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Originals

Involvement of K^+ Channels and Na^+, K^+-ATPase in Relaxant Actions of Selective Phosphodiesterase 3 Inhibitors on Airway and Vascular Smooth Muscles Isolated from Guinea-pigs

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SUMMARY

Milrinone or olprinone, a selective phosphodiesterase (PDE) 3 inhibitor, has relaxant actions on smooth muscles in addition to positive inotropic actions. The exact mechanism on vasodilating and bronchodilating actions of milrinone or olprinone has not been elucidated. In the present experiments, relaxant responses to PDE3 inhibitors were examined on the precontracted airway or pulmonary artery smooth muscle preparations to clarify their mechanism. Both milrinone and olprinone relaxed the airway smooth muscle preparation or the pulmonary artery preparation isolated from guinea-pigs in a concentration-dependent manner. In the airway smooth muscle, these relaxations were markedly blocked by iberiotoxin (a blocker of large conductance Ca^{2+}-activated K^+ channels). On the other hand, in the main pulmonary artery, the milrinone- and olprinone-induced relaxations were significantly blocked by iberiotoxin, and were more strongly blocked by ouabain (an inhibitor of Na^+, K^+-ATPase). In the right/left (R/L) pulmonary artery, ouabain also strongly blocked relaxant responses to milrinone and olprinone, but iberiotoxin did not modify these relaxations. Similar observations were seen on the bucladesine (a cyclic AMP mimic agent)-induced relaxation. In conclusion, milrinone and olprinone cause concentration-dependent relaxations of the isolated airway and pulmonary artery smooth muscles via an increase in intracellular cyclic AMP (cAMP). In the airway smooth muscle, large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels seem to play a crucial role for these relaxations. Relaxations of the main pulmonary artery induced by milrinone and olprinone are mediated predominantly by activation of Na^+, K^+-ATPase, and partly through BK_{Ca} channels. In the R/L pulmonary artery, vasorelaxant effects of milrinone and olprinone are more likely mediated by activation of Na^+, K^+-ATPase, but not BK_{Ca} channels.

Key Words : milrinone, olprinone, K^+ channel, Na^+, K^+-ATPase, smooth muscle

INTRODUCTION

It is well known that methylxanthines improve force expiratory volume in one second ($FEV_{1.0}$) in patients with chronic obstructive pulmonary disease[1] or asthma[2] . Methylxanthines also decrease mean pulmonary

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arterial pressure, implying a relaxation both of airway and vascular smooth muscle[3,4]. Although various mechanisms have been proposed for these actions, inhibition of cyclic nucleotide phosphodiesterase (PDE) is most widely accepted. PDE plays a role in modulating intracellular cyclic nucleotide levels. The existence of multiple isoenzymes of PDE in many tissues has been demonstrated[5,6]. Since PDE3 type, one of isoenzymes, is abundant in cardiac muscle and smooth muscle[5], selective PDE3 inhibitor is expected as a cardiac inotropic agent and a relaxant of smooth muscle[5~7].

In the isolated airway smooth muscle, inhibition of PDE3 reverses spasms induced by several agents including carbachol[8], methacholine[9,10], histamine[10], prostaglandin $F_{2\alpha}$[11] and leukotriene C_4[12]. Clinically, it has been reported that intravenous olprinone, a selective PDE3 inhibitor, produces a great increase in the $FEV_{1.0}$ than aminophylline in asthmatic patients[13]. Inhaled olprinone also produces bronchodilation without marked adverse effects[14]. Jones et al[15] have presented evidence that charybdotoxin-sensitive K^+ channels are involved in mediating relaxation of guinea-pig trachea induced by aminophylline (a non-selective PDE inhibitor). However, the mechanism on the bronchodilating action of selective PDE3 inhibitors has not yet been clear. Selective PDE3 inhibitors have been recognized as effective agents for the treatment of congestive heart failure[16]. PDE3 inhibitors elevate the intracellular cyclic AMP (cAMP) level which mediates positive inotropic actions[17,18]. PDE3 inhibitors are also known to possess vasodilator activity in rat[19] and human[20] pulmonary arteries. Olprinone improves right ventricular function in hypoxic pulmonary hypertension animals with pulmonary vasodilating effect[21]. In a porcine model of acute pulmonary hypertension[22], milrinone, another selective PDE3 inhibitor, decreases pulmonary vascular resistance and improves right ventricular contractility. Inhalation of milrinone produces a significant reduction of mean pulmonary arterial pressure and pulmonary vascular resistance in heart transplant candidates with pulmonary hypertension[23]. However, there is little information available on mechanisms of PDE3 inhibitor actions in the isolated pulmonary artery.

The aim of the present study is to clarify underlying mechanisms in relaxant effects of selective PDE3 inhibitors on airway and vascular smooth muscles isolated from the guinea-pig lungs.

METHODS

Animals

This study was approved by the Animal Ethics Committee of Dokkyo Medical University School of Medicine. Adult male guinea-pigs (Hartley strain ; 350 −500 g in weight) were used in the present study. Animals were housed under conditions of constant temperature and controlled illumination. Food and water were available *ad libitum*. Guinea-pigs were anesthetized with enflurane (Dai-Nippon Pharma. Co. Osaka, Japan) and were exsanguinated via the carotid artery. The thoracic cavity was opened, and then the tracheo-bronchial tree and lungs with pulmonary artery were rapidly removed. The tracheal, bronchial and pulmonary arterial ring preparations were prepared.

Preparations

The tracheo-bronchial tree was placed in an oxygenated modified Krebs solution with the following composition (mmol/l) : NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 14 and ascorbic acid 0.12. After removal of adhering tissue, two tracheal rings (length, 2.0−2.5 mm) and two bronchial rings (length, 1.5−2.0 mm) were prepared from each animal, according to our previous report[24]. The airway epithelium was removed from all test preparations by gently rubbing their mucosal surface with a fine stainless wire.

Lungs with pulmonary artery were rapidly excised and trimmed free of adherent connective tissue in an oxygenated modified Krebs solution. Two main pulmonary arterial rings (length, 1.5−2.0 mm) and right and/or left (R/L) pulmonary arterial rings (length, 1.0 −1.5 mm) were prepared from each animal[25]. All arterial ring preparations were denuded of endothelium by gently rubbing the internal surface with a fine stainless wire.

Organ Bath Experiments

Each ring preparation was suspended under 0.5 g load in a 5 ml organ bath containing modified Krebs solution which was bubbled with 95 % O_2 and 5 % CO_2

throughout the experiment. The temperature of the solution was maintained at 37℃. The mechanical activity of each ring preparation was recorded on a polygraph (RJG-4124, Nihon Kohden, Tokyo, Japan) with an isometric transducer (TB-651T, Nihon Kohden). Each ring preparation was equilibrated for 60 min and was washed with a fresh Krebs solution every 15 min. At the end of equilibration, the preparation was contracted with a single concentration of carbachol (1 μmol/l for tracheal and bronchial ring preparations) or phenylephrine (10 μmol/l for pulmonary arterial ring preparations). After washout and equilibration for a further 40 min, the experiment was started. In experiments on the responsiveness to PDE inhibitors (aminophylline, milrinone and olprinone) or bucladesine (dibutyryl cAMP), the ring preparation was precontracted with carbachol (1 μmol/l for airway ring preparations) or phenylephrine (10 μmol/l for pulmonary arterial ring preparations). The carbachol (1 μmol/l)-induced or the phenylephrine (10 μmol/l)-induced sustained contraction was approximately 70−90% of the maximum response to 30 μmol/l carbachol or 100 μmol/l phenylephrine, respectively. When the carbachol (1 μmol/l)- or phenylephrine (10 μmol/l)-induced sustained contraction had reached its plateau, each PDE inhibitor or bucladesine was added cumulatively. In some experiments on the responsiveness to PDE inhibitors or bucladesine, the airway or arterial ring preparation was precontracted with iso-osmotic high K^+ (60 mmol/l). Iso-osmotic high K^+ solution was made by displacing equimolar NaCl in Krebs solution with KCl. Responses to PDE inhibitors or bucladesine were expressed as a percentage of the precontraction plateau level of the preparation. Only one concentration-response curve for PDE inhibitors or bucladesine was generated in each ring preparation. To examine the mechanism of relaxant action of each PDE inhibitor, the ring preparation was pretreated with a large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel blocker iberiotoxin (0.1 μmol/l), a small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channel blocker apamin (0.1 μmol/l), a voltage-dependent K^+ (Kv) channel blocker α-dendrotoxin (0.1 μmol/l), an ATP-senitive K^+ (K_{ATP}) channel blocker glibenclamide (1 μmol/l), or a Na^+, K^+-ATPase inhibitor ouabain (1 μmol/l) for 30 min prior to the addition of PDE inhibitor.

Statistical analysis

Data are expressed as the mean ± SEM of n independent observations. Emax (the maximal relaxation induced by each relaxant), and pIC_{50} (-log molar IC_{50}) were calculated from the individual concentration-response curve. The IC_{50} was determined as the molar concentration of each relaxant required to relax the ring preparation by 50% of the precontraction plateau level. Statistical analysis was conducted by the Mann-Whitney U-test. P values less than 0.05 were considered to be statistically significant.

Drugs

The following drugs were used : aminophylline, olprinone hydrochloride (kindly donated by Eisai Co., Tokyo, Japan), milrinone (kindly donated by Astellas Pharma Inc., Tokyo, Japan), carbamylcholine chloride (carbachol), L-phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), iberitoxin, apamin (PEPTIDE Institute Inc., Osaka, Japan), α-dendrotoxin (LATOXAN, Valence, France), ouabain (g-strophanthin Krist., Merck, Darmstadt, Germany), glibenclamide (Wako Pure Chemical Industries Ltd., Osaka, Japan), bucladesine sodium (sodium N^6, 2'-O-dibutyryl adenosine 3', 5'-cyclic phosphate, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). Aminophylline, carbachol, phenylephrine and ouabain were dissolved in and diluted with 0.9% saline. Olprinone, iberiotoxin, apamin, α-dendrotoxin and bucladesine were dissolved in and diluted with distilled water. Milrinone (50 mg) was dissolved in distilled water (10 ml) containing 380 mg of glucose and 0.07 ml of lactic acid, and then was diluted with 0.9% saline. Glibenclaminde was dissolved in dimethyl sulfoxide (DMSO) and diluted with Krebs solution. The final concentration of DMSO in organ bath was 0.005%. The solvent at the concentration used in the present experiments did not affect responsiveness of preparations by themselves. All concentrations are expressed as final bath concentration.

RESULTS

Responsiveness to PDE inhibitors

All of aminophylline (1−300 μmol/l) , milrinone (1−300 μmol/l) and olprinone (1−300 μmol/l) caused concentration-dependent relaxations of the tracheal and bronchial preparations precontracted with carba-

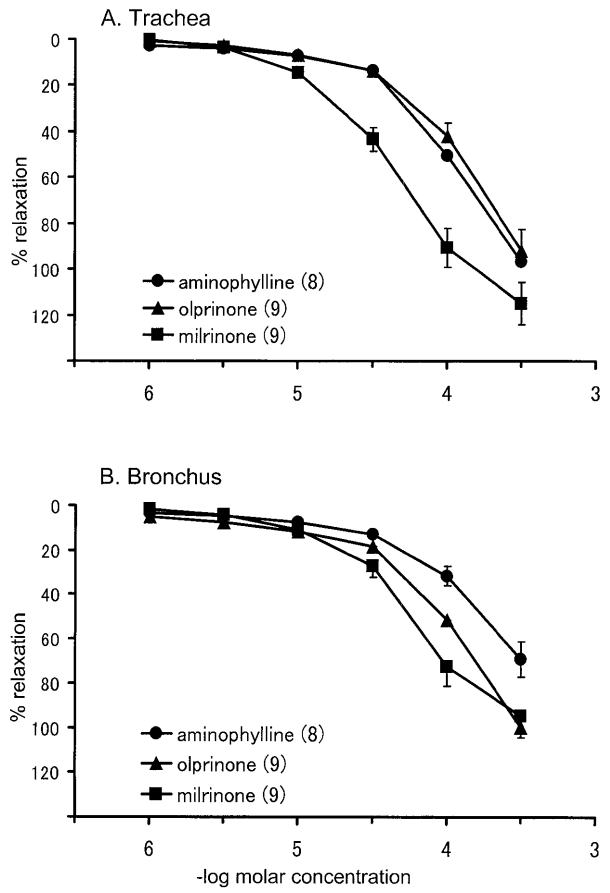

Fig. 1 Cumulative concentration-response curves for relaxant effects of PDE inhibitors on the carbachol (1 μmol/l)-contracted tracheal (A) and bronchial (B) ring preparations. The ordinate scales show the percent relaxation of the carbachol-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis.

chol (1 μmol/l) (Fig. 1). These relaxations were slow in onset (30 sec − 1 min) and reached the maximal relaxation at 4 − 10 min after the drug application. The rank order of the efficacy (Emax) was milrinone (114.7 ± 9.2 %) > aminophylline (96.5 ± 10.6 %) = olprinone (92.0 ± 9.4 %) in the tracheal preparation (Fig. 1A), and was olprinone (99.9 ± 4.3 %) = milrinone (94.6 ± 7.8 %) > aminophylline (69.2 ± 7.8 %) in the bronchial preparation (Fig. 1B). The potency (pIC_{50}) of these relaxant responses to PDE inhibitors for the tracheal and bronchial preparations is shown in Table 1. The rank order of the potency (pIC_{50}) was milrinone (4.44 ± 0.07) > aminophylline (4.00 ± 0.07) = olprinone (3.90 ± 0.05) in the tracheal preparation, and was milrinone

Table 1 pIC_{50} values of relaxants on the carbachol (1 μmol/l)-contracted guinea-pig airway smooth muscles

	Trachea	Bronchus
	pIC_{50}	pIC_{50}
Aminophylline	4.00 ± 0.07	3.85 ± 0.02
Olprinone	3.90 ± 0.05	4.03 ± 0.04**
Milrinone	4.44 ± 0.07##, +++	4.30 ± 0.04##, ++
Bucladesine	3.56 ± 0.10	3.56 ± 0.07

Each value represents mean ± SEM. pIC_{50} values were expressed as -log molar concentration of each relaxant required to relax the carbachol (1 μmol/l)-induced tone by 50 %.

*Aminophylline vs Olprinone, #Aminophylline vs Milrinone, +Olprinone vs Milrinone, **, ##, ++ $P < 0.01$, +++ $P < 0.001$.

Table 2 pIC_{50} values of relaxants on the phenylephrine (10 μmol/l)-contracted guinea-pig pulmonary artery

	Main Pulmonary Artery	R/L Pulmonary Artery
	pIC_{50}	pIC_{50}
Aminophylline	3.90 ± 0.08	3.78 ± 0.08
Olprinone	4.32 ± 0.19*	3.68 ± 0.07
Milrinone	4.78 ± 0.10##	4.66 ± 0.16#, ++
Bucladesine	3.04 ± 0.08	3.13 ± 0.05

Each value represents mean ± SEM. pIC_{50} values were expressed as -log molar concentration of each relaxant required to relax the phenylephrin (10 μmol/l)-induced tone by 50 %.

*Aminophylline vs Olprinone, #Aminophylline vs Milrinone, +Olprinone vs Milrinone, *, # $P < 0.05$, ##, ++ $P < 0.01$.

(4.30 ± 0.04) > olprinone (4.03 ± 0.04) > aminophylline (3.85 ± 0.02) in the bronchial preparation.

Aminophlline (1 − 300 μmol/l) and milrinone (0.1 − 300 μmol/l) relaxed the pulmonary artery preparation precontracted with phenylephrine (10 μmol/l) in a concentration-dependent manner. The concentration-response curve to aminophylline or milrinone was mono-phasic and parallel (Fig. 2). Olprinone (0.1 − 300 μmol/l) also caused a concentration-dependent relaxation of the pulmonary artery preparation, but the curve was biphasic (Fig. 2). The relaxant responses to

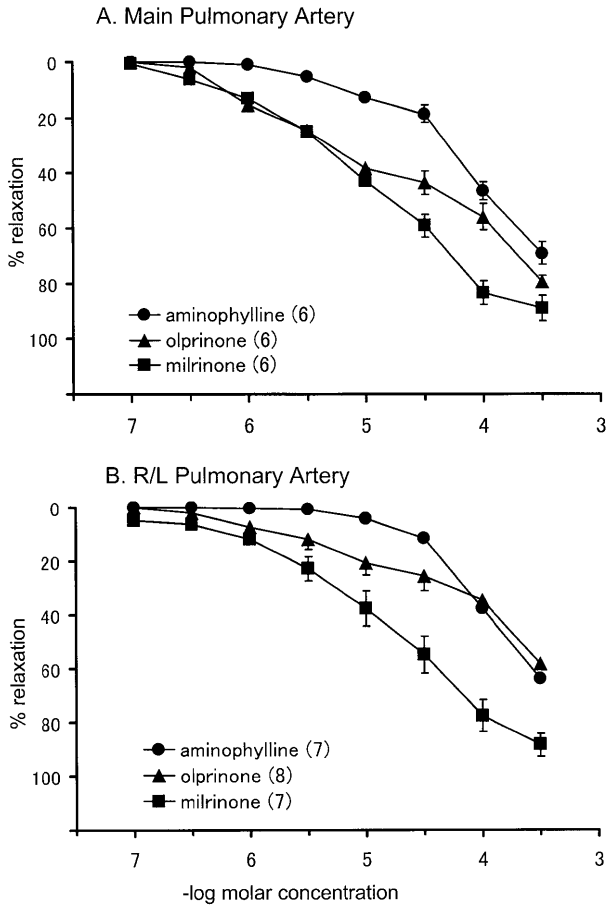

Fig. 2 Cumulative concentration-response curves for relaxant effects of PDE inhibitors on the phenylephrine (10 μmol/l)-contracted main (A) and R/L (B) pulmonary arterial ring preparations. The ordinate scales show the percent relaxation of the phenylephrine-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis.

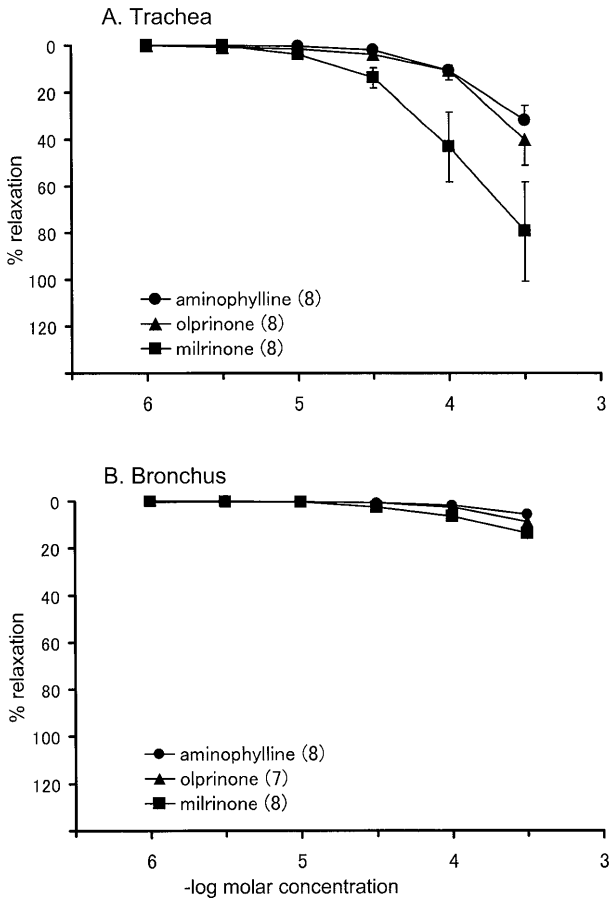

Fig. 3 Cumulative concentration-response curves for relaxant effects of PDE inhibitors on the high K^+ (60 mmol/l)-contracted tracheal (A) and bronchial (B) ring preparations. The ordinate scales show the percent relaxation of the high K^+-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis.

PDE3 inhibitors were slightly rapid in onset (about 30 sec) and reached the maximal relaxation after 2－6 min. The order of Emax was milrinone (88.8 ± 4.6 %) >olprinone (79.2 ± 2.2 %) >aminophylline (69.1 ± 4.0 %) in the main pulmonary artery (Fig. 2A), and was milrinone (88.4 ± 4.3 %) >aminophylline (63.9 ± 7.5 %) = olprinone (58.4 ± 5.9 %) in the R/L pulmonary artery (Fig. 2B). pIC_{50} values of these relaxant responses of the pulmonary artery preparations are shown in Table 2. The order of pIC_{50} was milrinone (4.78 ± 0.10) = olprinone (4.32 ± 0.19) >aminophylline (3.90 ± 0.08) in the main pulmonary artery, and was milrinone (4.66 ± 0.16) >aminophylline (3.78 ± 0.08) = olprinone (3.68 ± 0.07) in the R/L pulmonary artery.

Effects of PDE inhibitors on high K^+-contracted preparations

The tracheal and bronchial preparations exposed to high K^+ (60 mmol/l) produced a sustained contraction. Aminophylline (30－300 μmol/l), milrinone (10－300 μmol/l) and olprinone (30－300 μmol/l) caused concentration-dependent relaxations in the tracheal preparation precontracted with high K^+ (Fig. 3A). However, the concentration of each PDE inhibitor required to produce the equipotent relaxation was markedly higher than that effective on the carbachol (1 μmol/l)-induced sustained contraction, and the maximal relaxation was about 30－80 % of the high K^+-induced contraction (Figs. 1A, 3A). In the bronchial prepara-

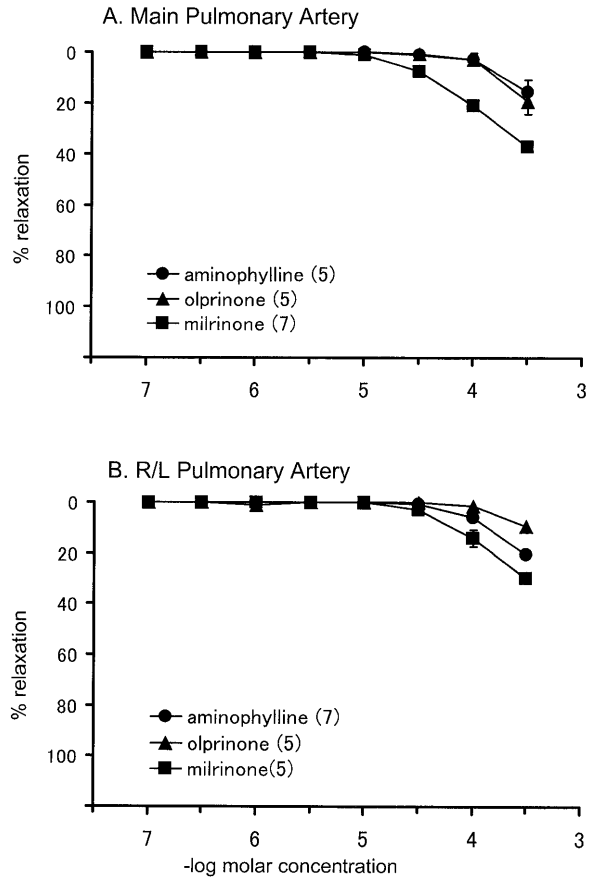

Fig. 4 Cumulative concentration-response curves for relaxant effects of PDE inhibitors on the high K^+ (60 mmol/l)-contracted main (A) and R/L (B) pulmonary arterial ring preparations. The ordinate scales show the percent relaxation of the high K^+-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis.

tion precontracted with high K^+, all PDE inhibitors tested here were almost inactive (Fig. 3B).

The main and R/L pulmonary artery preparations exposed to high K^+ (60 mmol/l) developed a sustained tension. Concentration-dependent relaxations induced by PDE inhibitors were also observed in these pulmonary artery preparations, but their efficacies were lower than the relaxation on the phenylephrine (10 μmol/l)-induced contraction (Figs. 2, 4). The maximal relaxations produced by these PDE inhibitors reached only 10－40% of the high K^+ (60 mmol/l)-induced tone (Fig. 4).

Influence of K^+ channel blockers on relaxation of airway smooth muscle

The relaxant responses to three PDE inhibitors of the tracheal preparation were markedly blocked by the pretreatment with iberiotoxin (0.1 μmol/l), and the extent of the maximal relaxation to aminophylline, milrinone or olprinone was reduced from the control response to 24.9 ± 4.2%, 21.9 ± 3.2% or 38.0 ± 13.0%, respectively (Fig. 5). The iberiotoxin-resistant relaxations elicited by PDE inhibitors were not blocked by additional treatment with other K^+ channel blocker (apamin, α-dendrotoxin or glibenclamide) (data not shown).

In the bronchial preparation, iberitoxin also significantly blocked the PDE inhibitor-induced relaxation, and caused a decrease of the maximal relaxation for each PDE inhibitor. After iberiotoxin treatment, the maximal relaxation induced by aminophylline, milrinne or olprinone was reduced to 17.2 ± 4.7%, 30.1 ± 3.8% or 44.7 ± 15.0%, respectively (Fig. 6). In contrast to the tracheal preparation, the iberiotoxin-resistant relaxation elicited by milrinone was blocked by additional treatment with glibenclamide (Fig. 6B) and that elicited by olprinone was blocked by an addition of α-dendrotoxin (Fig. 6C). However, combined treatments with iberiotoxin and other K^+ channel blocker (glibenclamide, α-dendrotoxin or apamin) cause no more changes of the relaxation-response curve to aminophylline (data not shown).

Influence of K^+ channel blockers on relaxation of pulmonary artery

In the main pulmonary artery preparation pretreated with iberiotoxin, the PDE inhibitor-induced maximal relaxation was reduced to approximately 40－60% (Fig. 7). After ouabain treatment, the PDE inhibitor-induced maximal relaxation was also reduced to approximately 30－40% (Fig. 7). Ouabain was more effective than iberiotoxin. Ouabain (1 μmol/l) itself very slightly increased the basal muscle tension by 1.9 ± 1.1% of the phenylephrine (10 μmol/l)-induced tone, but there were no significant differences in muscle force in response to phenylephrine (10 μmol/l) with and without ouabain.

In the R/L pulmonary artery preparation, the aminophylline-induced maximal relaxation was slightly

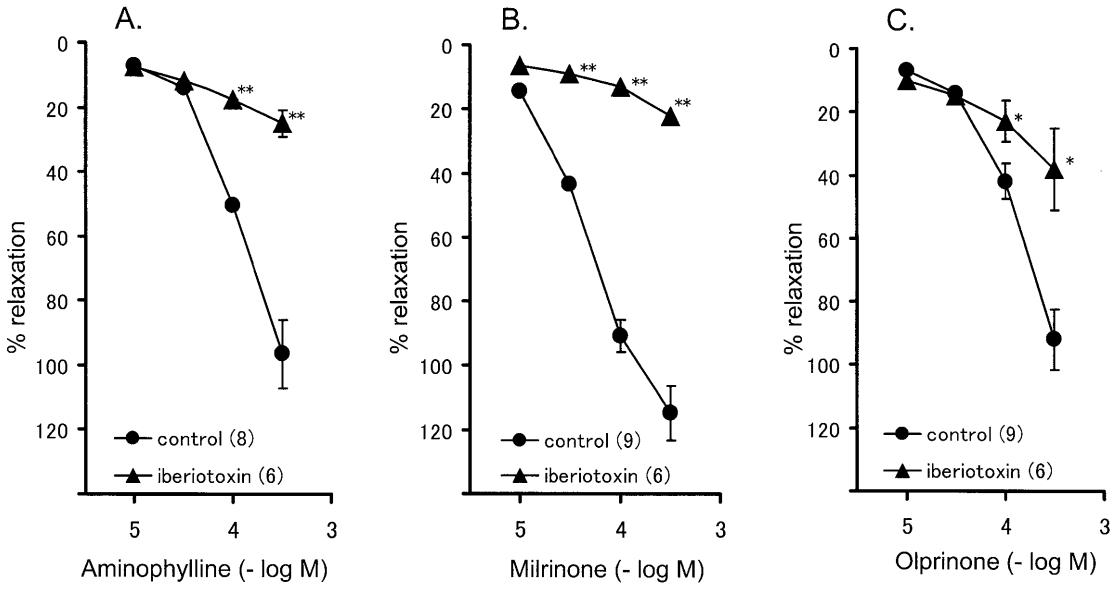

Fig. 5 Cumulative concentration-response curves for relaxant effects of aminophylline (A), milrinone (B) and olprinone (C) on the carbachol (1 μmol/l)-contracted tracheal ring preparations. ●, control responses ; ▲, responses after iberiotoxin (0.1 μmol/l). The ordinate scales show the percent relaxation of the carbachol-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis. * $P < 0.05$, ** $P < 0.01$, significant differences from control response.

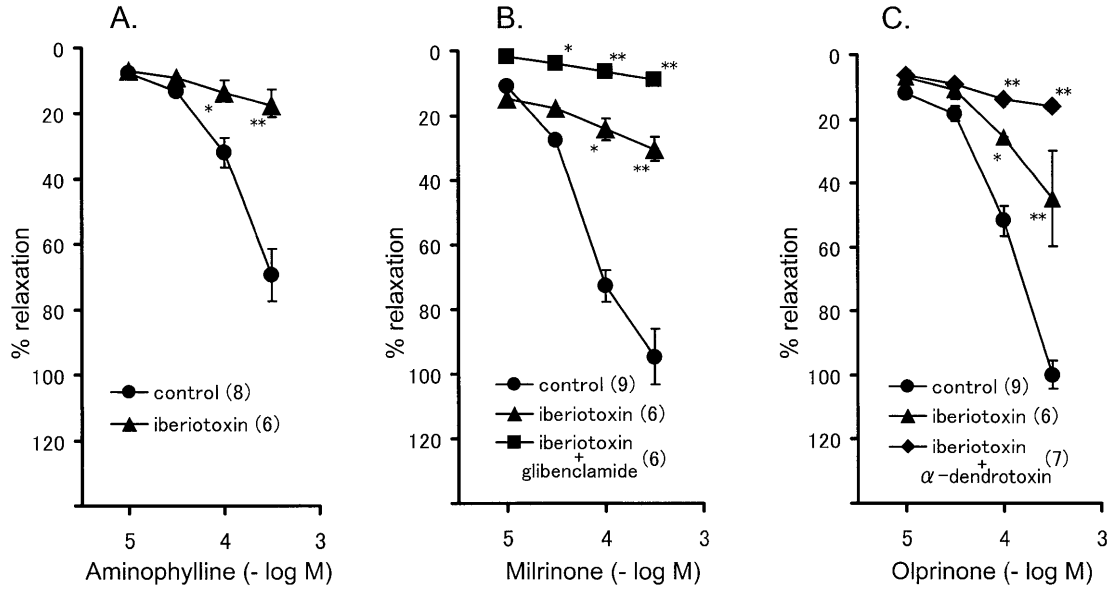

Fig. 6 Cumulative concentration-response curves for relaxant effects of aminophylline (A), milrinone (B) and olprinone (C) on the carbachol (1 μmol/l)-contracted bronchial ring preparations. ●, control responses ; ▲, responses after iberiotoxin (0.1 μmol/l) ; ■, responses after iberiotoxin (0.1 μmol/l) and glibenclamide (1 μmol/l) ; ◆, responses after iberiotoxin (0.1 μmol/l) and α-dendrotoxin (0.1 μmol/l). The ordinate scales show the percent relaxation of the carbachol-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis. * $P < 0.05$, ** $P < 0.01$, significant differences from control response.

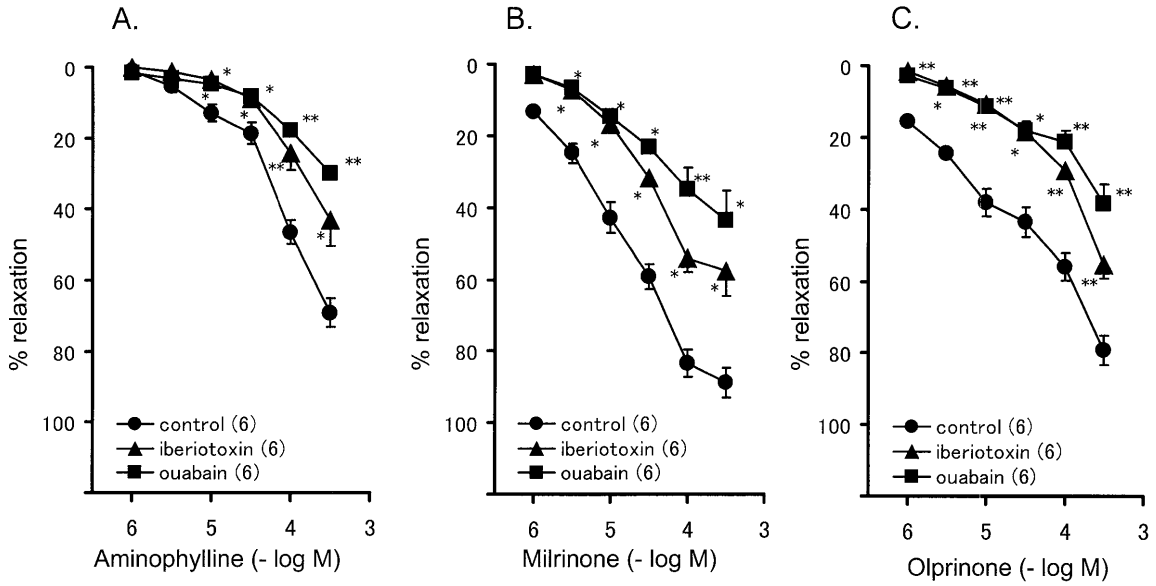

Fig. 7 Cumulative concentration-response curves for relaxant effects of aminophylline (A), milrinone (B) and olprinone (C) on the phenylephrine (10 μmol/l)-contracted main pulmonary arterial ring preparations. ●, control responses ; ▲, responses after iberiotoxin (0.1 μmol/l) ; ■, responses after ouabain (1 μmol/l). The ordinate scales show the percent relaxation of the phenylephrine-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences from control response.

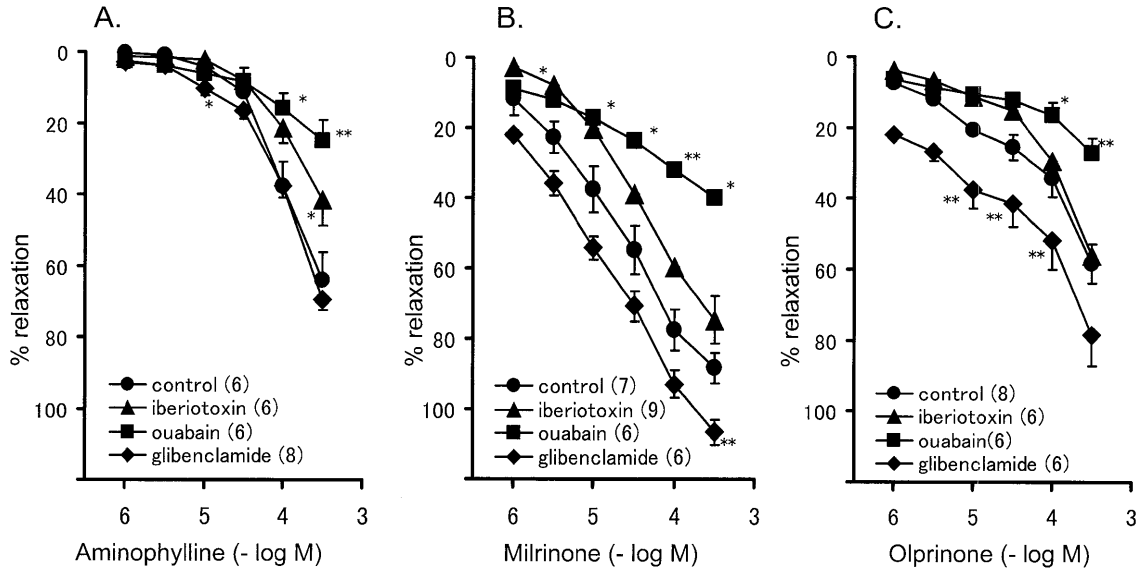

Fig. 8 Cumulative concentration-response curves for relaxant effects of aminophylline (A), milrinone (B) and olprinone (C) on the phenylephrine (10 μmol/l)-contracted R/L pulmonary arterial ring preparations. ●, control responses ; ▲, responses after iberiotoxin (0.1 μmol/l) ; ■, responses after ouabain (1 μmol/l) ; ◆, responses after glibenclamide (1 μmol/l). The ordinate scales show the percent relaxation of the phenylephrine-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences from control response.

blocked by iberiotoxin (Fig. 8A). Pretreatment with iberiotoxin showed a tendency to inhibit the relaxant response to milrinone or olprinone, but the difference was not statistically significant (Fig. 8B, C). In contrast, the aminophylline-, milrinone- or olprinone-induced maximal relaxation was reduced to 24.9 ± 5.7 %, 40.0 ± 5.7 % or 27.4 ± 5.0 %, respectively, in the ouabain pretreatment (Fig. 8). Other K^+ channel blockers, α-dendrotoxin and apamin, had no effect on the relaxant response to milrinone or olprinone in the R/L pulmonary artery preparation (data not shown). Glibenclamide slightly augmented the relaxant responses to three PDE inhibitors, but never reduced the responses to those inhibitors (Fig. 8).

Influence of K^+ channel blockers on the bucladesine-induced relaxation

Selective PDE3 inhibitors lead to an increase in intracellular cAMP concentrations of tracheal smooth muscle. Since bucladesine is well known as a cAMP mimic agent, the influence of various K^+ channel blockers on the bucladesine-induced relaxation was examined. Bucladesine (30 − 3000 μmol/l) caused a strong relaxation of the tracheal and bronchial preparations precontracted with carbachol (1 μmol/l) in a concentration-dependent manner (Fig. 9). The maximal relaxation produced by bucladesine reached over 100 % of the carbachol (1 μmol/l)-induced tone in airway preparations. Iberiotoxin pretreatment markedly blocked the bucladesine-induced relaxation of both the tracheal and bronchial preparations (Fig. 9). Combined treatments with iberiotoxin and other K^+-channel blocker (α-dendrotoxin, apamin or glibenclamide) no more caused significant changes of the relaxant response to bucladesine of the tracheal preparation (data not shown). In the bronchial preparation, however, the iberitoxin-resistant relaxation (about 20 %) was slightly but significantly blocked by the additive treatment with α-dendrotoxin (Fig. 9B) .

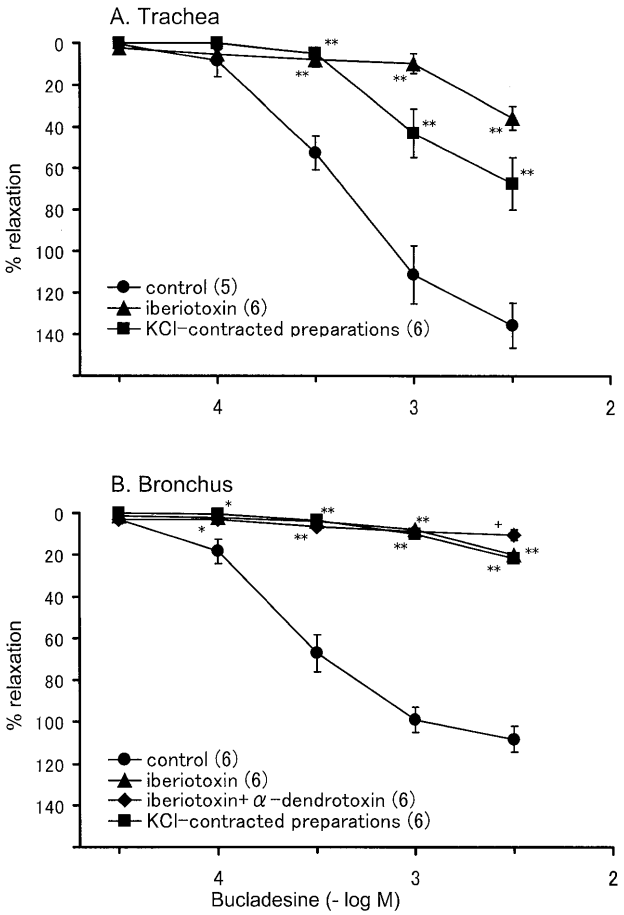

Fig. 9 Cumulative concentration-response curves for relaxant effects of bucladesine on the carbachol (1 μmol/l)- and high K^+ (60 mmol/l)-contracted airway ring preparations. A : trachea, B : bronchus. ●, control responses ; ▲, responses after iberiotoxin (0.1 μmol/l) ; ◆, responses after iberiotoxin (0.1 μmol/l) and α-dendrotoxin (0.1 μmol/l) ; ■, responses on the high K^+ (60 mmol/l)-contracted preparations. The ordinate scales show the percent relaxation of the carbachol- or high K^+-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis. $^*P < 0.05$, $^{**}P < 0.01$, significant differences from control response. $^+P < 0.05$, significant difference from response after iberiotoxin (0.1 μmol/l) .

Bucladesine, at concentrations higher than 100 μmol/l, also caused a concentration-dependent relaxation of the pulmonary artery preparations precontracted with phenylephrine (10 μmol/l). The Emax value for bucladesine was 88.2 ± 3.1 % in the main pulmonary artery and 74.4 ± 4.0 % in the R/L pulmonary artery (Fig. 10). Iberiotoxin slightly blocked the bucladesine-induced relaxation of the main pulmonary artery preparation, where the maximal relaxation was reduced to 68.4 ± 6.1 % after iberiotoxin treatment (Fig. 10A). In the R/L pulmonary artery preparation (Fig. 10B), iberiotoxin had no significant effects on the bucladesine-induced relaxation. In contrast to iberiotoxin, ouabain, a Na^+, K^+-ATPase inhibitor, strongly blocked the

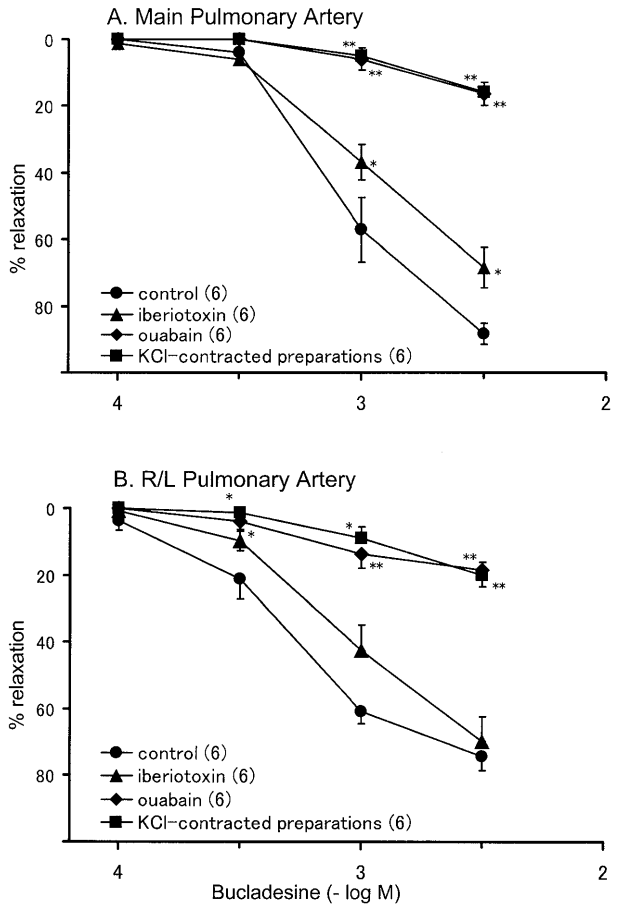

Fig. 10 Cumulative concentration-response curves for relaxant effects of bucladesine on the phenylephrine (10 μmol/l)- and high K^+ (60 mmol/l)-contracted arterial ring preparations. A : main pulmonary artery, B : right/left (R/L) pulmonary artery. ●, control responses ; ▲, responses after iberiotoxin (0.1 μmol/l) ; ◆, responses after ouabain (1 μmol/l) ; ■, responses on the high K^+ (60 mmol/l)-contracted preparations. The ordinate scales show the percent relaxation of the carbachol- or high K^+-induced tone. Each point represents mean ± SEM. The SEM lies within the symbol when it is small and not visible. Numbers of observations indicate in parenthesis. $^*P < 0.05$, $^{**}P < 0.01$, significant differences from control response.

relaxant response to bucladesine of the phenylephrine (10 μmol/l)-precontracted pulmonary artery preparations (Fig. 10A, B). In the presence of ouabain, the bucladesine-induced maximal relaxation was significantly reduced to less than 20%. In the airway and pulmonary artery preparations precontracted with high K^+ (60 mmol/l), the relaxant response to bucladesine was markedly reduced (Figs. 9, 10).

DISCUSSION

Responsiveness to PDE inhibitors

From the order of Emax and pIC_{50} values, milrinone was the most effective relaxant among three PDE inhibitors in both airway and pulmonary artery preparations, and olprinone was effective in the bronchial preparation and the main pulmonary artery preparation. The relaxation-response curve to olprinone was biphasic, in which the response to lower concentrations (0.1 − 10 μmol/l) was more sensitive to iberiotoxin than that to higher concentrations (Figs. 7C, 8C). These results suggest that BK_{Ca} channels have a dominant role in relaxant responses to lower concentrations of olprinone. Aminophylline was the lowermost potency in both airway and artery preparations. Without causing systemic adverse reaction, inhaled milrinone may be effective in improving asthmatic attack or pulmonary hypertension, and aerosol inhalation of olprinone may be useful for asthmatic responses.

In the present study, the onset time and the time course of relaxant responses to PDE3 inhibitors were slightly rapid in pulmonary artery preparation. These results are possibly attributed to the fact that PDE3 type is particularly abundant in pulmonary artery smooth muscle[7].

Mechanisms of relaxant action on airway smooth muscle

Aminophylline or theophylline induces hyperpolarization and suppresses the spontaneous tone of guinea-pig isolated trachea[26,27]. In a high K^+ solution, opening of K^+ channels cannot evoke hyperpolarization of smooth muscle cells because the K^+ equilibrium potential approaches 0 mV. In our present experiments, in the presence of the depolarizing concentration of KCl (60 mM), the relaxant effects of all PDE inhibitors on the tracheal and bronchial preparations were less potent than those in preparations precotracted with carbachol. These results raise the possibility that hyperpolarization as a result of opening various types of K^+ channels appears to be an important mechanism for the PDE inhibitor-induced relaxation of the airway smooth muscle. Iberiotoxin, a blocker of BK_{Ca} channels, markedly reduced the relaxant responses to three PDE inhibitors of both tracheal and bronchial prepara-

tions. These findings present pharmacological evidence that BK_{Ca} channels are largely involved in mediating relaxation of the guinea-pig airway smooth muscle induced by PDE inhibitors. Smooth muscles contract in response to an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$). A rise in $[Ca^{2+}]i$ activates BK_{Ca} channels on cell membrane. Activation of BK_{Ca} channels causes hyperpolarization and thereby opposes the contractile responses to various stimuli. BK_{Ca} channels on airway smooth muscle cells probably have an important role in negative feedback mechanism to airway obstruction.

Bucladesine caused a concentration-dependent relaxation of airway smooth muscles. In the tracheal preparation, iberiotoxin strongly blocked the bucladesine-induced relaxation as well as the PDE inhibitor-induced relaxation. These results indicate that BK_{Ca} channel activity is a very important factor in cAMP-dependent relaxations of tracheal smooth muscle, and that the relaxant effects of aminophylline, milrinone and olprinone are also mediated by an increase of intracellular cAMP concentrations. Furthermore, the iberiotoxin-resistant relaxations elicited by PDE inhibitors and bucladesine were no more modified by a further addition of apamin, α-dendrotoxin or glibenclamide. These findings indicate that, in the tracheal preparation, the iberiotoxin-resistant responses to PDE inhibitors or bucladesine are not accompanied with opening of K^+ channels. Iberiotoxin also strongly blocked the bucladesine-induced relaxation of the bronchial preparation. In contrast to trachea, the iberiotoxin-resistant relaxation elicited by bucladesine was blocked by α-dendrotoxin in the bronchial preparation. The bucladesine-induced relaxation of guinea-pig bronchus is probably mediated through opening predominantly BK_{Ca} channels and partly Kv channels. In the bronchial preparation, the iberiotoxin-resistant relaxation elicited by olprinone was also blocked by α-dendrotoxin. These results indicate that olprinone presumably opens both BK_{Ca} channels and Kv channels in bronchial smooth muscle via an increase of intracellular cAMP. On the other hand, the iberiotoxin-resistant relaxation of the bronchial preparation elicited by milrinone was blocked by glibenclamide but that elicited by aminophylline was not modified by other K^+ channel blockers. These results suggest that the relaxation to milrinone is partly mediated through opening K_{ATP} channels and the iberiotoxin-resistant response to aminophylline is not involved with opening of K^+ channels. Moreover, the iberiotoxin-resistant relaxations elicited by milrinone and aminophylline are presumably independent of the change in intracellular cAMP of bronchial smooth muscle. Subcellular mechanisms of cAMP-independent actions of milrinone have been suggested[28], but the exact mechanism is not yet clear. Methylxanthines are non-selective PDE inhibitors, and have several different actions including antagonism of adenosine receptors, modulation of intracellular Ca^{2+} level and inhibition of prostaglandin production[1]. The lack of effects of other K^+ channel blockers on the iberiotoxin-resistant relaxation elicited by aminophylline might be explained by its nonspecificity.

Our findings using the guinea-pig airway raise the possibility that selective PDE3 inhibitors relax the trachea or bronchus predominantly through activation of BK_{Ca} channels mediated by intracellular cAMP. Such BK_{Ca} channel activation leads to hyperpolarization of cell membrane, which inactivates the voltage-dependent L-type Ca^{2+} channels and/or directly affects intracellular signal transduction, including reduction in Ca^{2+} sensitivity of contractile proteins.

Mechanisms of relaxant action on pulmonary arterial smooth muscle

K^+ channels on vascular smooth muscle cell membrane are known to play an important role in the regulation of vascular tone[29,30], but K^+ channel blockers fail to modify the inhibitory action of milrinone and olprinone in various arterial preparations[31,32]. These previous studies indicate that K^+ channels do not appear to play an essential role in the milrinone- and olprinone-induced relaxations of arterial preparations. In the present experiments, iberiotoxin partly blocked the relaxant effects of three PDE inhibitors on main pulmonary artery preparations, but in R/L pulmonary artery preparations the relaxant responses to selective PDE3 inhibitors (milrinone and olprinone) were not significantly modified by iberiotoxin. Other K^+ channel blockers (apamin, α-dendrotoxin and glibenclamide) did not block the PDE inhibitor-induced relaxation. Similar results were also obtained on the bucladesine-induced relaxation. Our findings suggest that milrinone and ol-

prinone produce relaxations of the main pulmonary artery partly by opening BK_{Ca} channels via an increase in cAMP. In contrast, K^+ channels have hardly any roles for the vasorelaxant effects of these selective PDE3 inhibitors in the R/L pulmonary artery. On the other hand, the aminophylline-induced relaxation of the R/L pulmonary artery was significantly blocked by iberiotoxin. The result was not consistent with the effect of iberiotoxin on the bucladesine-induced relaxation of the R/L pulmonary artery. Since aminophylline has several different actions as described above, the aminophylline-induced relaxation is likely mediated partly by cAMP-independent mechanisms.

All PDE inhibitors tested here were less potent in pulmonary artery preparations precontracted with high K^+ than in preparations precontracted with phyenlephrine. This suggests that the PDE inhibitor-induced relaxation is accompanied by hyperpolarization. Vascular smooth muscle has an electrogenic Na^+, K^+-ATPase that contributes to membrane potential[33]. Na^+, K^+-ATPase activity has been related to the degree of hyperpolarization-relaxation of vascular smooth muscle[33,34]. Limas and Cohen[35] have reported that cAMP and theophylline activate Na^+, K^+-ATPase in microsomal fractions isolated from dog mesenteric arteries. Ouabain, a well-known inhibitor of Na^+, K^+-ATPase, inhibits the relaxant response produced by dibutyryl cAMP or theophylline in tail artery isolated from various animals[36]. In the present experiments, ouabain strongly blocked the relaxant responses to PDE inhibitors or bucladesine on pulmonary artery preparations. These results indicate that, in both main and R/L pulmonary artery preparations isolated from guinea-pigs, milrinone and olprinone cause cAMP-dependent relaxations predominantly by activating Na^+, K^+-ATPase. Hypoxic stress causes vasoconstrictions in the lung (i.e., hypoxic pulmonary vasoconstriction, HPV). Hypoxia can induce a decrease of oxidative ATP production and then inhibit Na^+, K^+-ATPase activity in pulmonary arterial smooth muscles[37]. It is likely that the inhibition of Na^+, K^+-ATPase facilitates vasoconstrictions under hypoxic conditions and intracellular cyclic nucleotides modulate HPV via Na^+, K^+-ATPase.

Previous studies and our present results allow the following formulation as possible mechanisms involved in the relaxant effects of selective PDE3 inhibitors. Selective PDE3 inhibitors, milrinone and olprinone, increase intracellular cAMP concentrations of pulmonary arterial smooth muscle which activates Na^+, K^+-ATPase of smooth muscle. The Na^+, K^+-ATPase activation hyperpolarizes the smooth muscle cell membrane, and subsequently inhibits intracellular Ca^{2+}-mobilization and/or decreases myofilament Ca^{2+}-sensitivity of smooth muscle. The Na^+, K^+-ATPase activation also causes a decrease in the intracellular levels of Na^+ available for exchange with Ca^{2+} by the Na^+/Ca^{2+} exchanger, therefore the intracellular Ca^{2+} concentration is decreased. These cellular events reduced the developed tension of smooth muscles. In the present experiments, however, ouabain did not completely block the selective PDE3 inhibitor- or bucladesine-induced relaxation of pulmonary artery. The mechanism of the ouabain-resistant relaxation remains to be clarified.

In conclusion, selective PDE3 inhibitors relax the airway or pulmonary arterial smooth muscles isolated from guinea-pigs via an increase in intracellular cAMP. BK_{Ca} channels seem to play an important role for PDE3 inhibitor-induced relaxation of the airway smooth muscle. Relaxations of the main pulmonary artery induced by selective PDE3 inhibitors are mediated predominantly by activation of Na^+, K^+-ATPase, and partly through opening BK_{Ca} channels. In the R/L pulmonary artery, vasorelaxant effects of selective PDE3 inhibitors are mediated by activation of Na^+, K^+-ATPase, but not of BK_{Ca} channels.

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