80633 (Hanifin et al., 1996). These effects may limit the therapeutic potential of the PDE4 inhibitors. Thus, a PDE4 inhibitor with little or no emetogenicity has been sought.

The mechanisms by which PDE4 inhibitors induce these side effects are uncertain, but studies demonstrating the potentiation of apomorphine-induced emesis in dogs by RO20-1724 (Carpenter et al., 1988) suggest that the nausea and vomiting are likely to be produced, at least in part, via the emesis centers in the brain. Other researchers also showed that these side effects are produced as an extension of the pharmacological mechanism of action of PDE4 inhibitors, and are caused primarily by inhibition of PDE4 in CNS (Duplantier et al., 1996). It is believed that various PDE4 isoforms can adopt distinct conformations detected by very different sensitivities to inhibition of rolipram (Souness et al., 1997). It is reported that an interaction with the conformation showing high affinity for rolipram (HAR-conformation) may be strongly cor-

ABBREVIATIONS: PDE4, phosphodiesterase type 4; CNS, central nervous system; HAR-conformation, conformation with high affinity for rolipram; YM976, 4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-H]pyrimidin-2(1H)-one; DMSO, dimethyl sulfoxide; MC, methylcellulose; BCG, bacillus Calmette-Guerin; LPS, lipopolysaccharide; ILMP, formyl-methionine-leucyl-phenylalanine; CB, cytochalasin B; LTC4, leukotriene C4; MED, minimal effective dose; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; MNED, maximal non-emetic dose; AUC, area under the curve; LAR-conformation, conformation with low affinity for rolipram.
related to emesis (Duplantier et al., 1996). On the other hand, a family of PDE4 is divided into at least four subtypes, and some differences are reported in the distribution of each subtype. These appear to have very similar catalytic sites and are inhibited similarly by a variety of PDE4 inhibitors (Muller et al., 1996; Houssay et al., 1998). Although the relationship between the side effects and subtype function is expected, there are no evidences, in our knowledge, indicating this relationship. Therefore, the interaction with HAR-conformation and the selectivity for PDE4 subtypes have also been discussed as candidates for the mechanisms for the side effects.

We previously reported that YM976, 4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-d]pyrimidin-2(1H)-one, was a novel and selective PDE4 inhibitor having a different structure from rolipram (Fig. 1), and that orally administered YM976 inhibited neutrophil infiltration induced by carrageenan (Aoki et al., 2000a), and inhibited antigen-induced airway responses in guinea pigs (Aoki et al., 2001). Therefore, YM976 is expected to become a new useful therapeutic drug for inflammatory diseases such as asthma. In the present study, we evaluated the anti-inflammatory activities and emetogenicity of YM976 compared with those of rolipram. In addition, we tried to elucidate one of the possible mechanisms by which the dissociation of YM976 was manifested.

Materials and Methods

Animals. Male BALB/c mice and male ferrets were purchased from Charles River Japan (Atsugi, Japan). C57Black/6 mice, ICR mice, and Wistar rats were purchased from SLC (Hamamatsu, Japan). All animals were maintained in ordinary animal cages in a constant 12-h light/dark cycle, with food and water available ad libitum. The rats and mice were housed in groups of 6 and 10 per cage, respectively, and ferrets were housed one or two per cage. The animals were fasted for 16 h before each experiment.

Drugs and Chemicals. YM976, (±)-rolipram, RP73401, and CDP840 were synthesized by the Department of Chemistry, Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan); Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan); and Nacalai Tesque (Kyoto, Japan), respectively. In in vitro experiments, all compounds were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was less than 0.1%. In in vivo experiments, these compounds were suspended in 0.5% methylcellulose (MC) solution, and were administered orally at a volume of 10 ml/kg, except for ferrets (3 ml/kg). The control groups were treated only with vehicle (0.5% MC).

The reagents and chemicals used were as follows: reserpine purchased from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan); heparin from Shimizu Seiyaku (Shimizu, Japan); DMSO, CaCl2, methanol, chloroform, perchorlic acid, and K2CO3 were purchased from Kanto Chemical Co. (Tokyo, Japan); BCG from Japan BCG Laboratory (Tokyo, Japan); lipopolysaccharide (LPS; Escherichia coli serotype 0.55:B5) from Paesel + Lorei GmbH & Co (Hanau, Germany); Ficoll solution, dextran, and (O-methyl-[3H] rolipram from Amersham Pharmacia Biotech (Uppsala, Sweden); Hanks’ balanced salt solution without calcium chloride or magnesium sulfate, ML, cytoclasin B (CB), bovine serum albumin, glucose, sodium acetate, Tris-HCl, EDTA, EGTA, Bis-Tris, and cAMP from Sigma Chemical Co. (St. Louis, MO); HEPES buffer, fatal bovine serum, and RPMI medium from Life Technologies (Rockville, MD); brevier thioyleolate from Difo Laboratories (Detroit, MI); and Al(OH)3 (Inject, Alum) from Pierce (Rockford, IL).

Isolation of Human Peripheral Eosinophils. Granulocytes were isolated from the peripheral blood of healthy human volunteers using the density gradient centrifugation (Ficoll solution) as described previously (Boyum, 1962; Aoki et al., 2000b). Eosinophils were purified from the granulocyte fraction with a Magnetic Cell Sorting System (column type BS, 3.5 ml/min; Milteny Biotech, Bergisch-Gladbach, Germany) (Miltienny et al., 1990). The final cell preparation consisted of >98% eosinophils.

LTC4/D/E4 (Cysteinyl-Leukotrienes) Release. In eosinophil samples isolated from five different donors, the effects of YM976 (0.1–1000 nM) and rolipram (0.1–1000 nM) on LTC4/D/E4 release stimulated by CB plus fMLP were studied. Human eosinophils (3 × 106 cells/ml) suspended in a buffer containing 20 mM HEPES, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, 0.025% bovine serum albumin, 1 mM CaCl2, and 1 mM MgCl2 (pH 7.4) were treated with YM976 and rolipram, and placed in a incubator at 37°C (95% O2, 5% CO2) for 25 min. CB (5 μg/ml) was then added, and 5 min later, the cell suspension was stimulated with fMLP (2.5 μM). Fifteen minutes after the stimulation, the reaction was stopped by placing the sample on ice water. The samples were centrifuged at 500g for 5 min, and the LTC4/D/E4 contents of the supernatant were measured by an LTC4/D/E4 enzyme immunoassay kit (Amersham Pharmacia Biotech).

LPS-Induced Plasma TNF-α Production. The experiment was conducted according to the method of Christ et al. (1995). The C57Black/6 mice were intravenously pretreated with BCG (2 mg/mouse). Fourteen days after the pretreatment, the animals, which were fasted for 16 h, were intravenously injected with LPS (3 μg/mouse) to elicit TNF-α production. YM976, rolipram, and CDP840 were orally administered 30 min before the LPS injection. Prednisolone was orally administered 2 h before the LPS. The saline group was injected with physiological saline instead of LPS. The peripheral blood was obtained 1 h after the elicitation. After centrifugation at 10,000g, the plasma TNF-α content was measured using a mouse TNF-α ELISA kit (Amersham Pharmacia Biotech).

Measurement of [3H](±)-Rolipram Binding to Brain Membranes. The specific radioactivity of the [3H](±)-rolipram was 3.0 TBq/mmol. Wistar rat brain membranes were prepared as described by Schneider et al. (1986). The binding assay was performed using 2 nM [3H](±)-rolipram and membranes from 0.6 mg of brain tissue according to the method described by Barronet et al. (1995). IC50 values were determined from graphs of percentage of inhibition versus concentration.

Cloning of PDE4 Subtypes. To obtain the full-length of PDE4 subtypes, reverse transcription-PCR was performed. Transcription name (accession number) of each PDE4 subtype used in this study is as follows: PDE4A, HSPDE4A4B (L20965); PDE4B,
HSPDE4B1A (L20966); PDE4C; HSPDE4C1B (Z46632); and PDE4D, HSPDE4D3A (L20970). Reverse transcription-PCR was carried out using specific primers and the human brain cDNA as a template for 35 cycles at 96°C for 30 s, 60°C for 30 s, and 74°C for 3 min. Specific primers were synthesized based on the report (Houslay et al., 1998). Forward primer and reverse primer of each subtypes are as follows: PDE4A (5'-TGTAGGTGGGAAAAGGC-3', 5'-TTGAGGCAAGGAGAGGAGTG-3'), PDE4B (5'-GGGGCGTATGGGTAAGAAAGGAAAGATGGTTGCTCACC-3', 5'-GGGGCATGGTATGGTATGGTGTTATGCACG-3'), and PDE4D (5'-GGGGCGTATGGTATGGTATGGTGTTATGCACG-3', 5'-GGGGCATGGTATGGTATGGTGTTATGCACG-3'). PCR product was subcloned into pEF-BOS expression vector and sequenced. This vector was kindly provided by Dr. Shigekazu Nagata (Osaka Bioscience Institute, Osaka, Japan) (Mizushima and Nagata, 1990).

**Determination of Inhibitory Effect for PDE4 Subtypes.** The inhibitory effects for each PDE4 subtype were determined by a previously reported method (Aoki et al., 2000b). Briefly, each test compound was incubated at 30°C for 10 min in a reaction mixture containing cAMP, [3H]cAMP, and the PDE4 subtype. The reaction was stopped by the addition of methanol. The -nucleotidase, and then incubated at 30°C for 30 min. The reaction was stopped by the addition of methanol. The solution was passed through a Dowex 1-X8 (Bio-Rad, Richmond, CA) column to adsorb unhydrolyzed materials and then the radioactivity in the elution was measured.

**Emetogenic Effects in Conscious Ferrets.** The emetogenic activities of the compounds were examined in ferrets fasted overnight. Each test compound was administered orally at a volume of 3 ml/kg. The number of episodes of emesis for each ferret was recorded for 8 h from just after the administration. The emetogenic effects of the test compounds were expressed as the identity number and the incidence of ferrets showing emesis. The control group was treated with 0.5% MC (3 ml/kg p.o.). The maximal non-emetic dose (MNED) was defined as the maximal dose showing no emesis.

**Reserpine-Induced Hypothermia.** The experiment was performed according to a modification of the method described in the previous report (Koe et al., 1983). Male ICR mice were housed in a plastic chamber. The animals were injected subcutaneously with reserpine at a dose of 2 mg/kg and maintained at 25°C for 18 h. Rectal temperatures were then measured with an electric thermometer (Thermistor type PV, Shibaura Electronics Co., Ltd., Tokyo, Japan) as the pre values, and the animals were orally administered YM976 (3, 10, and 30 mg/kg) or rolipram (1, 3, and 10 mg/kg) at a volume of 10 ml/kg. Then, at 0.5, 1, 1.5, 2, and 2.5 h after the administration, the rectal temperatures were again measured.

Data were expressed as changes of rectal temperature, and the effects on the hypothermia were determined using areas under the curve (AUC0–2.5, °C × h). MED (minimum effective dose) was defined as the lowest dose at which a statistically significant increase in AUC0–2.5 occurred in comparison with the control.

**LPS-Induced TNF-α Production in Mice Pretreated with Thioglycollate.** Male BALB/c mice (23–28 g) were intraperitoneally injected with a brewer thioglycollate solution (4%, 1 ml/animal). Four days later, TNF-α production was elicited by an intraperitoneal injection of LPS (1 μg). One hour after the LPS injection, the peritoneal cavity was lavaged with 3 ml of saline containing heparin (1 unit/ml). The lavage fluid was divided into three fractions. One fraction was used for measuring intracellular CAMP levels. It was quickly placed in boiling water for 2 min, and centrifuged at 10,000g. CAMP levels in the supernatant were measured with a cAMP ELISA kit (Amersham Pharmacia Biotech). Another fraction was used for counting cell numbers in the lavage fluid. The main cell type found was macrophages (85–95%). The other fraction was centrifuged, and the supernatant TNF-α content was measured using the mouse TNF-α ELISA kit.

**cAMP Levels of Mice Brain.** For comparison with the CAMP levels of the peripheral tissue (peritoneal macrophages), brain CAMP levels were measured in mice pretreated with thioglycollate as described above. YM976 and rolipram were orally administered to the mice at 10 mg/kg, and 30 min later the heads of the mice were subjected to microwaves (4.6 kW, 1.4 s) from a microwave applicator (TMW-6402A; Toshiba, Tokyo, Japan) to inactivate the PDEs. Then, the whole brain was removed and placed on dry ice. After the weight of the brain was determined, it was homogenized in perchloric acid with a cell disruptor (Sonifier model W-200P, Branson Ultrasonics Corporation, Danbury, CT). The sample was centrifuged at 10,000g for 30 min, and the supernatant was obtained and neutralized with K2CO3. After the second centrifugation, the CAMP levels in the supernatant were measured using the cAMP ELISA kit.

**Data Analysis.** Data were expressed as the mean ± S.E. or the mean with 95% confidence limits. Statistical significance of differences between means of groups was determined by Dunnett’s multiple range test or Student’s t test. Probabilities of <0.05 were considered significant. A dose (ED50) or a concentration (IC50) causing 50% inhibition was determined by nonlinear curve fitting using an Statistical Analysis System (SAS Institute Inc., Cary, NC).

**Results**

**LTC4/D4/E4 Release from Eosinophils.** Stimulation with CB plus fMLP induced the release of LTC4/D4/E4 from the eosinophils (240 ± 18 pg/ml, n = 5). YM976 and rolipram concentration dependently increased the release at 0.1 to 1000 nM, and the IC50 values were 3.9 nM (95% confidence limit, 1.1–12) and 12 nM (4.5–32), respectively (Table 1).

**LPS-Induced Plasma TNF-α Production.** YM976 inhibited LPS-induced plasma TNF-α elevation with an ED50 value of 2.8 (2.0–4.2) mg/kg p.o. (Fig. 2 and Table 1). Rolip-
ram and prednisolone also suppressed TNF-α elevation with ED$_{50}$ values of 3.5 (1.9–5.0) and 1.5 (1.1–2.2) mg/kg p.o., respectively. On the other hand, CDP840 failed to show any clear inhibition up to 30 mg/kg p.o.

**Emetogenicity.** As shown in Table 2, YM976 caused no emesis at 3 and 10 mg/kg p.o., but it did induce emesis at 30 mg/kg and more p.o. Rolipram, RP73401, and CDP840 elicited no emesis at 1, 1, and 10 mg/kg p.o., respectively. The MNED of YM976, rolipram, RP73401, and CDP840 were 10, 1, 1, and 10 mg/kg p.o., respectively.

**Inhibition of PDE4 Subtypes and Displacement of [³H]Rolipram Binding.** As shown in Table 3, YM976 inhibited the enzymatic activities of PDE4A, B, and D subtypes stronger than that of PDE4C, which were similar to rolipram, RP73401, and CDP840. YM976 showed displacement of [³H]rolipram binding, with an IC$_{50}$ value of 2.6 nM. Rolipram, RP73401, and CDP840 also displaced rolipram binding.

**Reserpine-Induced Hypothermia.** As shown in Fig. 3, the rectal temperatures of the saline-treated mice were $36.5 \pm 0.27°C (n = 10)$, the reserpine-treated mice exhibited temperatures of $28.6 \pm 0.17°C (n = 40)$. YM976 significantly restored the decreased AUC$_{0-2.5}$ of the rectal temperatures at 30 mg/kg p.o. Rolipram also significantly induced the elevation of AUC$_{0-2.5}$ of the temperatures at 1 and 3 mg/kg p.o. Thus, the MEDs of YM976 and rolipram were 30 and 1 mg/kg p.o., respectively.

**Effects on TNF-α Production and cAMP Elevation in Mice Pretreated with Thioglycollate.** YM976 and rolipram dose dependently inhibited TNF-α production in the peritoneal cavity of mice pretreated with thioglycollate. The ED$_{50}$ values of YM976 and rolipram were 3.5 (95% confidence limit, 2.2–4.7) and 5.4 (2.9–21) mg/kg p.o., respectively. In the same experiments, both YM976 and rolipram at 10 mg/kg p.o. significantly elevated the intracellular cAMP level of peritoneal leukocytes, which were mainly macrophages ($p < 0.05$; Fig. 4).

**cAMP Levels of Mouse Brain.** YM976 did not significantly raise the brain cAMP levels in mice at 10 mg/kg p.o.,

### Table 2

<table>
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<th>Compound</th>
<th>Dose (mg/kg p.o.)</th>
<th>n</th>
<th>0–0.5 h</th>
<th>0.5–8 h</th>
<th>Incidence (%)</th>
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<td>8</td>
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<td>a, b</td>
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</tr>
<tr>
<td></td>
<td>30</td>
<td>6</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4</td>
<td>c, c</td>
<td>c, d</td>
<td>50</td>
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<td>5</td>
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<td></td>
<td>10</td>
<td>5</td>
<td>b, h, j, k</td>
<td>b, i, j</td>
<td>80</td>
</tr>
<tr>
<td>RP73401</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td>0</td>
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<td>10</td>
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<td>p, q, r</td>
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<tr>
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<td>6</td>
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<td>r, s, t</td>
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<td>2</td>
<td>r, s, t, u, v</td>
<td>w, x</td>
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<tr>
<td>CDP840</td>
<td>10</td>
<td>4</td>
<td></td>
<td></td>
<td>0</td>
</tr>
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<td></td>
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**Fig. 2.** Effects of YM976, rolipram, CDP-840, and prednisolone on plasma TNF-α elevation induced by LPS in BCG-pretreated mice. Mice were pretreated with BCG, and 14 days later LPS was intravenously injected. One hour after the LPS, plasma TNF-α levels were measured with an ELISA kit. Compounds were orally administered 30 min before the LPS, except for prednisolone, which was orally administered 2 h before the LPS. ***p < 0.001 for the difference between the saline and control groups (Student’s t test). ****p < 0.001 for difference between the control group and each treatment group (Dunnett’s multiple range test).
in comparison with the vehicle control group \( (p > 0.09; \text{Fig. 5}) \). On the other hand, rolipram significantly elevated brain cAMP levels at 10 mg/kg p.o. \( (p < 0.001) \). The difference between the effect of YM976 and that of rolipram was also statistically significant \( (p < 0.01) \).

**Discussion**

Asthma is a chronic inflammatory disease of the airways, whose main features are nonspecific airway hyperreactivity, inflammatory cell infiltration, and airway edema. Eosinophils are predominant among the inflammatory cells infiltrating into airways and play a critical role in the pathogenesis of asthma. Thus, one therapeutic strategy would be to target the mechanisms involved in the accumulation and activation of eosinophils in the airways. PDE4 is contained in eosinophils, whose inhibition is associated with an increase in intracellular cAMP levels and suppression of eosinophil functions (Giembycz and Dent, 1992). Based on the putative role of cAMP in inflammatory cells, PDE4 has been identified as a molecular target for novel antiasthmatic agents (Barrett, 1999). Clinical utility of the first-generation inhibitors, such as rolipram, is limited by the associated nausea and vomiting following administration to human subjects (Bertolino et al., 1988; Palfreyman and Souness, 1996). Consequently, much effort has been made in the development of a new generation of PDE4 inhibitors that retain the therapeutic activity, but have an improved therapeutic ratio (Souness and Rao, 1997).

YM976 is a novel PDE4 inhibitor, whose structure is totally different from those of existing compounds, in terms of its lack of the 3-cyclopentyloxy-4-methoxyphenyl group, which is shared by rolipram, RP73401, and CDP840. YM976 is a novel PDE4 inhibitor, whose structure is totally different from those of existing compounds, in terms of its lack of the 3-cyclopentyloxy-4-methoxyphenyl group, which is shared by rolipram, RP73401, and CDP840 (Fig. 1). Our previous study demonstrated that YM976 significantly inhibited antigen-induced asthmatic responses in guinea pigs (Aoki et al., 2001). In the present study, the in vitro and in vivo effects of YM976 and rolipram were first evaluated. In vitro experiments indicated that YM976 and rolipram suppressed LTC4/D4/E4 release from human eosinophils, and in vivo experiments demonstrated that these inhibitors also inhibited the elevation of plasma TNF-\( \alpha \) level with ED50 values of 2.8 and 3.5 mg/kg p.o., respectively. Concerning the emetic effects, MNEDs of YM976 and rolipram were 10 and 1 mg/kg p.o., respectively. These results suggested that YM976 showed dissociation between its anti-inflammatory activity and emetogenicity compared with rolipram. Although the precise mechanism remains to be determined, at least two hypotheses for this reduced emetogenicity can be considered.

**Table 3**

<table>
<thead>
<tr>
<th>IC50</th>
<th>YM976</th>
<th>Rolipram</th>
<th>RP73401</th>
<th>CDP840</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Rolipram</td>
<td>2.6 ± 0.18</td>
<td>1.2 ± 0.092</td>
<td>0.40 ± 0.081</td>
<td>15 ± 1.4</td>
</tr>
<tr>
<td>PDE4A</td>
<td>3.5 ± 0.48</td>
<td>690 ± 84</td>
<td>0.90 ± 0.11</td>
<td>27 ± 3.9</td>
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<td>PDE4B</td>
<td>1.0 ± 0.065</td>
<td>270 ± 67</td>
<td>0.32 ± 0.079</td>
<td>10 ± 3.6</td>
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<tr>
<td>PDE4C</td>
<td>13 ± 1.9</td>
<td>1900 ± 150</td>
<td>4.8 ± 0.57</td>
<td>63 ± 19</td>
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<td>PDE4D</td>
<td>1.7 ± 0.46</td>
<td>260 ± 32</td>
<td>0.42 ± 0.062</td>
<td>14 ± 3.6</td>
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**Fig. 3.** Effects of YM976 and rolipram on reserpine-induced hypothermia in mice. Each compound was orally administered 18 h after the injection of reserpine. Top, changes in rectal temperature at 0.5, 1, 1.5, 2, and 2.5 h after administration. The *pre* value was measured before the administration. Data are expressed as the mean ± S.E. of five animals. Control group animals were administered 0.5% MC (A), and the saline group was injected with saline in place of reserpine (B). Doses of YM976 (A) were 3 ( ), 10 ( ■ ), and 30 ( ○ ) mg/kg p.o., and those of rolipram (B) were 0.3 ( □ ), 1 ( ▲ ), and 3 ( ○ ) mg/kg p.o. Bottom, areas under the curve between pre (0) and 2.5 h (AUC0 _– _2.5). Data are expressed as the mean ± S.E. of AUC0 _– _2.5 of five animals. *p < 0.05; **p < 0.01; and ***p < 0.001, for differences between the control group and each treatment group (Dunnett’s multiple range test).
namely, PDE4 subtype selectivity and binding for protein conformation with high affinity for rolipram.

It has become apparent that PDE4 is not just one enzyme but comprises a group of enzymes (PDE4A, B, C, and D), which are differently regulated and expressed in different cells (Muller et al., 1996). Recently, PDE4D expression was shown to be increased following short-term cAMP stimulation (Alvarez et al., 1995), while the suppressive effects of T cell proliferation and TNF-α production were shown to be regulated with PDE4A/B inhibition (Manning et al., 1999), demonstrating the correlation between cell function and subtype. In addition, several compounds were reported that displayed some specificity for the individual subtype(s): CDP840, a PDE4A-selective inhibitor (Hughes et al., 1996); a series of PDE4D selective inhibitors (Hersperger et al., 2000); and dual PDE4A/B selective inhibitors (Manning et al., 1999). Although SB207499, which showed therapeutic potency without emesis (Torphy et al., 1999), also demonstrated 10-fold selectivity for PDE4D (Torphy et al., 1997), there is, to our knowledge, no direct evidence showing a relationship between subtype and emetogenicity. In the present study, we evaluated the inhibitory effects against each PDE4 subtype to elucidate the relationship between subtype selectivity and emetogenicity. As shown in Table 3, YM976, rolipram, RP73401, and CDP840 showed inhibition of PDE4 subtypes, which were closely similar to those of the previous studies (Hughes et al., 1996), and all compounds failed to show a distinct preference for the PDE4 subtype. Indeed, these inhibitors, including YM976, had weaker inhibitory activities against PDE4C than against the other subtypes. Some inhibitors were reported to be less potent against this subtype (Muller et al., 1996), while the reasons for the weaker inhibition on PDE4C remained unclear. Although each PDE4 subtype additionally generates multiple isoforms (Houslay et al., 1998), obvious differences in subtype selectivity between YM976 and the other compounds were not found in our experiments. These results indicated that the predominant dissociation of YM976 might not be explained by the PDE4 subtype selectivity.

A family of PDE4 enzymes is known to exist two different conformational states, which can be distinguished by their different affinity for rolipram (Souness et al., 1997). The protein conformation with low affinity for rolipram is called LAR-conformation, and the conformation with high affinity is called HAR-conformation. Recent pharmacological studies suggest that the interaction of PDE4 inhibitors with LAR-conformation may relate to the anti-inflammatory effects (Barnette et al., 1996; Souness et al., 1996). On the other hand, the association with HAR-conformation strongly correlates with emesis, but not with anti-inflammatory effects (Duplantier et al., 1996). We then measured the binding activities for HAR-conformation of YM976 and the other PDE4 inhibitors, and compared them with their MNEDs. Rolipram showed displacement of [3H]rolipram (IC50 = 1.2 nM), which could be supported by the previous studies (Souness et al., 1996). YM976 exhibited the displacement of [3H]rolipram with an IC50 value of 2.6 nM, which was almost identical to that of rolipram. The MNEDs of YM976 and rolipram in ferret emetogenicity were 10 and 1 mg/kg, respectively, and the in vivo inhibitory activities of YM976 on inflammatory responses, such as TNF-α production (Table 1) and carrageenan-induced pleurisy (Aoki et al., 2000b), were closely similar to those of rolipram. These results suggest that the affinity for HAR-conformation may not be related to the weak emetogenicity of YM976.

Rolipram has been developed as an antidepressant drug acting on the CNS (Horowski and Sastre-y-Hernandez, 1985) and is likely distributed in the brain. Heaslip and Evans (1995) reported that the emetogenicity of PDE4 inhibitors might be induced by their CNS effects, and the CNS effects of YM976 and rolipram were therefore evaluated. In an earlier study, rolipram had been shown to reverse the reserpine-induced hypothermia by an action beyond postsynaptic monoamine receptors (Wachtel and Schneider, 1986). We therefore examined CNS effects using this model. Both compounds reversed the hypothermia, with MEDs of YM976 and rolipram of 30 and 1 mg/kg p.o., respectively (Fig. 3). The MEDs of both compounds were apparently dissociated, sug-
gesting that YM976 has little effects on the CNS. We interpreted these results to mean that YM976 had a poor brain penetration compared with rolipram.

We next measured cAMP content of peripheral tissue and the brain using the same protocol. Peritoneal macrophages were used as the peripheral tissue, and the anti-inflammatory activity was evaluated simultaneously. YM976 and rolipram dose dependently and significantly inhibited TNF-α production in the peritoneal cavity, with ED₅₀ values of 3.2 and 5.4 mg/kg p.o., respectively, when the compounds were administered 30 min before the elicitation. At that time, both compounds significantly elevated intracellular cAMP contents in the peritoneal leukocytes at an oral dose of 10 mg/kg. These results suggested that YM976 and rolipram showed the same levels of anti-inflammatory effect and PDE4 inhibitory activity. In a separate experiment using thioglycollate-treated mice, cAMP content of the whole brain was measured. The dose was set at 10 mg/kg, where the evident anti-inflammatory effects of both compounds were noted.

Additionally, the measurement time point was set at 30 min after administration, on the basis that YM976 and rolipram showed emesis within 30 min at higher doses, suggesting that these compounds were sufficiently absorbed in the gastrointestinal tract and transferred to the blood by this time. The pharmacokinetic data of YM976 also demonstrated that plasma level peaked at 15 to 60 min (unpublished data). Thirty minutes after administration, YM976 failed to induce significant elevation of brain cAMP, while rolipram significantly increased the brain cAMP content. Thus, YM976 showed little inhibition on brain PDE4 activities at doses showing anti-inflammatory activity, suggesting that YM976 has poor brain penetration compared with rolipram. The present study did not allow a definitive conclusion that the low emetogenicity of YM976 was derived from its poor brain penetration, because we have no direct evidence to indicate a relationship between brain cAMP content and emetogenicity. If the hypothesis that the emetogenicity of PDE4 inhibitors may contribute to their CNS effects is confirmed, PDE4 inhibitors with poor brain penetration probably have little emetogenic activity.

The anti-inflammatory activities of YM976 are apparently more dissociated from its emetogenicity than other PDE4 inhibitors such as rolipram. Neither affinity for HAR-conformation nor selectivity for PDE4 subtypes may explain the observed beneficial effect on emesis of YM976. Poor brain penetration could be more closely related to low emetogenicity. In conclusion, YM976 is a PDE4 inhibitor with little emetogenicity and is expected to become a useful novel therapeutic agent for inflammatory diseases such as bronchial asthma.

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