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Effects of Y-27632 on acetylcholine-induced contraction of intact and permeabilized intrapulmonary bronchial smooth muscles in rats

Yoshihiko Chiba*, Hisao Takeyama, Hiroyasu Sakai, Miwa Misawa

Department of Pharmacology, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan

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Abstract

In the present study, the effects of a selective Rho-associated coiled-coil forming protein kinase (ROCK) inhibitor, Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] on acetylcholine-induced contraction and Ca^{2+} sensitization of rat bronchial smooth muscle were examined. Intact and β-escin-permeabilized muscles of the third branch of intrapulmonary bronchi were used. In intact muscles, Y-27632 (10^{-6}–10^{-4} M) concentration-dependently inhibited acetylcholine-induced contractile responses. In acetylcholine (10^{-3} M)-precontracted intact muscles, the maximal relaxation (about 50% inhibition of contraction) was obtained by a concentration of 10^{-4} M Y-27632, which had no effect on the resting tone. In β-escin-permeabilized muscles, addition of acetylcholine (10^{-5}–10^{-3} M) plus GTP (100 μM) induced a further contraction, i.e., Ca^{2+} sensitization at a constant Ca^{2+} concentration of $pCa = 6.0$. The acetylcholine-induced Ca^{2+} sensitization was completely blocked in the presence of 10^{-4} M Y-27632, whereas the Ca^{2+}-induced contraction itself was not affected by Y-27632. Immunoblot study revealed the expression of ROCK-I and ROCK-II proteins in the intrapulmonary bronchi of rats. These findings suggest that Y-27632 dilates acetylcholine-mediated contraction of rat bronchial smooth muscle by inhibiting RhoA/ROCK-mediated Ca^{2+} sensitization.

Keywords: Bronchial smooth muscle, intrapulmonary; Ca^{2+} sensitization; Rho; ROCK; Y-27632; β-Escin

1. Introduction

In general, smooth muscle contraction has been thought to be induced by an increase in cytosolic Ca^{2+} via the activation of plasma membrane Ca^{2+} channels and/or Ca^{2+} release from sarcoplasmic reticulum. However, additional mechanisms have been suggested in agonist-induced smooth muscle contraction by studies which used the simultaneous measurements of force development and intracellular Ca^{2+} concentration (Sato et al., 1988), and chemically permeabilized preparations (Fujita et al., 1995) in various types of smooth muscles including airways (Ozaki et al., 1990; Chiba et al., 1999b). It has been demonstrated that agonist stimulation increases myofilament Ca^{2+} sensitivity in β-escin-permeabilized smooth muscles of the rat coronary artery (Satoh et al., 1994), guinea pig vas deferens (Fujita et al., 1995), canine trachea (Bremerich et al., 1997) and rat bronchus (Chiba et al., 1999b). Although the detailed mechanism is not fully understood, a participation of Rho protein, a monomeric GTP binding protein, in the agonist-induced Ca^{2+} sensitization has been suggested by many investigators (e.g., Fujita et al., 1995; Otto et al., 1996; Gong et al., 1997; Chiba et al., 1999b).

Recently, several target proteins have been identified as effectors of RhoA, including Rho-associated coiled-coil forming protein kinase-I (ROCK-I) (Narumiya et al., 1997) and ROCK-II (Nakagawa et al., 1996). The importance of ROCKs in the induction of smooth muscle Ca^{2+} sensitization has been demonstrated in various types of smooth muscle (Fu et al., 1998; Iizuka et al., 1999; Yoshii et al., 1999; Swärd et al., 2000; Yamagata et al., 2000) by using a selective ROCK inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632; Uehata et al., 1997). The RhoA/ROCK-mediated Ca^{2+} sensitization has also been reported in airway smooth muscle (Iizuka et al., 1999; Yoshii et al., 1999; Yamagata et al., 2000). In permeabilized rabbit tracheal smooth muscle, carbachol (in the presence of

* Corresponding author. Tel.: +81-3-5498-5786; fax: +81-3-5498-5787.

E-mail address: chiba@hoshi.ac.jp (Y. Chiba).

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GTP)- and guanosine-5′-*O*-(3′-thiotriphosphate) (GTPγS)-induced contractions at a constant Ca^{2+} concentration, which are mediated by Ca^{2+} sensitization, were completely inhibited by Y-27632 (Iizuka et al., 1999; Yoshii et al., 1999). Furthermore, in intact (nonpermeabilized) rabbit tracheal smooth muscle, Y-27632 completely reversed carbachol-induced contraction (Yoshii et al., 1999). Similar results have also been reported using human bronchial smooth muscle (Yoshii et al., 1999; Yamagata et al., 2000). Thus, it is suggestive that inhibition of RhoA/ROCK pathway may become a new strategy to treatment of bronchial asthma.

We recently revealed the existence of acetylcholine-induced Ca^{2+} sensitization in rat bronchial smooth muscle contraction by using β-escin-permeabilized muscle strips (Chiba et al., 1999b). The acetylcholine-induced Ca^{2+} sensitization was completely blocked by treatment with C3 exoenzyme, indicating an involvement of RhoA in the acetylcholine-induced Ca^{2+} sensitization of rat bronchial smooth muscle. However, there is no information about the downstream pathway of RhoA in bronchial smooth muscle of rats. Moreover, it is of interest whether the RhoA/ROCK system is inherent in smooth muscle over species. Therefore, in the present study, the effects of Y-27632 on acetylcholine-induced contractile response of rat intrapulmonary small bronchi were examined to elucidate the downstream pathway of acetylcholine-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in rats.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (170–190 g, specific pathogen-free) were used. Animals were killed by exsanguination from the abdominal aorta under anesthetization by chloral hydrate (400 mg/kg, i.p.). The third branch of the intrapulmonary bronchus was isolated by the method described previously (Chiba et al., 1999b). In brief, the tissue was carefully cleaned of lung parenchyma and adhering connective tissue, and then cut into ring strips (about 200 μm width, 500 μm diameter). The epithelium was removed by gently rubbing with keen-edged tweezers under a stereomicroscope (Chiba et al., 1999b).

2.2. Intact (nonpermeabilized) smooth muscle

The resultant tissue ring preparation was suspended in a 400-μl organ bath at a resting tension of 50 mg. The isometric contraction of the circular smooth muscle was measured with a force-displacement transducer (T7-8-240, Orientec, Japan) and recorder (FBR-252A, Toa Electronics, Japan). The organ bath contained modified Krebs–Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0 (pH 7.4). The buffer solution was maintained at 37 °C and oxygenated with 95% O_2–5% CO_2. During an equilibration period in the organ bath, the tissues were washed four times at 15- to 20-min intervals and were equilibrated slowly to a baseline tension of 50 mg. After the equilibration period, a concentration–response curve to acetylcholine (10^{-7}–10^{-3} M) was constructed cumulatively.

2.3. β-Escin-permeabilized smooth muscle

The ring strips were permeabilized by a 30-min treatment with 10 μM β-escin (Sigma) at room temperature in relaxing solution. Relaxing solution contained: 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 7.1 mM Mg^{2+}-dimethanesulfonate, 108 mM K^+-methanesulfonate, 2 mM EGTA, 5.875 mM Na_2ATP, 2 mM creatine phosphate, 4 U/ml creatine phosphokinase, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 1 μg/ml *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64; pH 6.8) containing 10 μM A23187. Free Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^6 M^{-1} (Hori et al., 1993). The permeabilized muscle strip was then suspended in a 400-μl organ bath at room temperature. The contractile force developed was measured by an isometric transducer (T7-8-240, Orientec) under a resting tension of 50 mg.

2.4. Western blot analysis

To quantify the expression of ROCK-I and ROCK-II proteins, Western blot was performed in the homogenates of intrapulmonary bronchi that were dissected free from lung parenchyma. Briefly, the samples (10 μg of total protein per lane) were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were then electrophoretically transferred to a nitrocellulose membrane. After blocking with 3% gelatin, the nitrocellulose membrane was incubated with primary antibody (polyclonal goat anti-human ROCK-I [carboxy terminus] or anti-rat ROCK-II [carboxy terminus]; 1:2000 dilution, respectively; Santa Cruz). Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G (IgG) (1:8000 dilution; Amersham), detected by an enhanced chemiluminescent system (Amersham).

2.5. Data analyses

All the data are expressed as means ± S.E.M. Statistical significance of difference was determined by Dunnett's multiple analysis.

3. Results

In intact (nonpermeabilized) intrapulmonary bronchial smooth muscle of rat that was precontracted by 10^{-3} M acetylcholine (which induced the maximal contraction), cumulatively applied Y-27632 (10^{-6}–10^{-4} M) induced a concentration-dependent relaxation (Fig. 1). The plateau responses induced by respective concentrations of Y-27632 were reached 15–20 min after the application. The effective concentration of Y-27632 was 10^{-5}–10^{-4} M and no further relaxation was observed at the concentration of 10^{-3} M (Fig. 1). Thus, 10^{-5} and 10^{-4} M concentrations of Y-27632 were used in the present study. Fig. 2 shows the effects of pretreatment with Y-27632 on acetylcholine-induced contractile responses of intact muscle strips. Y-27632 was pretreated 20 min before administration of the first concentration of acetylcholine (10^{-7} M) and was then present throughout the experiment. Acetylcholine (10^{-7}–10^{-3} M) elicited a concentration-dependent contractile response in intact bronchial smooth muscle. No further contraction was observed by higher concentrations of acetylcholine (data not shown). The acetylcholine-induced contraction was attenuated in the presence of Y-27632 (10^{-5} and 10^{-4} M) in a concentration-dependent manner (Fig. 2). In the presence of Y-27632, no further contraction was observed at a higher concentration of 10^{-2} M acetylcholine. Neither concentration of Y-27632 had any effect on the resting tension.

In all tissue preparations permeabilized by 10 μM β-escin (for 30 min), the application of free Ca^{2+} (pCa = 6.5, 6.3, 6.0, 5.5 and 5.0) induced concentration-dependent reproducible contractile responses, indicating successful permeabilization (under these conditions, no such contractile effect was observed in nonpermeabilized muscles). Y-27632 was added to the bath 20 min before application of the first pCa and was then present throughout the experiment. As shown in Fig. 3, the Ca^{2+}-induced contractile responses were not significantly changed in the presence of Y-27632 (10^{-4} M). The upper panel of Fig. 4 shows a typical recording of acetylcholine-induced Ca^{2+} sensitization of the β-escin-permeabilized bronchial smooth muscle. When the Ca^{2+} concentration was clamped at pCa = 6.0, cumulative application of acetylcholine (10^{-5}–10^{-3} M) in the presence of 100 μM GTP caused a further contraction (even though the Ca^{2+} concentration was constant, i.e., acetylcholine-induced Ca^{2+} sensitization) in a concentration-dependent manner. No further contraction was observed by 10^{-2} M acetylcholine (data not shown). In the presence of Y-27632 (10^{-5} and 10^{-4} M), the

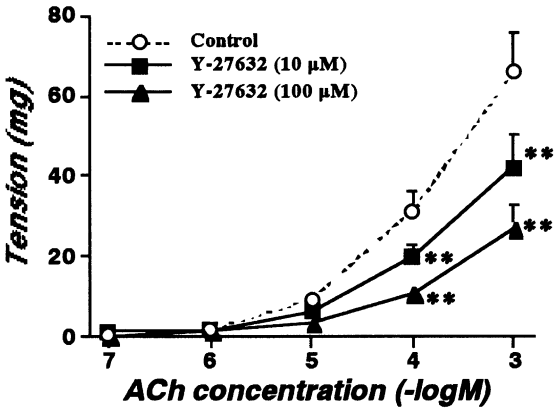

Fig. 2. Effect of pretreatment with Y-27632 on acetylcholine-induced contractile responses of rat intact (nonpermeabilized) intrapulmonary bronchial smooth muscle. Twenty minutes after Y-27632 or its vehicle (Control) treatment, acetylcholine (10^{-7}–10^{-3} M) was cumulatively administered in the presence of Y-27632 or vehicle. Data represent the mean ± S.E.M. from six experiments. $^{**}P < 0.01$ vs. control by Dunnett's multiple analysis.

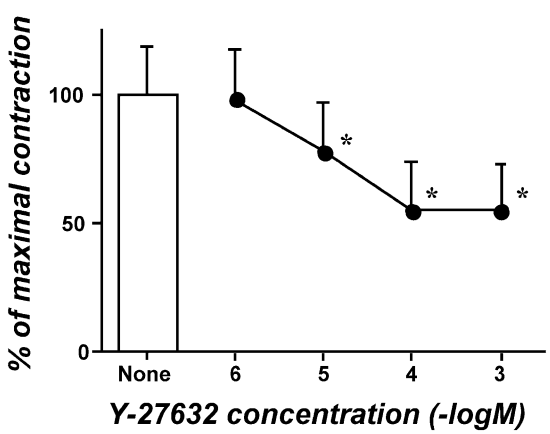

Fig. 1. Effect of Y-27632 on rat intact (nonpermeabilized) intrapulmonary bronchial smooth muscle precontracted with acetylcholine (10^{-3} M). After the acetylcholine (10^{-3} M)-induced contractile response reached plateau (None), Y-27632 (10^{-6}–10^{-4} M) was cumulatively applied. Data represent the mean ± S.E.M. from six experiments. $^{*}P < 0.05$ vs. None by Dunnett's multiple analysis.

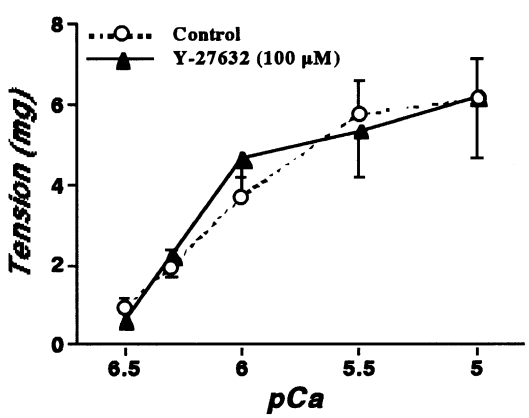

Fig. 3. Effect of Y-27632 on Ca^{2+}-induced contraction of β-escin-permeabilized rat intrapulmonary bronchial smooth muscle. After permeabilization with β-escin (for 30 min, at room temperature) in the presence of 10 μM A23187, the muscle strips were equilibrated. Twenty minutes after Y-27632 (10^{-4} M) or its vehicle (Control) treatment, Ca^{2+}-induced contractions were measured, cumulatively, in the presence of Y-27632 or vehicle. pCa; $-\log[Ca^{2+}]$. Data represent the mean ± S.E.M. from six experiments.

acetylcholine-induced Ca^{2+} sensitization of bronchial smooth muscle contraction was markedly and significantly inhibited in a concentration-dependent manner, although the initial contraction induced by $p\mathrm{Ca} = 6.0$ (in the absence of acetylcholine and GTP) was not affected by Y-27632. The acetylcholine-induced Ca^{2+} sensitization was completely inhibited by 10^{-4} M Y-27632 (the lower panel of Fig. 4). In the presence of Y-27632, no further contraction was observed by 10^{-2} M acetylcholine. The acetylcholine-induced Ca^{2+} sensitization was also completely antagonized by 10^{-6} M atropine (Chiba et al., 1999b).

To determine the expression of ROCKs, target proteins of Y-27632, in intrapulmonary bronchial smooth muscle of the rat, immunoblottings were performed in the homogenates of intrapulmonary bronchi that were dissected from lung parenchyma. As shown in Fig. 5, immunoblottings with the antibodies against ROCK-I and ROCK-II gave a single 160 kDa band, respectively, both of which were sensitive to respective blocking peptide, indicating the existence of ROCK-I and ROCK-II proteins in the intrapulmonary bronchi of rats.

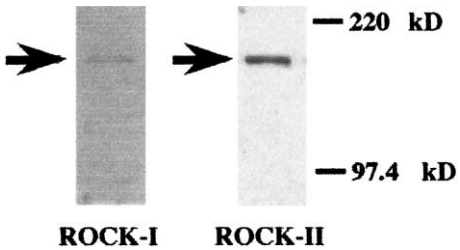

Fig. 5. Typical immunoblot of Rho-associated coiled-coil forming protein kinase-I (ROCK-I; left lane) and ROCK-II (right lane) in bronchial homogenates of rats. The homogenates (10 μg protein $lane^{-1}$) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were incubated with specific antibody against ROCK-I or ROCK-II (carboxy terminus, respectively). After further incubation with horseradish peroxidase-conjugated secondary antibody, a ~160 kDa band corresponding to ROCK-I or ROCK-II was detected by enhanced chemiluminescence. The data shown are representative for four sets of separate experiments. The bars indicate markers of standard molecular weight.

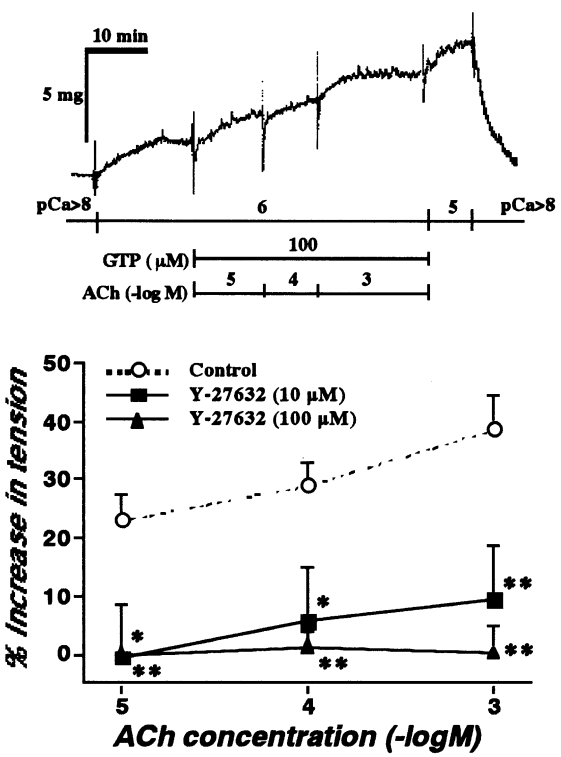

Fig. 4. Upper panel: A typical trace showing acetylcholine-induced Ca^{2+} sensitization of β-escin-permeabilized rat intrapulmonary bronchial smooth muscle. After the 10^{-6} M Ca^{2+} ($p\mathrm{Ca} = 6$)-induced contraction reached plateau, acetylcholine (10^{-5}–10^{-3} M) induced a concentration-dependent further contraction in the presence of 100 μM GTP, i.e., Ca^{2+} sensitization. Lower panel: Effect of Y-27632 (10^{-5} and 10^{-4} M) on the acetylcholine-induced Ca^{2+} sensitization of β-escin-permeabilized rat intrapulmonary bronchial smooth muscle. After permeabilization with β-escin (for 30 min, at room temperature) in the presence of 10 μM A23187, the muscle strips were equilibrated. Twenty minutes after Y-27632 or its vehicle (Control) treatment, Ca^{2+} ($p\mathrm{Ca} = 6$)-induced contraction was measured. When the Ca^{2+}-induced contraction reached plateau, acetylcholine (in the presence of GTP)-induced contractions were measured cumulatively, in the presence of Y-27632 or vehicle. $p\mathrm{Ca}$; $-\log[Ca^{2+}]$. Data represent the mean ± S.E.M. from six experiments. $^{**}P < 0.01$ vs. control by Dunnett's multiple analysis.

4. Discussion

We previously reported the existence of acetylcholine-induced Ca^{2+} sensitization in rat intrapulmonary bronchial smooth muscle contraction, which was sensitive to C3 exoenzyme (Chiba et al., 1999b). In the present study, the acetylcholine-induced Ca^{2+} sensitization of β-escin-permeabilized muscle was also inhibited by Y-27632, a selective inhibitor of ROCKs (Uehata et al., 1997). It is, therefore, suggested that like other airway smooth muscles (Iizuka et al., 1999; Yoshii et al., 1999; Yamagata et al., 2000), the RhoA/ROCK pathway might be a major signaling pathway of acetylcholine-induced Ca^{2+} sensitization in rat intrapulmonary bronchial smooth muscle. In fact, distinct expression of ROCK-I and ROCK-II, both of which are inhibited by Y-27632 (Ishizaki et al., 2000), was also demonstrated in rat bronchi by using immunoblot analysis. The expression of ROCK-I and ROCK-II and the inhibitory effect of Y-27632 on agonist-induced Ca^{2+} sensitization have also been reported in rabbit tracheal and human bronchial smooth muscles (Yoshii et al., 1999).

The phosphorylation of myosin light chain, which regulates smooth muscle contraction, is primarily maintained by two mechanisms, Ca^{2+}-dependent and independent pathways. An increase in cytosolic concentration of Ca^{2+} forms a $4Ca^{2+}$-calmodulin–myosin light-chain kinase complex and activates myosin light-chain kinase. The activated myosin light-chain kinase phosphorylates myosin light chain, resulting in smooth muscle contraction (Rodger and Pyne, 1992). On the other hand, inhibitory mecha-

nisms of myosin light-chain phosphatase, an enzyme which dephosphorylates phosphorylated myosin light chain, also increase smooth muscle contraction even at constant Ca^{2+} concentration (Somlyo and Somlyo, 2000), which is referred to as Ca^{2+} sensitization. To date, in smooth muscles including airways (Iizuka et al., 1999; Yoshii et al., 1999; Yamagata et al., 2000), it has been recognized that the inhibitory mechanism of myosin light-chain phosphatase is mainly regulated by serine/threonine kinases, ROCKs, which are activated by RhoA (Somlyo and Somlyo, 2000). Y-27632 is known as a putative Ca^{2+} sensitization modulator of smooth muscle contraction basing on its specific inhibitory effect of ROCKs (Uehata et al., 1997; Ishizaki et al., 2000). This compound inhibits ROCKs by binding to the catalytic site (Ishizaki et al., 2000). In our preliminary study, phorbol 12,13-dibutyrate-induced Ca^{2+} sensitization observed in the β-escin-permeabilized bronchial smooth muscle was not affected by Y-27632 at the concentrations used in the present study, indicating that Y-27632 had no effect on protein kinase C at the concentrations used. In addition to its inhibitory effect on ROCKs, however, only a slight inhibitory effect of Y-27632 on myosin light-chain kinase has been reported (Uehata et al., 1997).

In the present study, Ca^{2+}-induced contraction of β-escin-permeabilized rat intrapulmonary bronchus was not significantly affected even by a higher concentration of Y-27632 (Fig. 3). The finding is consistent with the report of β-escin-permeabilized rabbit mesenteric artery (Uehata et al., 1997), indicating that the Y-27632 concentrations used in the present study do not affect myosin light-chain kinase activity. The finding also suggests that rat intrapulmonary bronchial smooth muscle might have less basal activity of ROCKs, as has been reported in rabbit trachea (Yoshii et al., 1999) and human bronchus (Yamagata et al., 2000). No effect of Y-27632 on the resting tone of intact rat intrapulmonary bronchus (see Results) also supports this possibility.

In β-escin-permeabilized rat intrapulmonary bronchial smooth muscle, acetylcholine induced a further contraction in a concentration-dependent manner at constant Ca^{2+} concentration of $pCa = 6.0$ in the presence of 100 μM GTP (Fig. 4). The Ca^{2+}-sensitizing effect induced by acetylcholine was completely blocked in the presence of 10^{-4} M Y-27632 (Fig. 4), the concentration of which had no significant effect on the Ca^{2+}-induced contraction (Fig. 3). We also previously reported that the acetylcholine-induced Ca^{2+} sensitization was completely blocked by treatment with C3 exoenzyme, which selectively ADP-ribosylates to inhibit Rho family of proteins (Fujita et al., 1995), and that RhoA, but not RhoB, protein is expressed in rat bronchial smooth muscle (Chiba et al., 1999b). Taken together, the RhoA/ROCK pathway could be a major mechanism of induction of acetylcholine-induced Ca^{2+} sensitization of bronchial smooth muscle contraction in rats. The RhoA/ROCK-mediated agonist-induced Ca^{2+} sensitization was also suggested in rabbit tracheal (Yoshii et al., 1999; Iizuka et al., 1999) and human bronchial smooth muscles (Yoshii et al., 1999; Yamagata et al., 2000). Thus, the RhoA/ROCK system might be inherent in airway smooth muscles over species.

In the current study, the effect of Y-27632 on acetylcholine-induced contraction of intact (nonpermeabilized) smooth muscle of rat intrapulmonary bronchi was also examined. Y-27632 concentration-dependently inhibited acetylcholine-induced contractile responses (Fig. 2). Also, Y-27632 showed a dilatory effect on acetylcholine-precontracted intact bronchial smooth muscles in a concentration-dependent manner (Fig. 1). The maximal dilatation was found at a concentration of 10^{-4} M Y-27632 (see Results), the concentration of which completely inhibited acetylcholine-induced Ca^{2+} sensitization in β-escin-permeabilized bronchial smooth muscle (Fig. 4). The capacity of dilatation effect of Y-27632 on acetylcholine-precontracted rat bronchial smooth muscles (about 50%) (Fig. 1) is greater than that of a β-agonist, isoprenaline (the maximal relaxation was obtained at 10^{-5} M: about 20%) (Chiba and Misawa, 1996), suggesting that selective inhibition of airway ROCKs may become a good treatment for airway obstructive disease such as bronchial asthma. Although the remaining Y-27632-insensitive, acetylcholine-mediated contraction of intact muscles is not yet known here, Ca^{2+}-calmodulin–myosin light-chain kinase pathway might be thought to be involved in the remaining contraction by the following observations of rat bronchial smooth muscle. We have previously reported that the sustained tonic contraction induced by acetylcholine is completely blocked by extracellular Ca^{2+} removal (Chiba and Misawa, 1995). Secondly, acetylcholine stimulation induced a significant elevation of cytosolic Ca^{2+} concentration (measured by Fura-2) at the sustained tonic contraction (Chiba et al., 1999a). Thirdly, the Ca^{2+}-induced contraction of β-escin-permeabilized bronchial smooth muscle was not affected by Y-27632 (Fig. 3). Alternatively, Y-27632-insensitive Ca^{2+}-sensitizing pathway may also be involved in the remaining contraction of intact smooth muscle. Recent studies demonstrate a participation of a protein phosphatase inhibitory protein, CPI-17 (C-kinase-potentiated inhibitory protein of protein phosphatase-1, 17 kDa), in smooth muscle Ca^{2+} sensitization (Somlyo and Somlyo, 2000). It has been reported that CPI-17 is mainly phosphorylated and activated by protein kinase C and inhibits myosin phosphatase, resulting in Ca^{2+} sensitization of smooth muscle contraction (Somlyo and Somlyo, 2000). However, the CPI-17-mediated pathway is reported to be sensitive to β-escin permeabilization because of its relatively low molecular weight (Kitazawa et al., 1999). If the remaining contraction observed in our intact bronchial smooth muscle is mediated by CPI-17, the Ca^{2+} sensitization mediated by CPI-17 might not be found in our β-escin-permeabilized muscles, resulting in a complete inhibition of acetylcholine-induced Ca^{2+} sensitization by Y-27632. Further studies are needed to understand the

detailed mechanisms of acetylcholine-induced contraction and Ca^{2+} sensitization in airway smooth muscles.

In conclusion, Y-27632, a selective inhibitor of ROCKs, attenuates acetylcholine-induced contractile responses of rat intrapulmonary bronchial smooth muscle by inhibiting RhoA/ROCKs-mediated Ca^{2+} sensitization. Thus, selective inhibition of airway RhoA/ROCKs pathway might become a good treatment for airway obstructive disease such as bronchial asthma.

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