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Short communication

Y-27632 potentiates relaxant effects of β_2-adrenoceptor agonists in bovine tracheal smooth muscle

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Abstract

We examined how (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide (Y-27632), an inhibitor of Rho-associated coiled coil-forming protein kinase (ROCK I) and Rho kinase (ROCK II), affects the relaxant responses to β_2-adrenoceptor agonists in bovine tracheal smooth muscle preparations precontracted with methacholine. Y-27632 (0.3–30 μM) caused a concentration-dependent attenuation of precontraction with methacholine (0.3–3 μM). Pretreatment with Y-27632 (1 μM) significantly ($P < 0.05$) augmented salbutamol (0.3–100 nM) and terbutaline (0.3 nM–1 μM)-induced relaxations. These results suggest that the ROCK inhibitor could become a new type bronchodilator and its combination with β_2-adrenoceptor agonists may become a novel strategy for the long-term treatment of asthma.

Keywords: Airway; Bronchodilation; Rho-associated coiled coil-forming protein kinase; Rho; Smooth muscle

1. Introduction

Many asthmatic patients have an increased vagal tone and/or hyperresponsiveness to acetylcholine receptor agonists (Roberts et al., 1984). Acetylcholine receptor agonists act both to increase intracellular Ca^{2+} concentration and to enhance the effectiveness of Ca^{2+} for inducing contraction. The latter phenomenon has been referred as Ca^{2+} sensitization (Somlyo and Himpens, 1989). Although the mechanism underlying the Ca^{2+} sensitization has not been fully elucidated, it appears that inhibition of myosin light chain phosphatase due to a small GTPase Rho-associated coiled coil-forming protein kinase (ROCK I) and its isoform, ROCK II, is partly involved (Kimura et al., 1996; Uehata et al., 1997; Fu et al., 1998). Inhibition of myosin light chain phosphatase increases the level of myosin light chain phosphorylation and helps to develop and/or maintain tension. Therefore, increases in activity of myosin light chain phosphatase by blockade of Rho/ROCK-mediated signaling pathway would attenuate the contractile responses via the inhibition of Ca^{2+} sensitizing mechanism.

Recent studies showed that (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide (Y-27632), a selective ROCK inhibitor, could attenuate agonists-induced contractions of airway smooth muscle (Uehata et al., 1997; Yoshii et al., 1999). Thus, Rho/ROCK-mediated signaling pathway might become a novel target of drug therapy of asthma. However, involvement of ROCK in regulation of many other cellular functions (Uehata et al., 1997; Tominaga et al., 1998; Klages et al., 1999) leads us to predict side effects produced by chronic inhibition of ROCK.

β_2-adrenoceptor agonists are potent bronchodilators and are widely used in the treatment of asthma. On the other hand, the regular use of β_2-adrenoceptor agonists in asthmatic patients could cause the exacerbation of asthma (Sears et al., 1990; Cockcroft et al., 1993). Additionally, the continuous or repeated exposure to these drugs reduces the bronchodilation mediated by β_2-adrenoceptors. In these situations, to reduce the dose of β_2-adrenoceptor agonists, combining β_2-adrenoceptor agonists with methylxanthines (e.g., theophylline) has traditionally been conducted. The combination, however, could exert a synergistic effect on the cardiovascular system. Thus, combination with other drugs, which can potentiate the effects of β_2-adrenoceptor agonists on the airway smooth muscle, may become the useful clinical strategies to improve airflow limitation in asthma.

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The purpose of this study was to determine whether the combination of a ROCK inhibitor with a β_2-adrenoceptor agonist would become a novel therapeutic strategy of asthma. For this purpose, we evaluated the effect of Y-27632 on salbutamol- and terbutaline-induced relaxations of bovine tracheal smooth muscle.

2. Materials and methods

2.1. Preparation of bovine tracheal smooth muscle segments

We obtained freshly excised bovine tracheae from the local abattoir and transported to the laboratory immersed in cold Krebs–Ringer bicarbonate buffer (KRB) of the following composition (in mM): 118.5 NaCl, 4.47 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2.54 $CaCl_2$, 24.9 $NaHCO_3$, 10.0 glucose, and 1.0 pyruvic acid (pH 7.4). We carefully separated smooth muscle from cartilage, mucosa, and connective tissues while immersed in ice-cold KRB gassed with 95% O_2–5% CO_2 as described previously (Katsuki and Murad, 1977; Ishii and Murad, 1989).

2.2. Measurement of mechanical activity

We used segments of smooth muscles ($1–2 \times 10$ mm) for measurement of mechanical responses. Muscle tension was recorded isometrically. One end of each muscle was attached by cotton thread to a force displacement transducer (model TB-611T, Nihon Kohden, Tokyo, Japan), and the other end was tied to a stainless holder. We mounted muscle segments in 20 ml jacketed organ baths with KRB gassed with 95% O_2–5% CO_2 at 37°C. Muscles were allowed to equilibrate for 2 h under an initial tension of 0.75 g, and the buffer was changed every 15 min. The tension was changed to 0.5 g 10 min before the initiation of each experiment.

2.3. Experimental procedure

In the first series of experiments, we examined the effect of Y-27632 on the tension developed by methacholine (0.3, 1 or 3 μM). After confirming the plateau of the methacholine effect, we added cumulatively Y-27632 (0.3–30 μM) to the tissue baths.

In the second series of experiments, we examined the effect of Y-27632 on salbutamol (0.3–100 nM)- and terbutaline (0.3 nM–1 μM)-induced relaxations. When plateau tone was reached 15–20 min after the addition of methacholine (0.3, 1 or 3 μM), tissues were exposed to vehicle (KRB) or Y-27632 (1 μM). After an additional 15-min incubation period, we cumulatively added salbutamol or terbutaline to the tissue baths. Only one concentration–response curve was constructed with each preparation.

2.4. Data analysis and statistics

Data were expressed as the mean ± SEM. Relaxant responses were expressed as percentages of methacholine-induced tension obtained just before starting the cumulative addition of drugs. Changes in tension induced by Y-27632 were analyzed using analysis of variance (ANOVA) for repeated measures. IC_{50} values (the concentration required to decrease methacholine-induced tension by 50%) were calculated by linear regression analysis and were analyzed using Student's *t*-test. A P value smaller than 0.05 was considered statistically significant.

2.5. Drugs

Acetyl-β-methylcholine chloride (methacholine), salbutamol hemisulfate and terbutaline hemisulfate were obtained from Sigma (St. Louis, MO, USA). Y-27632 was a gift from Yoshitomi Pharmaceutical Industries, (Iruma-shi, Saitama, Japan).

3. Results

3.1. Precontraction with methacholine

Tensions developed by methacholine at concentrations of 0.3, 1 and 3 μM were 8.3 ± 0.8 g ($n = 15$), 14 ± 1.0 g ($n = 25$) and 16 ± 1.0 g ($n = 25$), respectively. These developed tensions were approximately 50%, 80% and 90%, respectively, of its own maximum tension of each bovine tracheal smooth muscle preparation (data not shown).

3.2. Relaxation to Y-27632

Y-27632 (0.3–30 μM) caused concentration-dependent decreases in the tension developed by methacholine (0.3, 1 or 3 μM) (Fig. 1). The relaxations caused by 30 μM Y-27632 in the preparations precontracted with 0.3, 1 and 3 μM methacholine were 87 ± 2.6%, 57 ± 4.1% and 39 ±

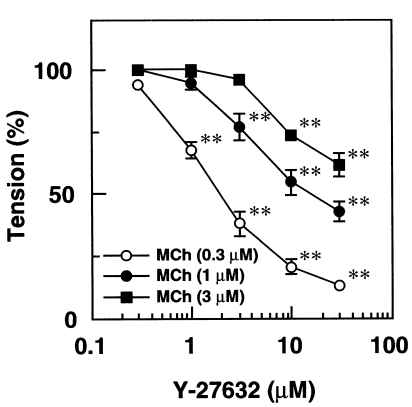

Fig. 1. Effect of Y-27632 on bovine tracheal smooth muscle precontracted with methacholine (MCh, 0.3, 1 or 3 μM). Each point represents the mean ± SEM from 5–6 separate preparations. $^{**}P < 0.01$ vs. corresponding 0.3 μM-values.

4.6%, respectively. Thus, the relaxant effects of Y-27632 were decreased with increased concentrations of methacholine. The IC_{50} values for relaxation with Y-27632 in the preparations precontracted with 0.3 and 1 μM methacholine were 2.4 ± 0.4 and 22 ± 4.8 μM, respectively.

3.3. Effect of Y-27632 on salbutamol-induced relaxation

We evaluated the influence of pretreatment of bovine tracheal smooth muscle with 1 μM Y-27632 on the relaxant responses to salbutamol and terbutaline. This concentration of Y-27632 significantly ($P < 0.01$) attenuated contractions induced by 0.3 μM methacholine, however, it had no significant relaxant effect on preparations precontracted with either 1 or 3 μM methacholine (Fig. 1).

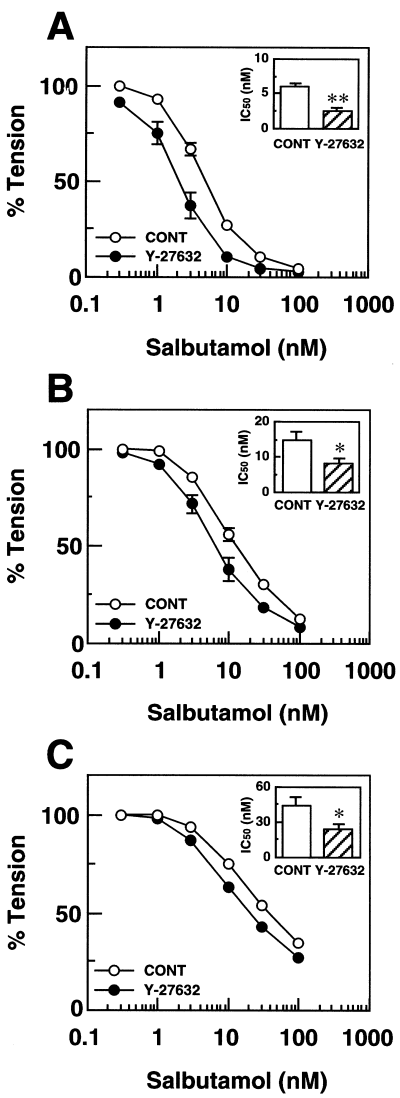

Fig. 2. Concentration–response curves for the relaxant responses to salbutamol in the absence (CONT) and presence of Y-27632 (1 μM). The bovine tracheal smooth muscle preparations were precontracted with 0.3 (Panel A), 1 μM (Panel B) or 3 μM (Panel C) methacholine. Insets show IC_{50} values for the responses to salbutamol. Each point or column with a vertical bar represents the mean ± SEM from 5–6 separate preparations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding control (CONT)-values.

Fig. 2 shows the effect of Y-27632 (1 μM) on salbutamol-induced relaxations in the preparations precontracted with methacholine (0.3, 1 or 3 μM). Salbutamol (0.3–100 nM) caused concentration-dependent relaxations. Pretreatment with Y-27632 caused the leftward shifts in the concentration–response curve for salbutamol-induced relaxant responses. When tissues were precontracted with 0.3 μM methacholine, the IC_{50} values for salbutamol-induced relaxations in the absence and presence of Y-27632 were 5.9 ± 0.6 and 2.5 ± 0.5 nM, respectively. The difference was statistically significant ($P < 0.01$) (Panel A). Also, in the preparations precontracted with either 1 or 3 μM methacholine, Y-27632 significantly ($P < 0.05$) decreased the IC_{50} values for salbutamol-induced relaxations (1 μM methacholine, 15 ± 2.2 vs. 8.2 ± 1.6 nM; 3 μM methacholine, 44 ± 6.2 vs. 24 ± 3.8 nM) (Panels B and C).

Similarly, Y-27632 significantly ($P < 0.05$) decreased the IC_{50} values for relaxant responses to terbutaline in the preparations precontracted with 1 or 3 μM methacholine (1 μM methacholine, 55 ± 5.3 vs. 37 ± 5.1 nM; 3 μM methacholine, 176 ± 34 vs. 81 ± 3.7 nM) ($n = 4–5$).

4. Discussion

Acetylcholine receptor agonists increase the sensitivity of contractile proteins to Ca^{2+} via activation of Rho (Croxton et al., 1998). The Ca^{2+} sensitization plays an important role in maintenance of the contractile responses in airway smooth muscle (Yoshii et al., 1999). We demonstrated that Y-27632 concentration-dependently relaxed the methacholine-precontracted bovine tracheal smooth muscle. The IC_{50} value of 2.4 μM for Y-27632-induced relaxation in preparations precontracted with 0.3 μM methacholine was close to those obtained in previous experiments using vascular and tracheal smooth muscle preparations (0.3–1.3 μM) (Uehata et al., 1997; Yoshii et al., 1999). In addition, 30 μM Y-27632, which was the highest concentration in this study, markedly (87%) attenuated methacholine (0.3 μM)-induced precontraction. These results suggest that Rho/ROCK-mediated Ca^{2+} sensitization considerably contributes to maintenance of tension developed by 0.3 μM methacholine.

The present study also demonstrated that Y-27632 (1 μM) significantly augmented the relaxant responses to β_2-adrenoceptor agonists in preparations precontracted with methacholine. Because Y-27632 per se can attenuate the contraction induced by methacholine, the decreased levels of precontraction might contribute to the augmentation of the responses to β_2-adrenoceptor agonists. However, Y-27632 (1 μM), which did not significantly affect the tensions developed by either 1 or 3 μM methacholine (Fig. 1), shifted the concentration–response curve for salbutamol-induced relaxation constructed under precontraction with either 1 or 3 μM methacholine to the left by 1.8-fold (Fig. 2B and C). Thus, it is unlikely that the decreased

levels of precontraction by Y-27632 alone account for the augmentation of responses to β_2-adrenoceptor agonists.

Y-27632 could prevent the inhibition of myosin light chain phosphatase via the Rho/ROCK-mediated signaling pathway. Therefore, pretreatment with Y-27632 would increase myosin light chain phosphatase activity. Under such conditions, relaxant responses to β_2-adrenoceptor agonists would be augmented, because relaxations occur as a result of decreases in the level of phosphorylation of myosin light chain by increased activity of myosin light chain phosphatase. Needless to say, this mechanism applies to responses to other relaxant drugs. Accordingly, if this interpretation is correct, Y-27632 may potentiate the relaxant effects irrespective of the pharmacological mechanisms to relax smooth muscle.

Recently, asthma has been recognized primarily as an inflammatory condition of airway. However, β_2-adrenoceptor agonists are still widely used for therapy of the asthmatic attack, despite several inconveniences to the treatment. Unlike the inhibition of phosphodiesterases by methylxanthines, it seems that ROCK inhibition per se has no stimulating effect on cardiac muscles. Combination of a β_2-adrenoceptor agonist with a ROCK inhibitor, therefore, may have a lower risk for the occurrence of tachycardia or dysrhythmia as compared with the traditional combination therapy with methylxanthines. Thus, the present results may provide an important clue to overcome the limitations of use of β_2-adrenoceptor agonists.

In conclusion, Y-27632 attenuates the methacholine-induced precontraction and potentiates the relaxant effects of β_2-adrenoceptor agonists in bovine tracheal smooth muscle preparations. Therefore, not only a ROCK inhibitor alone but also its combination with β_2-adrenoceptor stimulants may become a useful clinical strategy to improve airflow limitation in asthma.

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