Relaxation of Contracted Rabbit Tracheal and Human Bronchial Smooth Muscle by Y-27632 through Inhibition of Ca^{2+} Sensitization

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The mechanism of Ca^{2+} sensitization of contraction has not been elucidated in airway smooth muscle (SM). To determine the role of a small G protein, rhoA p21, and its target protein, rho-associated coiled coil–forming protein kinase (ROCK), in receptor-coupled Ca^{2+} sensitization of airway SM, we studied the effect of (+)-(R)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride, monohydrate (Y-27632), a ROCK inhibitor, on isometric contractions in rabbit tracheal and human bronchial SM. Y-27632 completely reversed 1 μM carbachol (CCh)-induced contraction of intact trachea with a concentration producing half-maximum inhibition of effect (IC_{50}) of 1.29 ± 0.2 μM ($n = 5$). Although 4β-phorbol 12,13-dibutyrate (1 μM)–induced Ca^{2+} sensitization was relatively resistant to Y-27632 in α-toxin–permeabilized trachea, CCh (100 μM) plus guanosine triphosphate (GTP) (3 μM)– and guanosine 5′-*O*-(3′-thiotriphosphate) (10 μM)–induced contractions were relaxed completely by Y-27632 with IC_{50} of 1.44 ± 0.3 ($n = 6$) and 1.15 ± 0.3 μM ($n = 6$). Endothelin-1 (1 μM) plus GTP (3 μM)–developed force was also reversed by Y-27632 with IC_{50} of 4.10 ± 1.1 μM ($n = 6$) in the α-toxin–permeabilized bronchus. Both the rabbit and human SM expressed rhoA p21, ROCK I, and its isoform ROCK II. Collectively, rho/ROCK–mediated Ca^{2+} sensitization plays a central role in the sustained phase of airway SM contraction, and selective inhibition of this pathway may become a new strategy to resolve airflow limitation in asthma. **Yoshii, A., K. Iizuka, K. Dobashi, T. Horie, T. Harada, T. Nakazawa, and M. Mori. 1999. Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. Am. J. Respir. Cell Mol. Biol. 20:1190–1200.**

An increase in smooth muscle (SM) tension and/or phosphorylation of 20-kD regulatory light chain of myosin (MLC_{20}) at a constant Ca^{2+} concentration is referred to as Ca^{2+} sensitization (1, 2). This phenomenon is thought to be mediated mainly by a G protein–coupled inhibition of SM phosphatase 1 associated with myosin (SMPP-1M) (3–5). RhoA p21 (6), a small guanosine triphosphatase (GTPase) of ras superfamily, and protein kinase C (PKC)–dependent pathways (7, 8) are the proposed mechanisms of the SMPP-1M inhibition. Ca^{2+} sensitization is observed not only in vascular but also in other visceral SM tissues, including airway SM. Indeed, receptor-dependent, G protein–mediated Ca^{2+} sensitization occurs in canine tracheal SM (9). We have also demonstrated that the extent of Ca^{2+} sensitization is highly dependent upon receptor type, and that guanosine 5′-*O*-(3′-thiotriphosphate) (GTPγS)–sensitive and PKC-sensitive pathways may diverge in permeabilized canine tracheal SM (10).

Recently, it has been reported that two target proteins of rhoA p21, rho-associated coiled coil–forming protein kinase (ROCK I [11], also called p160ROCK [12]) and its isoform, ROCK II (13) (also known as ROKα [14] or rho kinase [15]), play a key role in the rhoA p21–mediated Ca^{2+} sensitization. (+)-(R)-Trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride, monohydrate (Y-27632) effectively inhibited ROCK I as well as ROCK II both *in vitro* and *in vivo*; Y-27632 reversed

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Abbreviations: adenosine diphosphate, ADP; calmodulin, CaM; carbachol, CCh; concentration of drug producing half-maximum contraction, EC_{50}; endothelin-1, ET-1; normal relaxing solution, G1; guanosine 5′-*O*-(2′-thiodiphosphate), GDPβS; guanosine triphosphate, GTP; guanosine 5′-*O*-(3′-thiotriphosphate), GTPγS; 3-isobutyl-1-methylxanthine, IBMX; concentration of drug producing half-maximum inhibition of effect, IC_{50}; immunoglobulin, Ig; 20-kD regulatory light chain of myosin, MLC_{20}; myosin light chain kinase, MLCK; 4β-phorbol 12,13-dibutyrate, PDBu; phosphodiesterase, PDE; protein kinase C, PKC; rho-associated coiled coil–forming protein kinase, ROCK; smooth muscle, SM; SM phosphatase 1 associated with myosin, SMPP-1M; sarcoplasmic reticulum, SR; (+)-(R)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride, monohydrate, Y-27632.

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GTPγS-induced Ca^{2+} sensitization in β-escin–permeabilized mesenteric arteries, and decreased blood pressure in the hypertensive animal models (16). Similarly, intact guinea-pig trachea contracted by histamine also responded to Y-27632 (16), suggesting that inhibition of the rho/ROCK pathway may become a new treatment not only of hypertension but also of bronchial asthma. However, the efficacy of excitatory agonists for Ca^{2+} sensitization varied in canine tracheal SM (10), and the precise mechanism of action of the different agonists also varied in rabbit vascular and ileum SM; inactivation of rhoA p21 by epidermal cell differentiation inhibitor (EDIN)–induced adenosine diphosphate (ADP)-ribosylation effectively blocked Ca^{2+} sensitization evoked by guanosine triphosphate (GTP) alone, GTP plus carbachol (CCh), GTP plus endothelin-1 (ET-1), and fluoroaluminates, but not that evoked by GTP plus phenylephrine or GTPγS (17). Thus, it remains to be elucidated whether rho/ROCK signaling might contribute to Ca^{2+} sensitization in airway SM.

The aim of this study was to determine whether inhibition of rho/ROCK signaling by Y-27632 might reverse G protein–mediated and/or PKC-mediated Ca^{2+} sensitization of airway SM *in vitro*. We permeabilized rabbit tracheal SM with *Staphylococcus aureus* α-toxin (10, 18), and measured isometric tension at a constant free Ca^{2+} in response to CCh plus GTP, GTPγS, or a phorbol ester, 4β-phorbol 12,13-dibutyrate (PDBu), followed by cumulative application of Y-27632. The inhibitory effect of Y-27632 on ET-1 plus GTP-induced Ca^{2+} sensitization was also tested in human bronchial SM permeabilized with α-toxin (18). Further, we determined expression of rhoA p21, ROCK I, and ROCK II in rabbit tracheal and human bronchial SM by immunoblot analysis.

Materials and Methods

The trachea was removed from Japanese albino rabbits (weighing 2.5 to 3.0 kg) under halothane anesthesia. The anesthesia was administered by placing the animals in an anesthetic chamber until they became unresponsive to corneal reflex. When the tracheal tissue had been removed, the animals were killed by rapid exsanguination through the carotid artery, in accordance with the recommendations of the Council of Animal Care of Gunma University (Gunma, Japan), and dissected as previously described (18).

Human bronchial SM was prepared from a macroscopically normal part of the lung tissue, which was obtained at surgery for lung cancer. The surgically resected tissue was put in ice-cold Dulbecco's modified Eagle's medium, and small bronchi with an outer diameter of 2 to 4 mm were carefully dissected as previously described (18). Consent was obtained from each patient before surgery.

Isometric Force Measurement in Intact Rabbit Tracheal SM

The rabbit tracheal strips (approximately 3 mm wide and 10 mm long with cartilage and epithelium) were set between a hook and an isometric force transducer (strain gauge TB-612T; Nihon Kohden Ltd., Tokyo, Japan) connecting an amplifier (TB-611-T; Nihon Kohden) and a multipen recorder (R66; Rika Denki Ltd., Tokyo, Japan), and vertically mounted in a 10-ml Magnus tube filled with Tyrode's solution aerated continuously with 5% CO_2 in O_2. The composition of the solution was NaCl, 136.8 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1 mM; NaH_2PO_4, 0.4 mM; $NaHPO_3$, 11.9 mM; and glucose, 5.6 mM; and temperature was kept at 37°C. At the onset of each experiment, tissues were subjected to an imposed tension of 1.0 g and allowed to equilibrate for 60 min. The solution was changed at 20-min intervals. To confirm stability of the preparations, application of high potassium (154 mEq/liter) and its washing were repeated twice, followed by CCh-induced contraction. Ibuprofen (2 μM), a cyclooxygenase inhibitor, was present throughout both the intact and the permeabilized experiments (*see following sections*) to prevent spontaneous tone development due to release of cyclooxygenase products (10, 18).

Permeabilization with α-Toxin or Triton X-100

Small strips of rabbit tracheal SM (200 to 300 μm wide; 40 to 50 μm thick; 3 mm long) and human bronchial SM (150 to 200 μm wide; 20 to 30 μm thick; 3 mm long) were mounted on a bubble plate (400 μl per bubble), and isometric force development was measured using a force transducer (AE801; SensoNor, Horten, Norway). The muscle strips were stretched to 1.3 times rest length. The composition of the solutions has been reported elsewhere (10, 18). In brief, the normal relaxing solution (G1) contained 74.1 mM potassium methanesulfonate, 2 mM Mg^{2+}, 4.5 mM adenosine triphosphate (Mg^{2+} salt), 1 mM [ethylene-*bis*(oxyethylenenitrilo)]-tetraacetic acid (EGTA), 10 mM creatine phosphate, and 30 mM 1,4-piperazinebis (ethane sulfonic acid)–KOH (pH 7.1 at 24°C, ionic strength 0.2). The same solution containing 10 mM, rather than 1 mM, EGTA and various amounts of calcium methanesulfonate was used to achieve the desired concentration of free Ca^{2+}.

The tracheal strips were exposed to α-toxin (16.4 μg/ml) with calcium ionophore A23187 (10 μM) in the pCa 6.5 solution for 30 to 45 min at 30°C, and then washed in G1 (negative logarithm of free Ca^{2+} concentration) for 5 min (10). The calcium ionophore was used to deplete Ca^{2+} stores of sarcoplasmic reticulum (SR). The temperature was kept at 24°C after permeabilization to obtain a reproducible contractile response. For permeabilization with Triton X-100, tracheal SM was incubated with 0.1% Triton X-100 in G1 at 4°C for 30 min, after which the temperature was increased rapidly to 30°C by exchange of the cold bubble plate for one that had been prewarmed, and permeabilization was continued for an additional 15 min. Cold preincubation with Triton X-100 was useful in obtaining homogeneously permeabilized preparations as reported by Lee and colleagues (19). The experiments using Triton X-100–permeabilized tracheal SM were performed at 24°C in the presence of calmodulin (CaM; 1 μM) (5).

Intact Rabbit Tracheal SM

After reproducible responses to high potassium (154 mEq/liter) were obtained, tracheal SM was contracted with CCh that was cumulatively applied to the bath (0.01 to 100 μM). In another series of experiments, when CCh (1 μM)–induced contraction became stable (approximately 20 min after the CCh application), we added Y-27632 cumulatively

to the bath. Finally, complete relaxation was confirmed by a potent phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 100 μM).

α-Toxin–Permeabilized Preparations

The maximum contraction induced by pCa 5.0 and relaxation in G1 was repeated twice. The second pCa 5.0 response was employed as the maximum contraction to normalize the following tension developments in each strip. When submaximum contraction induced by pCa 6.5 plus GTP (3 μM) was stable, CCh (100 μM) was added to the α-toxin–permeabilized SM. At the peak of additional contractions (Ca^{2+} sensitization) Y-27632 was cumulatively added to the strip (except as shown in Figure 8b, where a single dose of Y-27632 was applied to the strip). Control experiments were run in parallel in which the same amount of the vehicle (water) for Y-27632 was added to the strips at the same intervals as the Y-27632 application. Similar protocol was carried out for ET-1 plus GTP–induced, GTPγS-induced, and PDBu-induced Ca^{2+} sensitization. We used CCh to evaluate agonist-induced Ca^{2+} sensitization in rabbit trachea because CCh was the most potent Ca^{2+}-sensitizing agonist in canine trachea (10), and muscarinic acetylcholine receptor–mediated signaling was preserved in α-toxin–permeabilized rabbit trachea (18). On the other hand, in human bronchial SM studies we preferred ET-1 to CCh because ET-1 response was more constantly observed than was CCh response. This is presumably due to sparse distribution of muscarinic acetylcholine receptor at the distal portion of bronchial tree (20), although human bronchial tree throughout expresses endothelin B–receptor subtype that mediates ET-1–induced contraction (21).

Triton X-100–Permeabilized Preparations

In the preliminary experiments with α-toxin–permeabilized SM, we observed that Ca^{2+}-induced contraction was partially reversed by Y-27632. To determine whether Y-27632 might affect the activity of myosin light chain kinase (MLCK) of airway SM, we applied Y-27632 to Triton X-100–permeabilized rabbit trachea. Although MLCK and SMPP-1M system was preserved, introduction of val14p21^{rhoA} (a constitutive active rho mutant) to the Triton X-100–permeabilized SM failed to evoke a contraction, indicating that the rho/ROCK–mediated Ca^{2+}-sensitizing system was removed in such heavily permeabilized strips (17). After the maximum contraction at pCa 5.0 with CaM (1 μM) was obtained, the strip was relaxed in G1 and then pCa 6.0 plus CaM (1 μM)–induced contraction was evoked. When the produced force was stable, GTPγS was added to the strip to ascertain functional removal of the rho/ROCK pathway from the Triton X-100–permeabilized strip, followed by cumulative application of Y-27632. Finally, calyculin A (300 nM), a potent phosphatase-1 and -2 inhibitor (19), was added to the strip.

Determination of rhoA p21, ROCK I, and ROCK II in Airway SM

Four to five dissected airway SM strips were placed in 60 μl of glycerol sample buffer (containing 1% sodium dodecyl sulfate [SDS], 10% glycerol, 20 mM dithiothreitol, and 100 μg/ml bovine serum albumin [BSA]) and manually ground with a small glass–glass homogenizer on ice. The amount of 10 μl of the homogenates was used for protein assay (Bio-Rad Protein Assay; Bio-Rad, South Richmond, CA) using BSA as a standard. The resultant 50-μl homogenates were transferred to a 1-ml centrifuge tube. To precipitate the protein, 694 μl of cold distilled water, 250 μl of 24% trichloroacetic acid, and 6 μl of 2% deoxycholate were added to the tube, followed by centrifuging for 10 min at 5,000 × *g*. Supernatant was discarded, the resultant pellet was neutralized with 1 M Tris base, and 20 μl of sample buffer (Laemmli) was added to the pellet. Approximately 4 μg protein was subjected to each well of SDS polyacrylamide gel electrophoresis (PAGE) (acrylamide, 12.5% for rhoA p21 and SM α-actin; 7.5% for ROCK I and ROCK II). The proteins were transferred to the nitrocellulose membrane, and immunostaining was carried out using the following antibodies and visualized with enhanced chemiluminescence (Amersham, Buckinghamshire, UK): monoclonal anti-rhoA p21 (1:500), monoclonal anti-ROCK II (1:1,000), polyclonal anti-ROCK I (1:1,000), and monoclonal anti-SM α-actin (1:5,000) were applied to the membrane for 4 h at room temperature. After rinsing the unbound primary antibodies and blocking the nonspecific binding with nonfat milk, we used treatment with sheep antimouse or donkey antirabbit immunoglobulin (Ig)G antibodies linked to horseradish peroxides (1:5,000) for 2 h at room temperature to detect the primary antibodies.

Reagents

S. aureus α-toxin was obtained from Research Biochemicals International (Natick, MA); CCh, IBMX, and CaM were from Sigma (St. Louis, MO); and ET-1, PDBu, and calyculin A were from Calbiochem (La Jolla, CA). Y-27632 was a gift from Yoshitomi Pharmaceutical Industries Ltd. (Osaka, Japan). Y-27632 was dissolved in distilled water as stock solution (10 mM), and stored at −20°C until use. Guanosine 5′-*O*-(2′-thiodiphosphate) (GDPβS), GTP, and GTPγS were from Boehringer Mannheim (Indianapolis, IN). Monoclonal anti-SM α-actin antibody was purchased from Sigma. A mouse monoclonal antibody against rhoA p21 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a mouse monoclonal antibody against ROKα (ROCK II) was purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-p160ROCK (ROCK I) polyclonal antibody was a gift from T. Ishizaki (Kyoto University, Kyoto, Japan) (22). The second antibodies (sheep antimouse or donkey antirabbit IgG) were obtained from Amersham.

Statistical Analysis

Unless noted otherwise, the developed force was normalized to the initial pCa 5.0 response in the same strip, and given as mean ± standard error. Data were compared by the Mann–Whitney U test or Student's *t* test with the Bonferroni correction for multiple comparisons. A P value of < 0.05 was considered to be statistically significant in the Mann–Whitney U test, and a significant level of $P < 0.05/m$ (where m is the number of comparisons) was used in the Bonferroni method.

Results

Intact Rabbit Tracheal SM

As shown in Figure 1a, CCh dose-dependently contracted the intact rabbit tracheal SM with a concentration of drug producing half-maximum contraction (EC_{50}) of 0.37 ± 0.1 μM (n = 5), and reached a peak (12.0 ± 1.2 mN, n = 5) at 100 μM CCh. The CCh (1 μM)–induced contractions were reversed completely by Y-27632 with a concentration of drug producing half-maximum inhibition of effect (IC_{50}) of 1.29 ± 0.2 μM (n = 5) (Figure 1b). IBMX did not reduce the tone further (data not shown).

Expression of rhoA p21, ROCK I, and ROCK II in Airway SM

Figure 2 shows that both rabbit tracheal and human bronchial SM expressed rhoA p21, ROCK I, and ROCK II. Although comparable densities of SM α-actin (Figure 2d) suggest that similar amounts of SM proteins of rabbit and human tissues were run in the gels, ROCK II (Figure 2b) and rhoA p21 (Figure 2c) showed a tendency of lower expression in human tissue than in rabbit tissue.

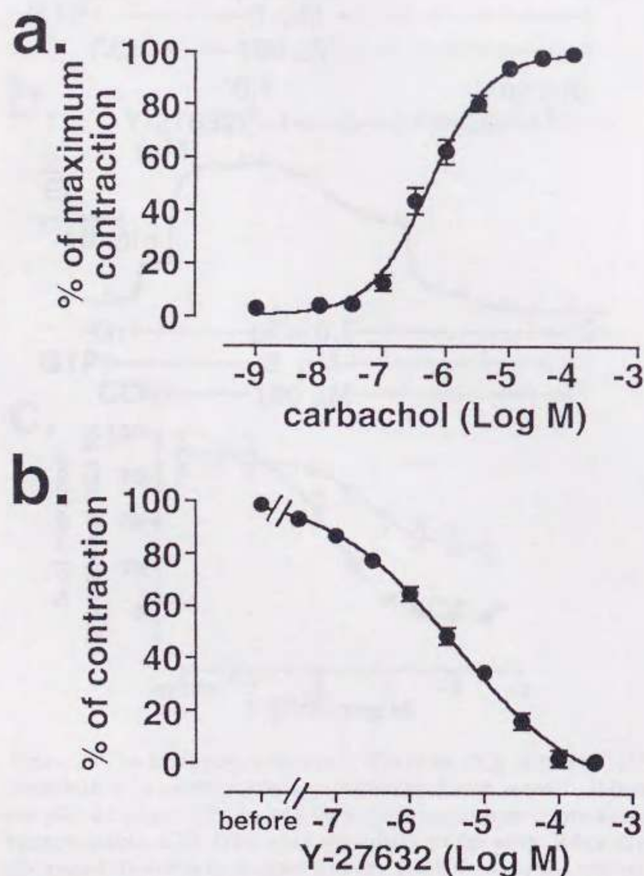

Figure 1. Dose–response curves for CCh and Y-27632 in intact rabbit trachea. CCh was applied to intact rabbit trachea (n = 5) cumulatively (*a*). After the CCh (1 μM) response became stable, Y-27632 was cumulatively added to the strip (n = 5) (*b*).

Effect of Post-Treatment with Y-27632 on Agonist-Induced Ca^{2+} Sensitization in Rabbit Tracheal and Human Bronchial SM Permeabilized with α-Toxin

The pCa 5.0–induced contractions before Ca^{2+} sensitization were 2.97 ± 0.1 mN in rabbit trachea (n = 45) and 1.92 ± 0.4 mN in human bronchial SM (n = 10). The stable contractions induced by pCa 6.5 plus GTP (3 μM) in trachea (Figure 3b) and by pCa 6.7 plus GTP (3 μM) in bronchus (Figure 4b) were 12.2 ± 5.3% (n = 6) and 21.9 ± 2.1% (n = 4), respectively. In the presence of GTP (3 μM), CCh (100 μM) induced an additional contraction at a fixed free Ca^{2+} concentration of pCa 6.5 in α-toxin–permeabilized trachea (Figure 3a). The peak force achieved 84.4 ± 3.0% (n = 6) of the initial contraction at pCa 5.0, followed by spontaneous decline of force in the control strips. The resultant force approximately 60 min after the CCh application was 34.1 ± 9.9% (n = 6). The resultant contractions were reversed to the initial submaximum level of contrac-

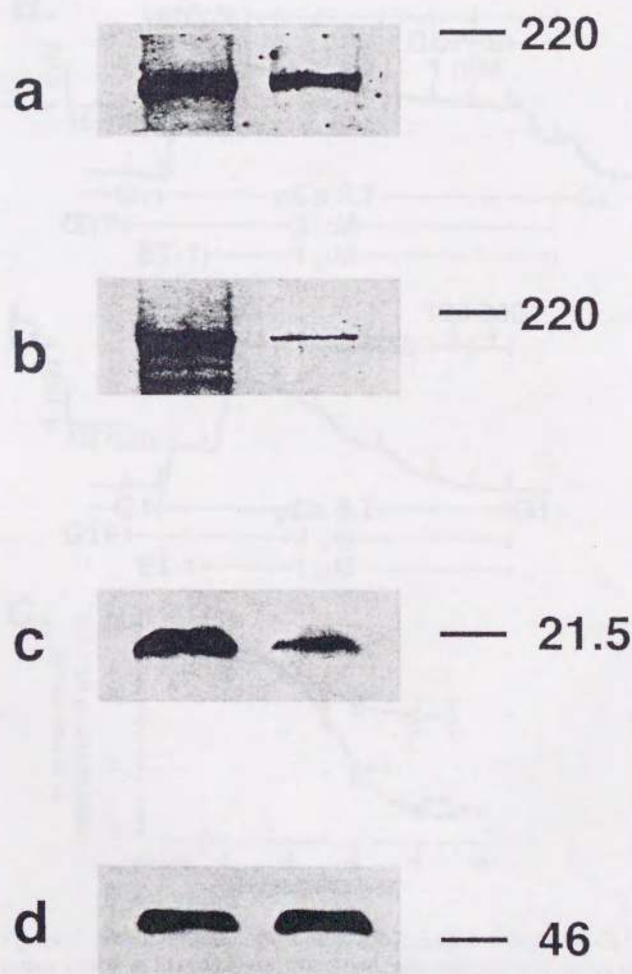

Figure 2. Immunoblot analysis of rhoA p21, ROCK I, and ROCK II in airway SM. *Arrows* indicate standard molecular weight. Crude homogenates of rabbit tracheal (*left lane*) and human bronchial SM (*right lane*) were run in SDS-PAGE. The data represent four sets of separate experiments. Panels *a*, *b*, *c*, and *d* indicate ROCK I, ROCK II, rhoA p21, and SM α-actin, respectively.

tion at pCa 6.5 by a nonpermeable G-protein inhibitor, GDPβS (1 mM), indicating that the CCh-induced additional contraction was due to G-protein–mediated Ca^{2+} sensitization in completely permeabilized preparations, and that the sensitization was at least partially retained at this point (Figure 3a). As shown in Figure 3b, Y-27632 also reversed the CCh (100 μM)–induced Ca^{2+} sensitization in a dose-dependent manner. The peak contraction of the CCh-induced Ca^{2+} sensitization was 86.9 ± 11.4% (n = 6). Note that the force returned to the basal tone at 100 μM of Y-27632 (−2.69 ± 3.0%, n = 6). The dose–response relationship is shown in Figure 3c. The IC_{50} value was 1.44 ± 0.3 μM (n = 6).

Similar findings were observed in ET-1–induced Ca^{2+} sensitization of the α-toxin–permeabilized human bronchial SM (Figure 4). The peak force developments induced by ET-1 in Y-27632–treated and nontreated (control) strips were 64.7 ± 4.1% (n = 6) and 59.0 ± 5.1% (n = 4), respectively. The ET-1–induced contractions were reversed to basal level (0.21 ± 1.0%, n = 6) by Y-27632 at 300 μM, whereas the resultant force before GDPβS application in control strips was 36.5 ± 6.8% (n = 4). Note that GDPβS relaxed the ET-1–induced contraction to the prior submaximum contraction level at pCa 6.7. Therefore, these results indicate again that the extent of ET-1–induced Ca^{2+} sensitization was reduced but still substantially retained at this point. The IC_{50} value for Y-27632 in ET-1–induced contraction of bronchial SM was 4.10 ± 1.1 μM (n = 6).

Selectivity of Y-27632 toward ROCK but Not MLCK

In α-toxin–permeabilized strips, the mechanism of the Ca^{2+} sensitizing system was retained almost completely. In

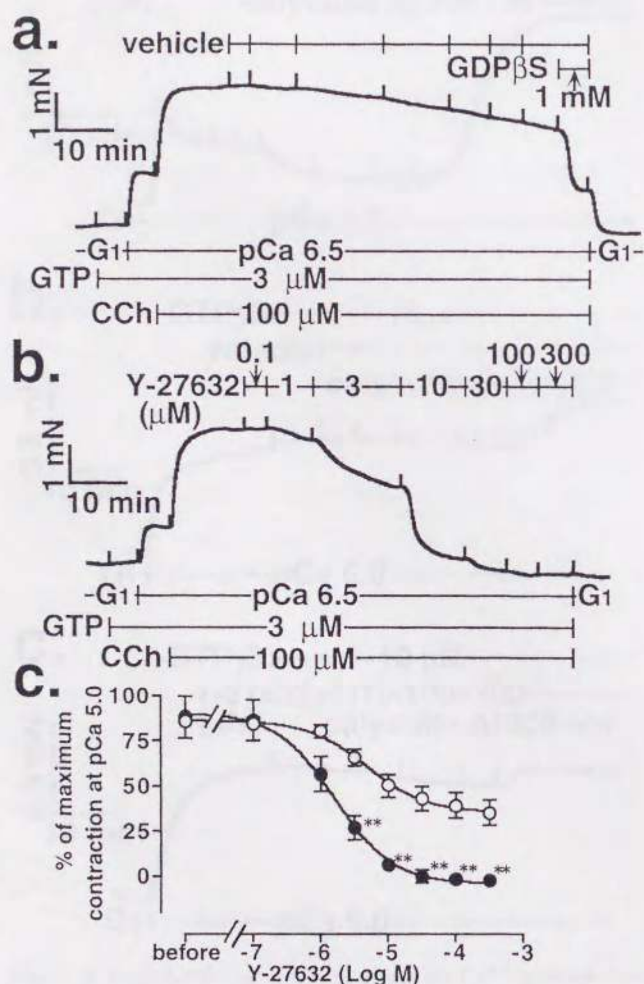

Figure 3. The inhibitory effect of Y-27632 on CCh-induced Ca^{2+} sensitization in rabbit trachea permeabilized with α-toxin. When the pCa 6.5 plus GTP (3 μM)–induced submaximum contraction became stable, CCh (100 μM) was added to the strip. After the additional contraction reached a peak, Y-27632 (*b*) or the vehicle (*a*) was cumulatively applied to the strip. A G-protein inhibitor, GDPβS, was added as indicated in control experiments (*a*). Data from six experiments are summarized in *c*. Y-27632 (*filled circle*) or the vehicle (*open circle*) was applied to the strip. $^{**}P < 0.01$ versus control.

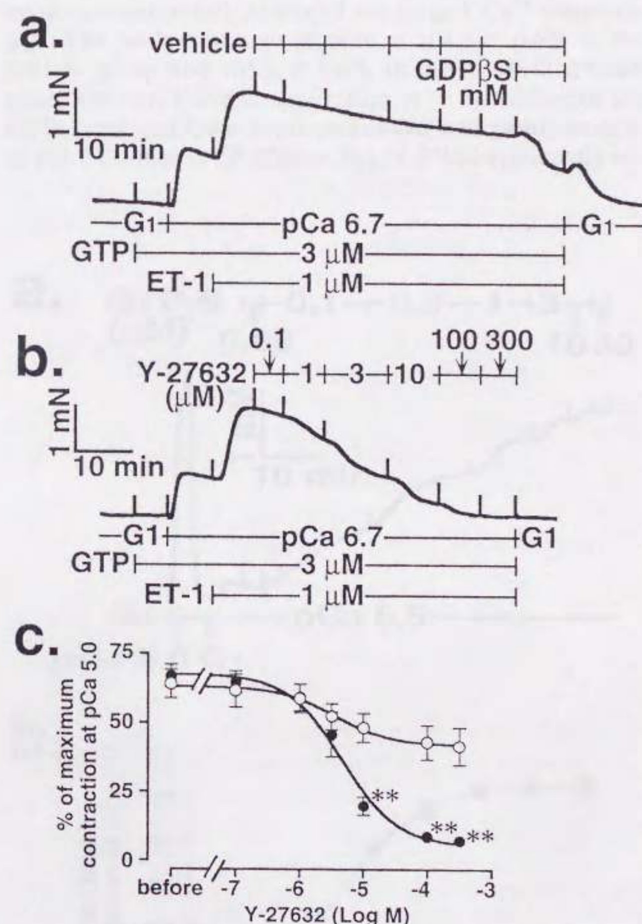

Figure 4. The inhibitory effect of Y-27632 on ET-1–induced Ca^{2+} sensitization in human bronchial SM permeabilized with α-toxin. When the pCa 6.7 plus GTP (3 μM)–induced submaximum contraction became stable, ET-1 (1 μM) was added to the strip. After the additional contraction reached a peak, Y-27632 (*b*) or the vehicle (*a*) was cumulatively applied to the strip. A G-protein inhibitor, GDPβS, was added as indicated in control experiments (*a*). Data from four to six experiments are summarized in *c*. Y-27632 (*filled circle*) or the vehicle (*open circle*) was applied to the strip. $^{**}P < 0.01$ versus control.

contrast, Triton X-100 permeabilization entirely disrupted the SMPP-1M regulatory system for Ca^{2+} sensitization while preserving MLCK and the function of calyculin A (a potent phosphatase inhibitor). As shown in Figure 5a, Y-27632 partially but significantly relaxed the pCa 6.0–induced contraction in the α-toxin–permeabilized strips; Y-27632 at 300 μM decreased the contraction to 32.1 ± 2.1% ($n = 3$), normalized to the initial contraction at pCa 6.0. The time-matched control strips did not show any decline of force (trace not shown). In Triton X-100–permeabilized strips, the maximum contraction at pCa 5.0 with CaM (1 μM) was 1.07 ± 0.2 mN ($n = 7$). There was no response to GTPγS, and no difference in force reduction between Y-27632–treated strips (5.6 ± 10.3%, normalized to the peak force at pCa 6.0, $n = 4$) and control strips (4.8 ± 3.7%, $n = 3$) before calyculin A application (Figures 5b and 5c). Calyculin A contracted both α-toxin– and Triton X-100–permeabilized strips either with or without Y-27632.

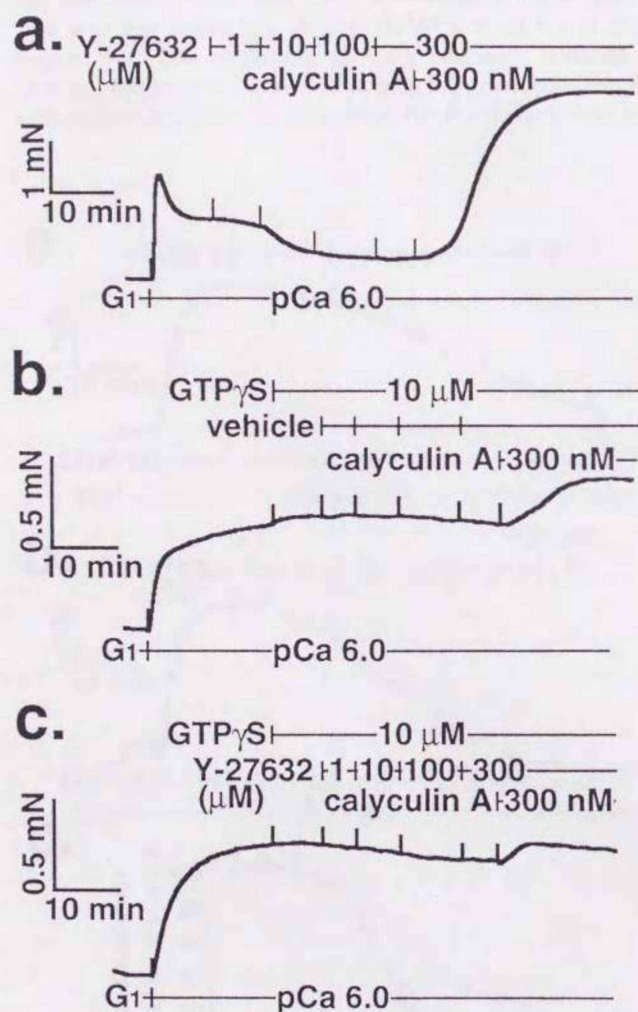

Figure 5. Comparison of Y-27632 effect on Ca^{2+}-induced contraction between α-toxin– and Triton X-100–permeabilized strips. When the pCa 6.0 response became stable, Y-27632 was applied to α-toxin– (*a*) or Triton X-100– (*c*) permeabilized rabbit tracheal SM. The same amount of vehicle was added to the time-matched control strip permeabilized with Triton X-100 (*b*). CaM (1 μM) was present in Triton X-100– (*b* and *c*) but not in α-toxin– (*a*) treated preparations. The final calyculin A (300 nM)–induced contraction was achieved to the initial maximum contraction level at pCa 5.0 in both α-toxin– (*a*) and Triton X-100– (*b* and *c*) permeabilized strips. The traces represent at least three sets of separate experiments.

GTPγS-Induced Ca^{2+} Sensitization

GTPγS, a stable GTP analogue, activates both trimeric and monomeric GTPase including rhoA p21. Indeed, GTPγS was a potent Ca^{2+} sensitizing agent in α-toxin–permeabilized canine trachea (10). When the pCa 6.5 response was stable, we cumulatively added GTPγS to the strip. GTPγS dose-dependently increased the Ca^{2+} sensitivity, and the force increased from 3.32 ± 0.5% before GTPγS application to 85.4 ± 3.6% ($n = 6$) at 30 μM of GTPγS (Figure 6a). The response to GTPγS reached a peak at 3 μM with EC_{50} of 0.16 ± 0.03 μM ($n = 6$) (Figure 6b). Single (noncumulative) application of GTPγS at 10 μM (a supramaximum concentration) produced the largest Ca^{2+} sensitization. The peak contractions were at 104.6 ± 6.6% in the control group and 105.3 ± 6.6% in the Y-27632–treated group (before Y-27632 application, $n = 6$). Although the GTPγS-induced force development declined spontaneously, as did CCh plus GTP (Figure 7a), Y-27632 apparently ac-

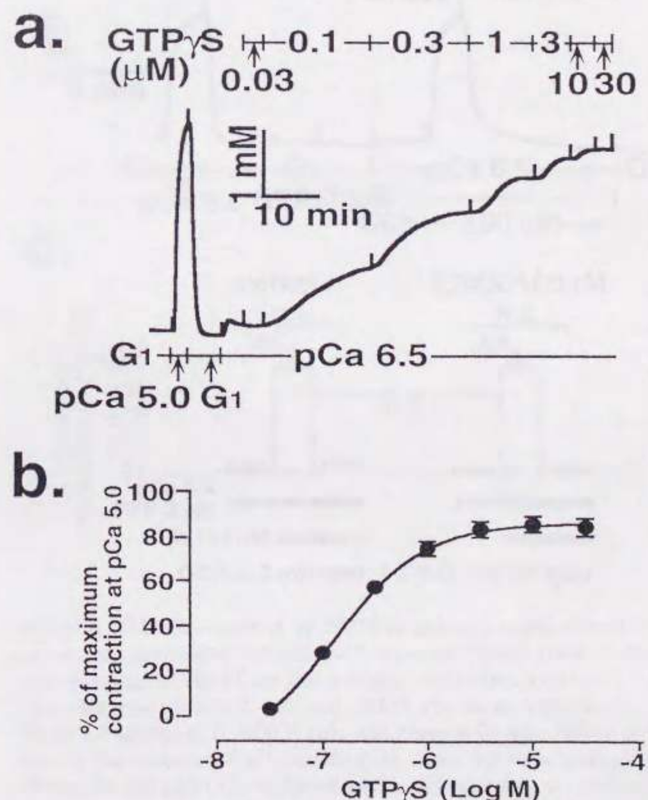

Figure 6. GTPγS-induced Ca^{2+} sensitization in rabbit trachea permeabilized with α-toxin. To determine the dose–response relationship in GTPγS-induced Ca^{2+} sensitization we cumulatively added GTPγS to the strip that was contracted by pCa 6.5 (*a*). Dose–response curves for GTPγS are shown in (*b*) ($n = 6$).

celerated relaxation with the IC_{50} value of 1.15 ± 0.3 μM (n = 6) (Figure 7b). Data are summarized in Figure 7c.

Rapid Relaxation of Ca^{2+}-Sensitized Airway SM by Noncumulative Application of Y-27632, and Lack of Effect of IBMX

To minimize the time-dependent factors, we added a supramaximum concentration of Y-27632 (100 μM) to the strips contracted by CCh (100 μM) at the peak in the presence of pCa 6.5 plus GTP (3 μM), resulting in a rapid relaxation (Figure 7b). The half-time ($t_{1/2}$) of relaxation was 70.6 ± 11.8 s (n = 4, $P < 0.01$) in the Y-27632–applied group and 80.9 ± 3.7 s (n = 4, $P < 0.01$) in the GDPβS-applied group. Note that $t_{1/2}$ of spontaneous force reduction was approximately 30 min (1,637.5 ± 44.5 s, n = 6, Figure 3a). The amplitude of CCh response reached a comparable level with the initial pCa 5.0–induced contraction, indicating that CCh sensitized the contractile machinery apparatus to near maximum level in the presence of GTP. The possibility of incomplete permeabilization was ruled out by the finding that GDPβS (2 mM) completely reversed the CCh response to the prior level (9.05 ± 2.1%, n = 4, Figure 8a). To examine whether the Y-27632 effect was due to inhibition of PDE activity, we treated the permeabilized strip with IBMX, a potent PDE inhibitor. As shown in Figure 8b, IBMX (100 μM) was present 20 min before and during the responses to CCh and Y-27632. The submaximum contraction induced by pCa 6.5 with GTP (3 μM) showed a tendency of reduction in the presence of IBMX (4.25 ± 0.3% in the IBMX-treated group and 5.96 ± 0.5% in the control group, n = 4). Even in the pres-

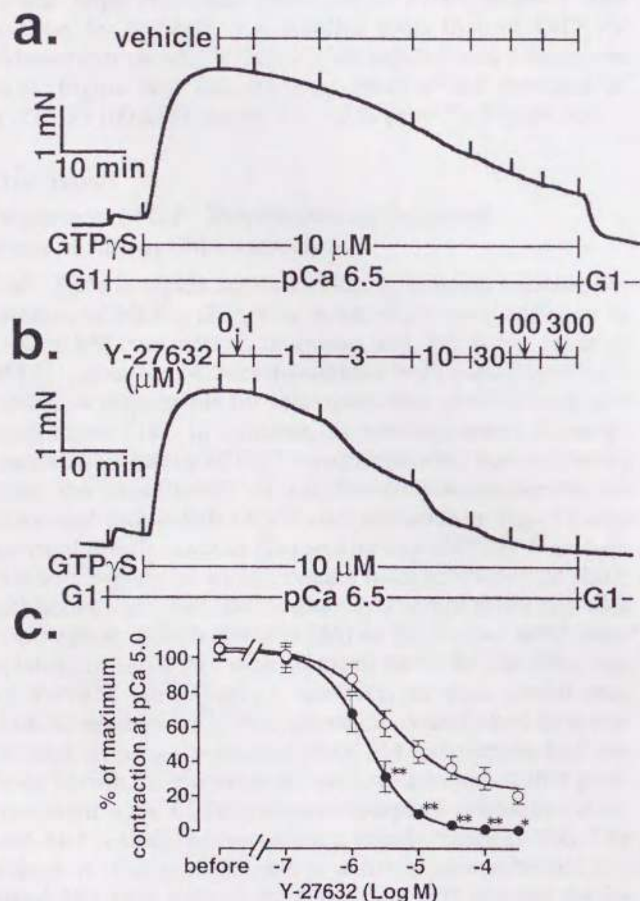

Figure 7. Inhibition of GTPγS-induced Ca^{2+} sensitization by Y-27632 in rabbit trachea permeabilized with α-toxin. When the 10 μM GTPγS–induced additional contraction reached a peak, we added the vehicle (*a*) or Y-27632 (*b*) to the strips. The traces represent six sets of separate experiments. Data from Y-27632–treated (*filled circle*, n = 6) and control strips (*open circle*, n = 6) are summarized in *c*. *$P < 0.05$ versus control; **$P < 0.01$ versus control.

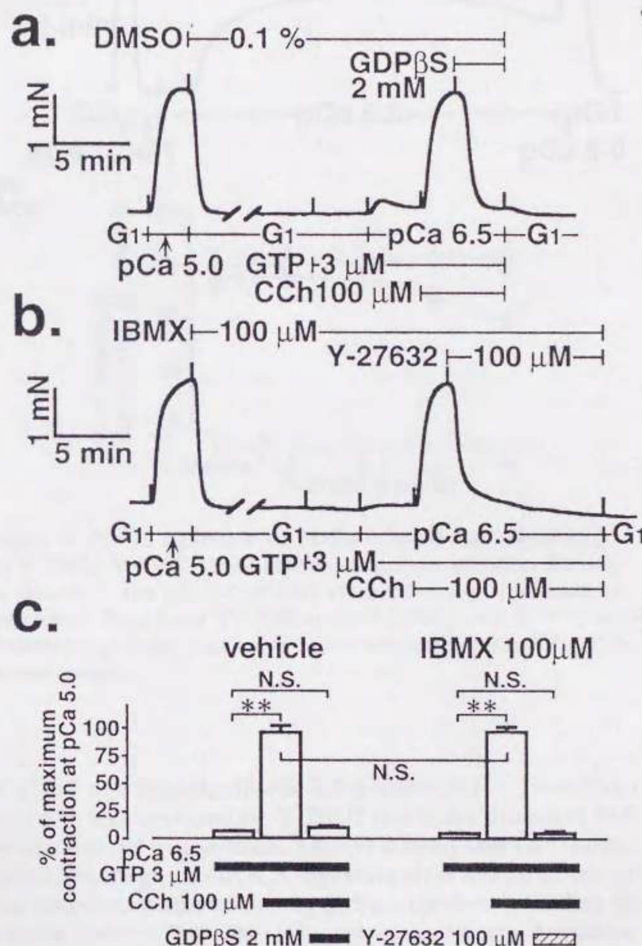

Figure 8. GDPβS-induced or Y-27632–induced rapid relaxation of α-toxin–permeabilized and Ca^{2+}-sensitized rabbit trachea, and lack of effect of IBMX on these events. After the initial pCa 5.0 response was obtained, 100 μM IBMX (*b*) or its vehicle, 0.1% dimethyl sulfoxide (DMSO) (*a*), was present 20 min before and during the following Ca^{2+} sensitization experiments as indicated. When the 100 μM CCh–induced additional contraction reached a peak, we added 2 mM GDPβS (*a*) or 100 μM Y-27632 (*b*) to the strips. The traces represent four sets of separate experiments. Data are summarized in *c*. **$P < 0.01$ versus the submaximum contractions induced by pCa 6.5 plus 3 μM GTP (Bonferroni method). The CCh response with or without IBMX was not significant (Mann–Whitney U test). N.S. indicates not significant.

ence of IBMX, however, CCh-induced full Ca^{2+} sensitization was still observed (95.2 ± 5.9% in the IBMX-treated group, 95.0 ± 5.9% in the control group, $n = 4$), and Y-27632 (100 μM) relaxed the contraction (4.24 ± 2.5%, $n = 4$). Thus, IBMX failed to affect both the CCh-induced Ca^{2+} sensitization and Y-27632–induced relaxation (Figure 8c).

PDBu-Induced Ca^{2+} Sensitization in Rabbit Tracheal SM Permeabilized with α-Toxin

The prior submaximum contractions at pCa 6.5 without GTP were 2.29 ± 0.4% in the control group ($n = 7$), and 2.00 ± 0.6% in the Y-27632–treated group ($n = 5$). A PKC activator, PDBu (1 μM), gradually caused Ca^{2+} sensitization without any spontaneous force reduction in control experiments of rabbit tracheal SM permeabilized with α-toxin (Figure 9a). The developed force became stable 35 to 45 min after the PDBu application. The peak amplitudes achieved were 68.9 ± 5.7% in the Y-27632–treated group ($n = 5$) and 75.0 ± 7.5% in the control group ($n = 7$), followed by cumulative addition of Y-27632 or vehicle to the strips. Although inhibition of PDBu-induced contraction by Y-27632 was smaller than that of GTPγS-induced contraction, Y-27632 dose-dependently relaxed the strip (Figure 9b). The resultant force in the presence of Y-27632 (100 μM) was 48.4 ± 4.2% ($n = 7$) (Figure 9c).

Discussion

Importance of Ca^{2+} Sensitization on Sustained Phase of Airway SM Contraction

Ca^{2+}–CaM complex activates MLCK, leading to phosphorylation of MLC_{20} (23). This is necessary and sufficient to initiate SM contraction. In airway SM, like other types of SM (1), inositol 1, 4, 5-triphosphate ($InsP_3$)–mediated Ca^{2+} release is responsible for this initial step of CCh-triggered contraction (18). In contrast, to maintain force developments, contribution of Ca^{2+} sensitization has been expected from the experiments of simultaneous measurement of force and intracellular Ca^{2+} concentration in intact (nonpermeabilized) vascular (24) and airway SM (25). Recently, this was supported by the results from inhibition of rhoA p21 activity in intact SM by glucosylation of rhoA p21 with *Clostridium difficile* toxin B (26) or by *in vivo* ADP-ribosylation of rhoA p21 with chimeric toxin DC3B (27). Until Y-27632 was reported, however, to what extent rho/ROCK–mediated Ca^{2+} sensitization contributed to maintenance of force in intact airway SM contraction had not been known. In the present study we confirmed that posttreatment with Y-27632 caused complete relaxation in intact and α-toxin–permeabilized rabbit tracheal SM. The values of IC_{50} in intact and in α-toxin–permeabilized tracheal SM were very close. When Y-27632 relaxed the intact strips contracted by CCh, mainly through inhibition of rho/ROCK–mediated Ca^{2+} concentration, the IC_{50} values should be comparable between intact and permeabilized strips. Thus, the similar IC_{50} values of intact versus permeabilized strips support the idea that relaxation of intact trachea was due to selective inhibition of the rho/ROCK pathway by Y-27632. Similar experiments were performed with human bronchial SM to estimate the clinical applicability of Y-27632 as a bronchodilator. ET-1–induced Ca^{2+} sensitization was also reversed by Y-27632 in human bronchial SM permeabilized with α-toxin. Thus, it is likely that Ca^{2+} sensitization through rho/ROCK signaling plays a central role in the sustained phase of airway SM contraction, including in human airway SM. The IC_{50} values in human bronchus were larger than those in rabbit trachea (IC_{50} for Y-27632: 4.10 μM versus 1.44 μM, respectively). A relatively small amount of expressed proteins, rhoA p21 and ROCK II (Figure 2), may account for the difference in the IC_{50} values. Further studies are required for qualitative and quantitative determination of these proteins in human airway SM.

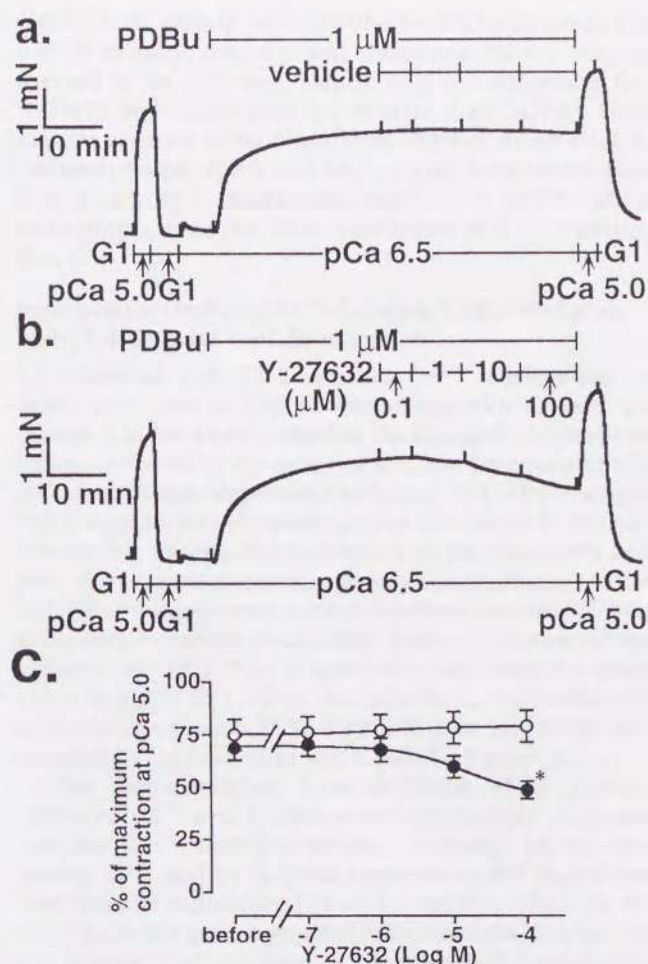

Figure 9. Partial inhibition of PDBu-induced Ca^{2+} sensitization by Y-27632. Y-27632 (*b*) or the vehicle (*a*) was added to the strips as indicated. The traces represent at least five sets of separate experiments. Data from Y-27632–treated (*filled circle*, $n = 7$) and control strips (*open circle*, $n = 5$) are summarized in *c*. $^*P < 0.05$ versus control.

Inhibitory Action of Y-27632 toward ROCK but Not MLCK

Although Ca^{2+}-induced contraction of β-escin–permeabilized rabbit mesenteric artery was not affected significantly by Y-27632 in the original report (16), Y-27632 ap-

parently relaxed the Ca^{2+}-induced contraction of α-toxin–permeabilized rabbit tracheal SM in the present study (Figure 5a). However, it is not likely that this was due to inhibition of MLCK by Y-27632 because Y-27632 had no apparent effect on Ca^{2+}-induced contraction of Triton X-100–treated strips (Figure 5c). Both Y-27632–treated and control strips showed approximately 5 to 6% decline of force, which is a "run down" phenomenon usually observed in preparations permeabilized by saponin, β-escin, and Triton X-100, but not α-toxin (5, 28). Complete loss of GTPγS response in the Triton X-100–treated strips suggests that rho/ROCK signaling was disrupted entirely by treatment with Triton X-100 (17). In contrast, the initial pCa 5.0 plus CaM–induced contraction and the final calyculin A–induced contraction indicate that the regulatory mechanism of MLC_{20} phosphorylation level by MLCK and SMPP-1M was preserved even in such heavily permeabilized strips (5).

Rho kinase (ROCK II) is known not only to inhibit phosphatase activity of SMPP-1M but also to directly phosphorylate Ser 19 of MLC_{20}, as does MLCK (29). In the β-escin–permeabilized strip, influx of 150-kD protein such as IgG and efflux of lactate dehydrogenase (approximately 140 kD) were observed (28), although proteins greater than 1 kD were not able to pass through the pores produced by α-toxin (10, 28). Thus, partial leakage of soluble compartment(s) of the rho/ROCK pathway may be the reason why Ca^{2+}-induced contraction of the β-escin–permeabilized strip was relatively insensitive to Y-27632, although this speculation should be examined further. The EC_{50} value for GTPγS in rabbit trachea permeabilized with α-toxin (the present study) was approximately five to six times smaller than that in bovine tracheal SM permeabilized with saponin (30) (EC_{50} for GTPγS: 0.16 versus 0.9 μM); whereas the 61-kD protein, Ca^{2+}-independent MLCK, was permeable as in β-escin–treated portal vein SM (28), supporting this possibility. Taken together, in α-toxin–permeabilized SM it is more likely that Y-27632 inhibited basal activity of ROCK I (and/or ROCK II) but did not affect MLCK activity, as reported (16).

Difference in the Effects of GDPβS and Y-27632

Why did Y-27632 more effectively reverse the agonist-induced Ca^{2+} sensitization than did GDPβS (Figures 3 and 4)? We propose a possibility that basal ROCK activity was present and GDPβS did not affect the activity. This idea is supported by the following reasons: (*1*) EDIN, which inactivates rhoA p21 via ADP-ribosylation, inhibited submaximal Ca^{2+}-induced contractions, whereas GDPβS did not affect the pCa-tension curves in the permeabilized rabbit mesenteric artery (17). This is very similar to the results of this study using Y-27632. (*2*) Amano and colleagues demonstrated that ROCK II phosphorylated MLC_{20} in the absence of rhoA p21, and that the GTPγS-bound form of rhoA p21 accelerated this reaction by approximately twice *in vitro* (29), suggesting that GTP may not be essential for the basal activity of ROCK. (*3*) Although GDPβs effectively reversed agonist- (this study) and fluoroaluminates-induced Ca^{2+} sensitization (10), GTPγS (10 μM)–induced Ca^{2+} sensitization was totally resistant to the GDPβS (1 mM) treatment (data not shown). Because Y-27632 effectively reversed the GTPγS response (Figure 7), efficacy, potency, and action sites of the reagents seemed to be different. Hence, it is not surprising that Y-27632 was a more effective relaxant than GDPβS. However, it remains to be elucidated whether direct ROCK-induced phosphorylation of MLC_{20} contributes to the basal ROCK activity *in situ* because inhibition of SMPP-1M has been proposed as the main mechanism of Ca^{2+} sensitization (1).

Spontaneous Decline of CCh-Induced, GTPγS-Induced, and ET-1–Induced Ca^{2+} Sensitization

CCh-induced and ET-1–induced Ca^{2+} sensitization reduced with time in SM permeabilized with α-toxin. Although it is not known whether the diminished agonist response occurred at the receptor site, the postreceptor site, or both, the time-dependent reduction in GTPγS-induced force suggests that the postreceptor site seems to be more responsible for the desensitization to the excitatory agonists. Gong and associates reported similar desensitization to GTPγS accompanied by translocation of rhoA p21 from cytoplasm to membrane fraction in α-toxin–permeabilized vascular SM (31). This is also the reason why the maximum response to GTPγS was smaller in the cumulative application protocol (85%; Figure 6) than that in the noncumulative application protocol (105%; Figure 7).

The time-dependent force reduction of G protein–mediated Ca^{2+} sensitization was a physiologic phenomenon but not a technical artifact. Although an approximately 30% decline of force appeared in the phenylephrine-induced contractions of intact vascular SM (16), the IC_{50} values are quite comparable between the original and our studies. Unfortunately, a time-matched control trace of the GTPγS response was not shown in the original paper (16). Our traces of post-treatment with Y-27632 were very similar to that in the original paper. Therefore, the time-dependent force reduction of the GTPγS response should be observed in β-escin–permeabilized vascular SM. However, the extent of force reduction might be only to a lesser extent because relative and/or absolute Ca^{2+} sensitization would be small in β-escin–permeabilized SM (*see below*). Furthermore, pCa 6.0 response in the original paper also showed an approximately 30% decline of force (16). By contrast, the responses to pCa 6.0 (data not shown) and to PDBu (Figure 9) in this study were well maintained, and the absolute force of contraction was much larger in the present study than in the original study. These differences were presumably caused not only by the preparations (differences in strip size and in SM types, vascular versus airway SM) but also by the permeabilization method (β-escin versus α-toxin). β-escin– but not α-toxin–permeabilized strips show "run down," as up to 150-kD–size proteins leak from the pores produced by β-escin (28). So far, the most successful permeabilization method for the long-term maintenance of both receptor coupling and contractility is by α-toxin (18, 28). Indeed, the amplitude of contractions at pCa 5.0 and also at pCa 6.5 with Ca^{2+} sensitizing agents in permeabilized preparations was larger than CCh (100 μM)–induced maximum contractions in intact preparations before permeabilization of the same strips (10). The extents of Ca^{2+} sensitization before

Y-27632 application in the α-toxin–permeabilized airway SM were 86.9 ± 11.4% (CCh, $n = 6$), 59.0 ± 5.1% (ET-1, $n = 4$), and 105.3 ± 6.6% (GTPγS, $n = 6$) (normalized to the initial pCa 5.0 response). In the original paper, pCa 5.0 response was not shown (16). Personal communication with the authors, Uehata and coworkers, confirmed that the extent of GTPγS-induced contraction was 35.6 ± 6.5% ($n = 4$) in their β-escin–permeabilized vascular SM. These are reasonable values because they are consistent with data from using the β-escin–permeabilized vascular SM (28); however, they are much smaller than those of α-toxin–permeabilized airway SM in previous (10) and present studies. Thus, one possible explanation for the substantial force reduction in this study is presumably the initial large response to the Ca^{2+} sensitizing agents (e.g., approximately three times larger GTPγS response: this study [105%] versus the original report [36%]) in the α-toxin–permeabilized SM, where preservation of the signal transduction system for Ca^{2+} sensitization was nearly complete. Another possibility is that rabbit trachea is a more phasic type SM than rabbit mesenteric artery, because we succeeded in Ca^{2+} release experiments from the SR with α-toxin–permeabilized rabbit trachea (18). In general, it is difficult to carry out the Ca^{2+} release protocol using tonic-type SM such as rabbit femoral artery. Hence, serious changes in tension with G protein–mediated Ca^{2+} sensitizing agents reveal the time course of the G protein–mediated Ca^{2+} sensitization in the most successfully permeabilized airway SM rather than technical error.

In this case it is important to determine the statistical significance between the time-matched control group and the Y-27632–treated group because time-dependent factors were involved in both groups. At 3 μM and more in rabbit and 10 μM and more in human airways, Y-27632 significantly decreased the force developments. Further, it should be noted that in the control strips GDPβS induced force reduction, indicating that agonist-triggered Ca^{2+} sensitization still remained at this point. Therefore, the IC_{50} data with statistical significance to time-matched strips are useful in comparing our data with the original data, and our data will provide further information on the time course of G protein–mediated Ca^{2+} sensitization in airway SM.

To minimize the time-dependent factors, especially diffusional delays, we applied a single dose of Y-27632 (100 μM) to the sensitized strip at the peak response to CCh (100 μM). As a consequence, the relaxation induced by Y-27632 became more apparent (Figure 8). The extent of CCh (100 μM) response was comparable with the pCa 5.0 response, and relaxant effects of GDPβS and Y-27632 were also comparable. These results indicate that CCh-induced full Ca^{2+} sensitization was reversed completely by GDPβS at the G-protein(s) level and by Y-27632 at the ROCK level.

Interestingly, PDBu-induced Ca^{2+} sensitization was well maintained in α-toxin–permeabilized trachea. It is not clear whether the different time course of produced force between PDBu and other Ca^{2+} sensitizing agents we used was due to qualitative or quantitative difference in PKC activity in rabbit tracheal SM (*see* below). However, complete relaxation of agonist-induced Ca^{2+} sensitization in both rabbit and human tissues by Y-27632 suggests that a common mechanism through the rho/ROCK pathway is generally present in the sustained phase of agonist-evoked airway SM contraction.

PDBu-Induced Ca^{2+} Sensitization

Introduction of activated PKC caused Ca^{2+} sensitization in single SM (7). A PKC activator, PDBu, also evoked Ca^{2+} sensitization in various types of SM (7, 8), including airway SM (10), and this is mediated mainly by inhibition of SMPP-1M (8). Although we could not exclude the possibility of inhibition of PKC activity by Y-27632, especially at high concentrations (16), partial inhibition by Y-27632 of PDBu-induced Ca^{2+} sensitization suggests a minor role of rho/ROCK in signaling downstream of PKC. However, Y-27632 inhibited Ca^{2+}-induced contraction of α-toxin–permeabilized SM in this study, raising another possibility: that the partial inhibition of PDBu-induced force might be due to a decrease in the basal ROCK activity. Further studies are required to clarify this point. Nevertheless, because Y-27632 completely reversed agonist- and GTPγS-induced Ca^{2+} sensitization, rho/ROCK–mediated signaling plays a much more important role in receptor-dependent, G protein–mediated Ca^{2+} sensitization than does PDBu-sensitive PKC in airway SM. This interpretation is supported by the findings that several PKC inhibitors failed to block acetylcholine-induced Ca^{2+} sensitization of canine trachea permeabilized with β-escin (32). However, we could not rule out the possibility that atypical PKC (insensitive to phorbol ester) may contribute to the agonist-evoked Ca^{2+} sensitization in SM (33).

Putative Other Mechanisms of Y-27632 Effect

In α-toxin–permeabilized and A23187-treated strips, the actions of ion channels in plasma membranes and of SR for Ca^{2+} storage were functionally removed. Direct evidence of agonist-induced Ca^{2+} sensitization has been established by the receptor-coupled permeabilization techniques with α-toxin and β-escin (1). Under these experimental conditions, putative other mechanisms of Y-27632 effect were limited. Myosin light chain phosphorylation theory indicates that MLCK and SMPP-1M activity ratio is the major mechanism of force development, although thin-filament proteins such as caldesmon and calponin may contribute to force generation (1). However, we have demonstrated that Y-27632 did not affect the MLCK activity *in situ* (in this study); we also reported that phosphorylation of caldesmon by mitogen-activated protein kinase (a proposed mechanism of increase in caldesmon activity *in situ*) had no effect on tension (34), and that contribution of calponin to Ca^{2+} sensitization was minor (only 25%) in canine trachea (10). We also observed that neither a tyrosine kinase inhibitor, genistein, nor phospholipase A_2 inhibitors (quinacrine and manoalide) prevented CCh-induced Ca^{2+} sensitization of rabbit airway SM (unpublished observation). Further, Y-27632 did not affect the calyculin A–induced contraction (16 and Figure 5a), indicating that this compound did not inhibit adenosine triphosphatase activity of myosin. Taken together, it is substantially reasonable that Y-27632 mainly affected the SMPP-1M activity via inhibition of the rho/ROCK pathway, although selectivity of Y-27632 toward other kinases, especially toward atypical PKC *in situ*, remains to be elucidated.

Conclusion

In conclusion, rho/ROCK–mediated Ca^{2+} sensitization is important for the sustained phase of contraction in airway SM, and Y-27632 selectively blocks this pathway, leading to complete relaxation of airway SM. Hence, inhibition of rho/ROCK signaling may become a new strategy to resolve airflow limitation in diseases such as bronchial asthma.

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Blue Cyan Green Yellow Red Magenta White 3/Color Black

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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19