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## Inhibition of geranylgeranyltransferase inhibits bronchial smooth muscle hyperresponsiveness in mice

Yoshihiko Chiba,<sup>1</sup> Shunsuke Sato,<sup>1</sup> Motohiko Hanazaki,<sup>1,2</sup> Hiroyasu Sakai,<sup>1</sup> and Miwa Misawa<sup>1</sup>

<sup>1</sup>Department of Pharmacology, School of Pharmacy, Hoshi University, Tokyo; <sup>2</sup>Department of Anesthesiology and Resuscitology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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**Chiba Y, Sato S, Hanazaki M, Sakai H, Misawa M.** Inhibition of geranylgeranyltransferase inhibits bronchial smooth muscle hyperresponsiveness in mice. *Am J Physiol Lung Cell Mol Physiol* 297: L984–L991, 2009. First published August 28, 2009; doi:10.1152/ajplung.00178.2009.—Recent studies revealed an involvement of RhoA/Rho-kinase in the contraction of bronchial smooth muscle (BSM), and this pathway has now been proposed as a new target for asthma therapy. A posttranslational geranylgeranylation of RhoA is required for its activation. Thus selective inhibition of geranylgeranyltransferase may be a novel strategy for treatment of the BSM hyperresponsiveness in asthmatics. To test this hypothesis, we investigated the effect of a geranylgeranyltransferase inhibitor, GGTI-2133, on antigen-induced BSM hyperresponsiveness by using mice with experimental asthma. Mice were sensitized and repeatedly challenged with ovalbumin antigen. Animals also were treated with GGTI-2133 (5 mg/kg ip) once a day before and during the antigen inhalation period. Repeated antigen inhalation caused a BSM hyperresponsiveness to acetylcholine with the increased expressions of RhoA and the anti-farnesyl-positive 21-kDa proteins, probably geranylgeranylated RhoA. The in vivo GGTI-2133 treatments significantly inhibited BSM hyperresponsiveness induced by antigen exposure. In another series of experiments, BSM tissues isolated from the repeatedly antigen-challenged mice were cultured for 48 h in the absence or presence of GGTI-2133. Under these conditions, the putative geranylgeranylated RhoA was decreased in a GGTI-2133 concentration-dependent manner. The in vitro incubation with GGTI-2133 also inhibited BSM hyperresponsiveness induced by antigen exposure. These findings suggest that GGTI-2133 inhibits antigen-induced BSM hyperresponsiveness, probably by reducing downstream signal transduction of RhoA. Selective geranylgeranyltransferase inhibitors may be beneficial for the treatment of airway hyperresponsiveness, one of the characteristic features of allergic bronchial asthma.

airway hyperresponsiveness; RhoA; geranylgeranylation; GGTI-2133

INCREASED AIRWAY NARROWING in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. This abnormality is an important sign of the disease, although the pathophysiological variations leading to the hyperresponsiveness are unclear now. Several mechanisms have been suggested to explain the airway hyperresponsiveness (AHR), such as alterations in the neural control of airway smooth muscle (2), increased mucosal secretions (15), and mechanical factors related to remodeling of the airways (39). In addition, it also has been suggested that one of the factors that contribute to the exaggerated airway narrowing in individuals with asthma is an abnormality of the properties of airway smooth muscle (21, 29). Rapid relief from airway limitation in patients with asthma by  $\beta$ -stimulant inhalation

also may suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction.

There is increasing evidence that a monomeric GTP-binding protein, RhoA, and its downstream target, Rho-kinases, are involved in the  $\text{Ca}^{2+}$ -independent contraction (termed  $\text{Ca}^{2+}$  sensitization) of airway smooth muscles (7, 10, 14, 40). When the RhoA/Rho-kinase system is activated by contractile agonists, the activity of myosin light chain (MLC) phosphatase is reduced and the level of phosphorylated MLC is then increased, resulting in an augmentation of contraction. Recent studies demonstrated that the agonist-induced, RhoA/Rho-kinase-mediated  $\text{Ca}^{2+}$  sensitization of bronchial smooth muscle (BSM) contraction is augmented in rats (7) and mice (10) with allergic bronchial asthma. An importance of the RhoA/Rho-kinase system also has been demonstrated in human BSM (40), and the signaling of RhoA and its downstream Rho-kinases are now considered as a therapeutic target for the treatment of AHR in asthma (11, 17, 26, 27).

Posttranslational geranylgeranylation of RhoA protein is required for its activation and/or membrane translocation (32, 33). Indeed, statins, which indirectly inhibit posttranslational prenylation by depleting the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) downstream metabolites farnesylpyrophosphate and geranylgeranylpyrophosphate, can inhibit translocation of RhoA to plasma membrane, resulting in an inhibition of RhoA-mediated function in cultured tumor cell lines (18, 41). Our previous studies also revealed that lovastatin can ameliorate BSM hyperresponsiveness by reducing the membrane-associated RhoA in animal models of allergic bronchial asthma (3, 6). Thus selective inhibition of geranylgeranyltransferase (GGTase) may be one of the novel strategies for treatment of the augmented airway responsiveness in allergic asthmatics. To test the hypothesis, we investigated the effects of a GGTase inhibitor, GGTI-2133 (37), on antigen-induced BSM hyperresponsiveness by using mice with experimental asthma.

### METHODS

**Animals and treatments.** Male BALB/c mice were purchased from the Charles River Japan (Kanagawa, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of the Hoshi University (Tokyo, Japan).

Preparation of a murine model of allergic bronchial asthma, which has an in vivo AHR (16), was performed as described previously (5, 6, 10). In brief, BALB/c mice (8 wk of age) were actively sensitized by intraperitoneal injections of 8  $\mu\text{g}$  of ovalbumin (OA; Seikagaku, Tokyo, Japan) with 2 mg of Imject Alum (Pierce Biotechnology, Rockford, IL) on day 0 and day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/ml) for 30 min on days 12, 16, and 20. The OA aerosol was generated with an ultrasonic nebulizer (Nihon Kohden, Tokyo, Japan) and introduced to a Plexi-

Address for reprint requests and other correspondence: Y. Chiba, Dept. of Pharmacology, School of Pharmacy, Hoshi Univ., Shinagawa-ku, Tokyo 142-8501, Japan (e-mail: chiba@hoshi.ac.jp).

glas chamber box (130 × 200 mm, 100 mm in height) in which the mice were placed. Animals also received intraperitoneal injection with GGTI-2133 [ $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  *N*-[4-(imidazol-4-yl)methylamino]-2-(1-naphthyl)benzoyl]leucine trifluoroacetate salt; Sigma-Aldrich, St. Louis, MO} (42) or its vehicle (50% DMSO in PBS) once a day for 10 days from *day 11* to *day 20*. Twenty-four hours after the last OA challenge, mice were killed by exsanguination from the abdominal aorta under urethane anesthesia (1.6 g/kg ip; Sigma).

**Determination of BSM responsiveness.** Mice were killed by exsanguination from the abdominal aorta under urethane anesthesia (1.6 g/kg ip), and the airway tissues under the larynx to lungs were immediately removed. About a 3-mm length of the left main bronchus (~0.5 mm in diameter) was isolated, and the epithelium was removed by gently rubbing with sharp tweezers (5, 6, 10). The resultant tissue ring preparation was then suspended in a 5-ml organ bath by two stainless steel wires (0.2 mm in diameter) passed through the lumen. For all tissues, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T; Nihon Kohden) for the measurement of isometric force. A resting tension of 0.5 g was applied. The buffer solution contained modified Krebs-Henseleit solution with the following composition (in mM): 118.0 NaCl, 4.7 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 25.0  $\text{NaHCO}_3$ , 1.2  $\text{KH}_2\text{PO}_4$ , and 10.0 glucose. The buffer solution was maintained at 37°C and oxygenated with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ . After the equilibration period, the concentration-response curve to ACh ( $10^{-7}$ - $10^{-3}$  M in

final concentration) was constructed cumulatively. The BSM responsiveness to high- $\text{K}^+$  depolarization was also measured by applying isotonic  $\text{K}^+$  solution cumulatively (10–90 mM in final concentration) in the presence of atropine and indomethacin (both  $10^{-6}$  M). In some experiments, the isolated BSM tissues were incubated with GGTI-2133 (1 or 3  $\mu\text{M}$ ) or its vehicle (0.1% DMSO) in serum-free DMEM for 48 h at room temperature before the measurement of BSM responsiveness or immunoblot analyses.

**Protein samples of bronchial tissues.** The airway tissues below the main bronchi to lungs were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The bronchial tissue (containing the main and intrapulmonary bronchi) segments were prepared as described previously (3) and quickly frozen with liquid nitrogen, and the tissue was crushed to powder with a mortar. The tissue powder was homogenized in ice-cold tris(hydroxymethyl)aminomethane (Tris; 10 mM, pH 7.5) buffer containing 5 mM  $\text{MgCl}_2$ , 2 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20  $\mu\text{g}/\text{ml}$  leupeptin, 20  $\mu\text{g}/\text{ml}$  aprotinin, 1% Triton X-100, and 1% sodium cholate. The tissue homogenate was then centrifuged (3,000 g, 4°C for 15 min), and the resultant supernatant was stored at  $-85^\circ\text{C}$  until use.

**Western blot analyses.** Protein samples were subjected to 15% SDS-PAGE, and the proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After being blocked with 3% gelatin, the PVDF membrane was incubated with the

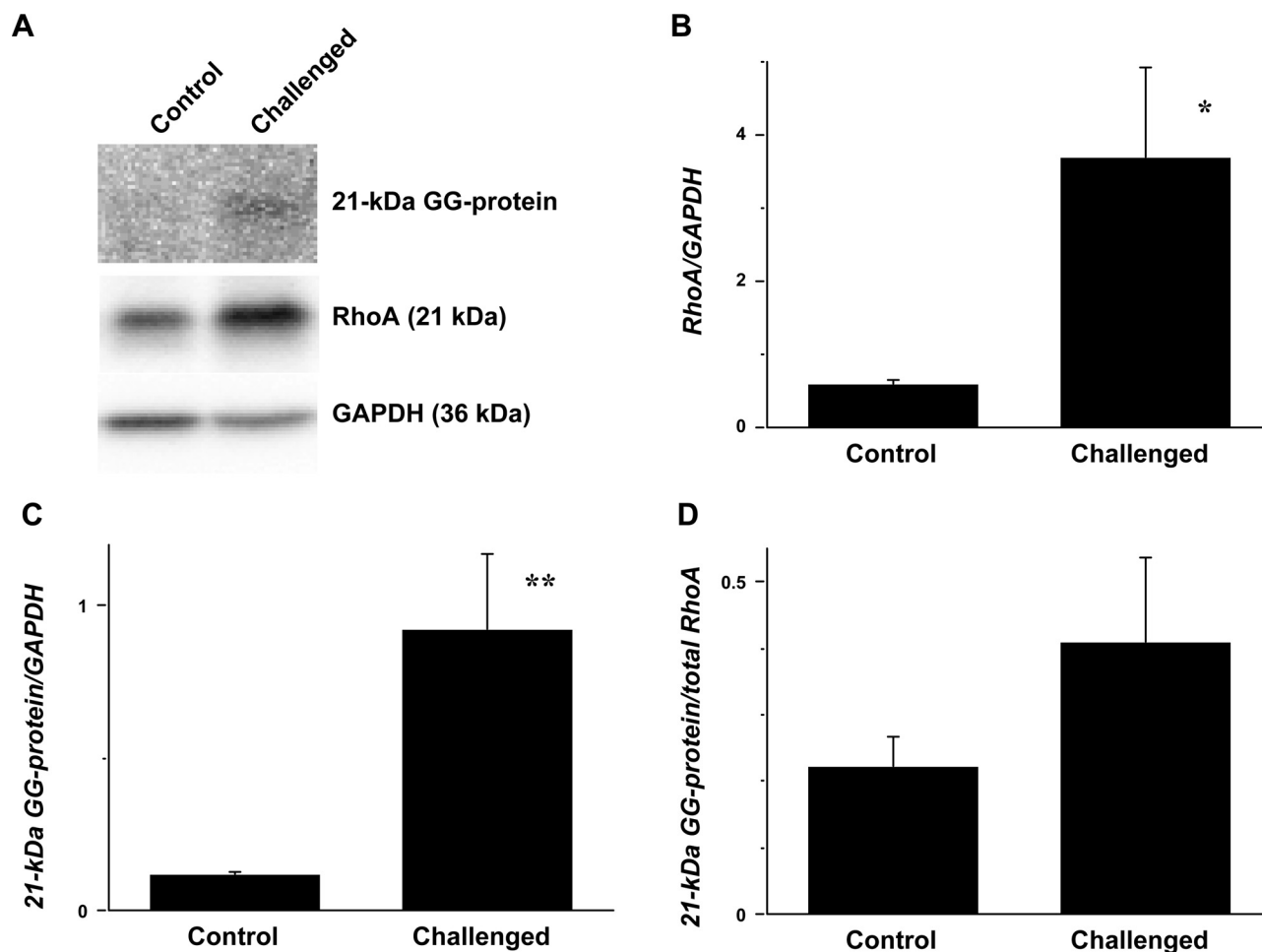


Fig. 1. Effects of repeated antigen exposure on the expression levels of RhoA and 21-kDa geranylgeranylated protein (GG-protein; measured as putative GG-RhoA; see RESULTS) in bronchial smooth muscle (BSM) tissues of mice. A: typical bands for 21-kDa GG-protein, RhoA, and GAPDH. The ratios of intensities of RhoA (B) and 21-kDa GG-protein (C) to the corresponding GAPDH bands were calculated. D: the 21-kDa GG-protein/RhoA ratios were also calculated. Each column represents the mean  $\pm$  SE from 6 different animals. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. respective control groups (by Bonferroni/Dunn's test).

primary antibody. The primary antibodies used in the present study were polyclonal rabbit anti-RhoA (1:2,500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-farnesyl antibodies (1:1,000 dilution; Calbiochem, Gibbstown, NJ). The membrane was then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2,500 dilution; Amersham Biosciences, Piscataway, NJ), detected using an enhanced chemiluminescence system (Amersham Biosciences), and analyzed using a densitometry system. Detection of housekeeping gene was also performed on the same membrane by using monoclonal mouse anti-GAPDH (1:10,000 dilution; Chemicon International, Temecula, CA) and horseradish peroxidase-conjugated sheep anti-mouse IgG (1:2,500 dilution; Amersham Biosciences) to confirm the same amount of proteins loaded.

**Statistical analyses.** All data are means  $\pm$  SE. Statistical significance of differences was determined using unpaired Student's *t*-test or two-way analysis of variance (ANOVA) with a post hoc Bonferroni/Dunn test (StatView for Macintosh version 5.0; SAS Institute, Cary, NC). A value of  $P < 0.05$  was considered significant.

## RESULTS

**Effects of repeated antigen exposure on the levels of total and geranylgeranylated RhoA in BSM tissues.** Figure 1 shows the results of immunoblottings for RhoA and geranylgeranylated proteins (GG-proteins). We used a polyclonal anti-farnesyl antibody, since antibodies against GG-RhoA and/or geranylgeranyl moiety are not commercially available to date. Different groups of investigators have reported that polyclonal anti-farnesyl antibodies also recognize geranylgeranyl moiety (1, 19, 20). It also has been reported that RhoA is geranylgeranylated, rather than farnesylated, by GGTase (31). As shown in Fig. 1A, immunoblot analysis using the anti-farnesyl antibody showed a weak but distinct band at 21 kDa, the molecular mass corresponding to RhoA protein. The anti-farnesyl-positive 21-kDa protein (21-kDa GG-protein) was measured as the putative GG-RhoA in the present study.

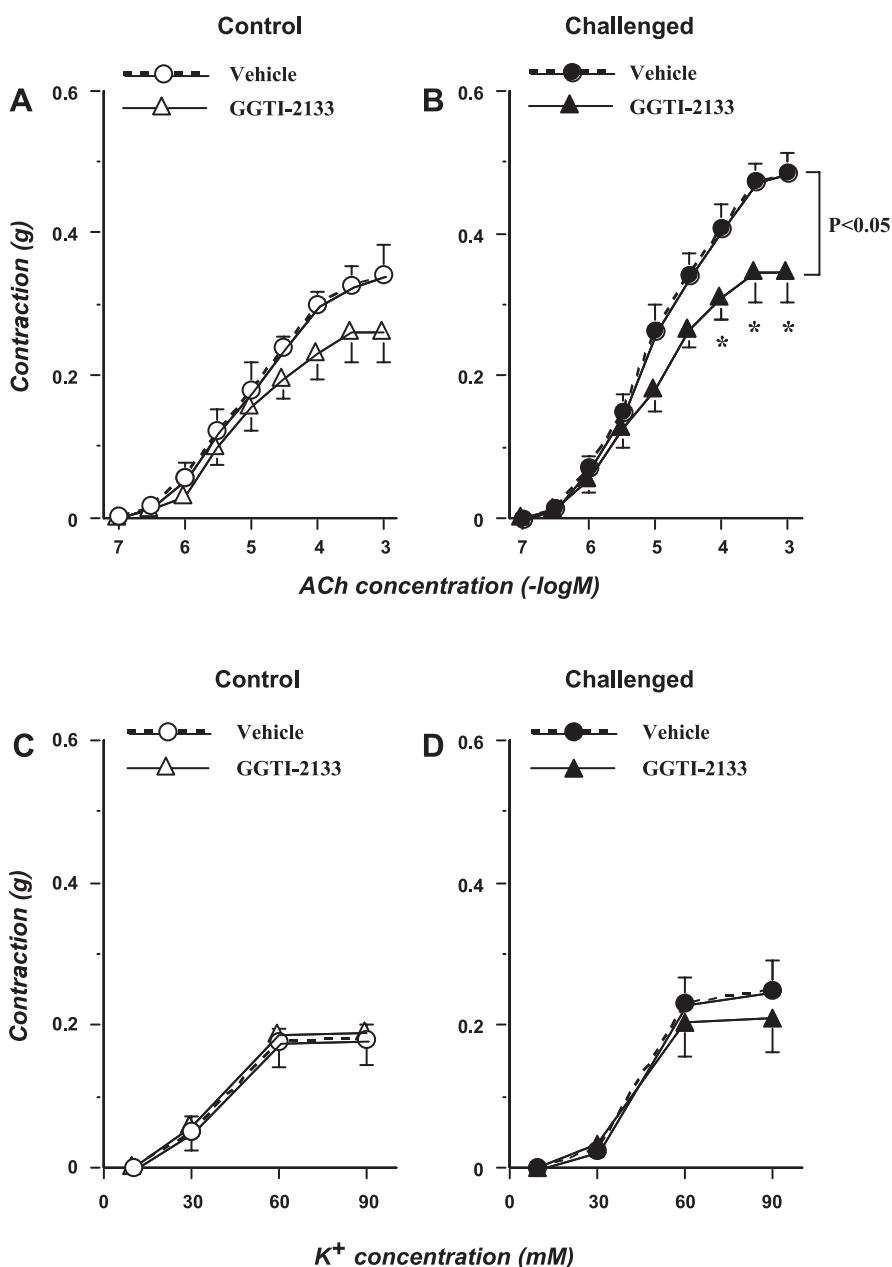


Fig. 2. Effects of systemic treatments with a geranylgeranyltransferase inhibitor, GGTI-2133 (GGTI), on the BSM hyperresponsiveness induced by repeated antigen exposure. The acetylcholine (ACh; A and B)- and high-K<sup>+</sup> depolarization (C and D)-induced contractions of BSMs isolated from nonsensitized normal control (A and C) and repeatedly antigen-challenged mice (B and D) were measured isometrically. GGTI (5 mg·kg<sup>-1</sup>·day<sup>-1</sup>) or its vehicle (50% DMSO in PBS) was also pretreated intraperitoneally for 10 days as described in MATERIALS AND METHODS. Each point represents the mean  $\pm$  SE from 5–6 different animals. \* $P < 0.05$  vs. vehicle-treated group (by Bonferroni/Dunn's test).

Both the expression levels of RhoA protein (Fig. 1B) and 21-kDa GG-protein (Fig. 1C) in the BSM tissues of the antigen-challenged mice were significantly increased compared with those of the nonsensitized normal control animals. In BSM tissues of the antigen-challenged group, an increase in the ratio of the 21-kDa GG-protein to total RhoA protein was also observed, whereas the difference was not statistically significant.

**BSM hyperresponsiveness in repeatedly antigen-challenged mice.** Figure 2 shows the acetylcholine (ACh) responsiveness of BSMs isolated from nonsensitized normal control (A) and repeatedly antigen-challenged mice (B). The nonsensitized normal control animals were used as controls because our previous studies demonstrated that the sensitization procedure alone has no effect on the BSM contractility and the expression and activation of RhoA (6, 10). Application of ACh ( $10^{-7}$ – $10^{-3}$  M) to isolated BSMs elicited a concentration-dependent contraction in all animals used. The contractile response to ACh of BSMs from the repeatedly antigen-challenged mice was markedly augmented, i.e., BSM hyperresponsiveness, compared with that in those from the control animals: the ACh concentration-response curve was significantly shifted upward by the antigen exposure (control vehicle group in Fig. 2A vs. challenged vehicle group in Fig. 2B;  $P < 0.05$  by 2-way ANOVA). On the other hand, no significant change in the contractile response induced by high- $K^{+}$  depolarization was observed between the groups (Fig. 2, C and D). These findings are consistent with our previous report (10) that demonstrates an augmented agonist-induced  $Ca^{2+}$  sensitization of contraction in the repeatedly antigen-challenged mice.

**Effects of systemic treatment with GGTI-2133 on antigen-induced BSM hyperresponsiveness.** To determine the effect of systemic treatment with GGTI-2133, an inhibitor of GGTase (37), on the augmented BSM responsiveness observed in the repeatedly antigen-challenged mice, animals also underwent

intraperitoneal injections with GGTI-2133 ( $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) (42) or its vehicle (50% DMSO in PBS) before and during the period of repeated antigen exposure. In both the control and challenged animals, Western blot analyses of BSM homogenates indicated no distinct band corresponding to the 21-kDa GG-protein (data not shown), suggesting that the treatments effectively inhibited GGTase *in vivo*. The GGTI-2133 treatments slightly inhibited the ACh-induced contraction of BSMs in the control mice, but the effect was not statistically significant compared with the vehicle-treated animals (Fig. 2A). In contrast, the augmented ACh-induced contraction, i.e., BSM hyperresponsiveness, observed in the repeatedly antigen-challenged mice was markedly and significantly inhibited by the systemic treatments with GGTI-2133 (Fig. 2B;  $P < 0.05$  by 2-way ANOVA). The BSM responsiveness to ACh of the antigen-challenged animals returned to the control level almost completely: no significant difference was observed between the challenged-GGTI-2133 (Fig. 2B) and the control vehicle (Fig. 2A) groups. In both the control and antigen-challenged animals, the systemic treatments with GGTI-2133 had no effect on the BSM contractility to high- $K^{+}$  depolarization (Fig. 2, C and D).

**Effects of depletion of geranylgeranylated RhoA *in vitro* on the antigen-induced BSM hyperresponsiveness.** It has been reported that incubation of isolated smooth muscle strips with another GGTase inhibitor, GGTI-297, caused a depletion of geranylgeranylated RhoA without affecting the expression of total RhoA protein (25). In the present study, isolated BSM tissues were incubated with GGTI-2133 to determine the role of geranylgeranylated RhoA in the augmented BSM contractility of the antigen-challenged mice. Because a slow turnover of isoprenylation has been suggested (25, 30), the BSM tissues were incubated for 48 h in the absence or presence of GGTI-2133. As shown in Fig. 3, incubation of BSMs with GGTI-2133 for 48 h caused a decrease in the level of 21-kDa

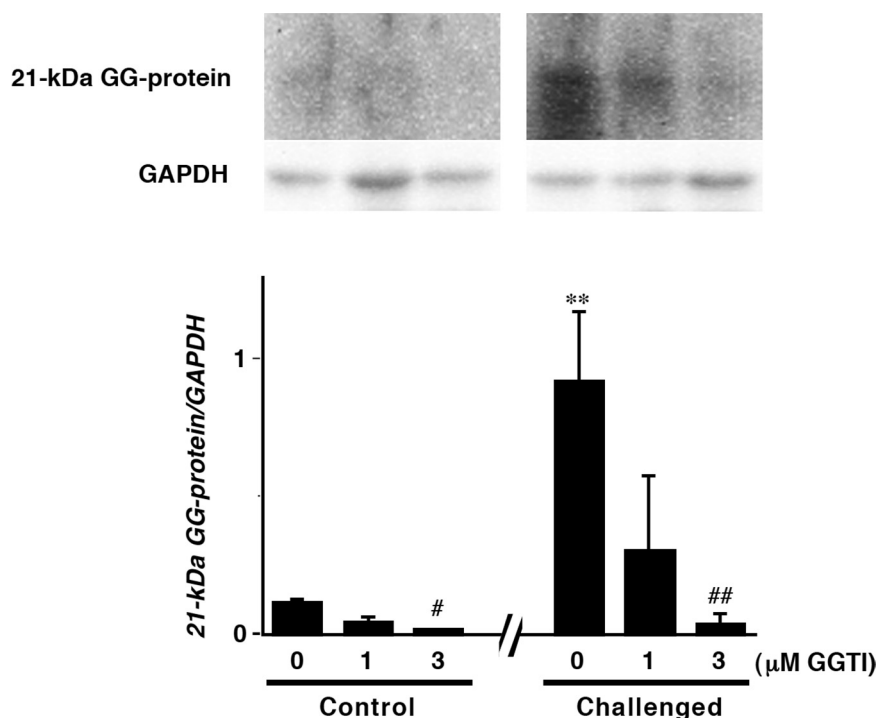


Fig. 3. Effects of *in vitro* incubation with GGTI on the expression of 21-kDa GG-protein (measured as putative GG-RhoA; see RESULTS) in BSM tissues of mice. The BSMs isolated from control and repeatedly antigen-challenged mice were incubated in the absence (0  $\mu\text{M}$ ; vehicle only) or presence (1 or 3  $\mu\text{M}$ ) of GGTI for 48 h, and then the tissues were subjected to Western blot analyses. *Top*: typical bands for 21-kDa GG-protein and GAPDH. The ratios of intensities of the 21-kDa GG-protein to the corresponding GAPDH bands were calculated and summarized at *bottom*. Each column represents the mean  $\pm$  SE from 3–5 independent experiments. \*\* $P < 0.01$  vs. control. # $P < 0.05$ ; ## $P < 0.01$  vs. respective 0  $\mu\text{M}$  GGTI (by Bonferroni/Dunn's test).

GG-protein, in a GGTI-2133 concentration-dependent manner, in both the control and the antigen-challenged animals. Almost complete depletion was observed when tissues were incubated with 3  $\mu\text{M}$  GGTI-2133 for 48 h (Fig. 3).

Figure 4 shows the effect of depletion of 21-kDa GG-protein on BSM contractility. The in vitro treatment with GGTI-2133 (1 or 3  $\mu\text{M}$  for 48 h) had no effect on the contractility to ACh (Fig. 4A) or high- $\text{K}^+$  depolarization (Fig. 4C) in BSMs of the control animals. However, the augmented ACh-induced contraction observed in the repeatedly antigen-challenged mice was significantly attenuated by the in vitro incubation with GGTI-2133 (Fig. 4B). The BSM responsiveness to ACh of the antigen-challenged group returned to the control level when the tissues were incubated with 3  $\mu\text{M}$  GGTI-2133: no significant

difference was observed between the challenged GGTI-2133 (3  $\mu\text{M}$ ; Fig. 4B) and the control vehicle (Fig. 4A) groups. On the other hand, the in vitro incubation with GGTI-2133 had no effect on the BSM contractility to high- $\text{K}^+$  depolarization in the antigen-challenged group (Fig. 4D).

Figure 5 shows the results of BSM relaxation induced by Y-27632, a Rho-kinase inhibitor. Y-27632 ( $10^{-8}$ – $10^{-5}$  M) was applied cumulatively when the BSM contraction induced by  $10^{-3}$  M ACh reached to plateau. In both the control and the antigen-challenged mice, Y-27632 induced concentration-dependent relaxing responses. The Y-27632-induced relaxation of BSMs in the antigen-challenged group was significantly augmented compared with the control group (challenged vehicle group in Fig. 5B vs. control vehicle group in Fig. 5A;  $P <$

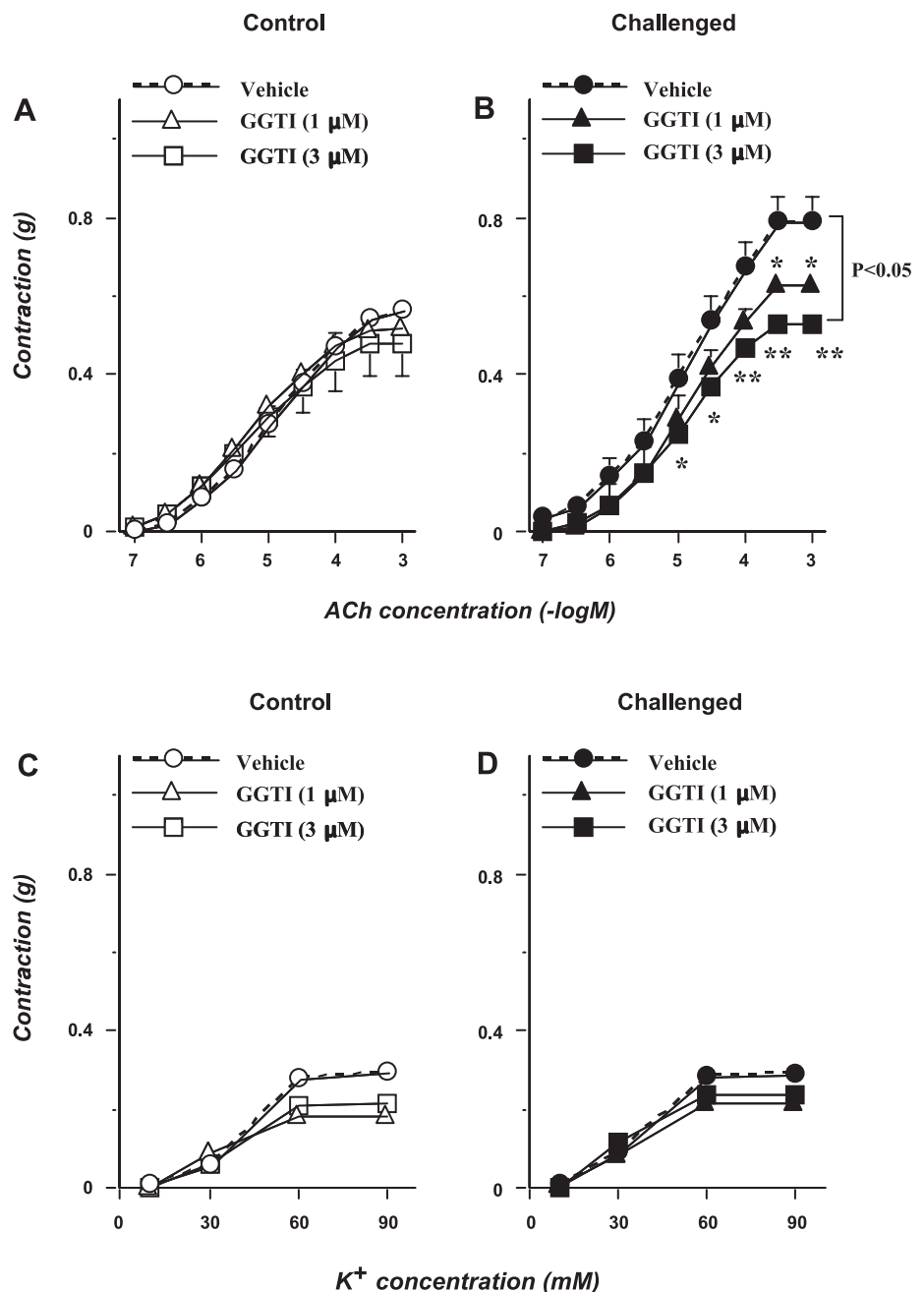


Fig. 4. Effects of in vitro incubation with GGTI on the BSM hyperresponsiveness induced by repeated antigen exposure. The BSMs isolated from control (A and C) and repeatedly antigen-challenged mice (B and D) were incubated in the absence (vehicle only) or presence (1 or 3  $\mu\text{M}$ ) of GGTI for 48 h. The ACh (A and B)- and high- $\text{K}^+$  depolarization (C and D)-induced contractions were measured isometrically. Each point represents the mean  $\pm$  SE from 5 different animals. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. vehicle-treated group (by Bonferroni/Dunn's test).

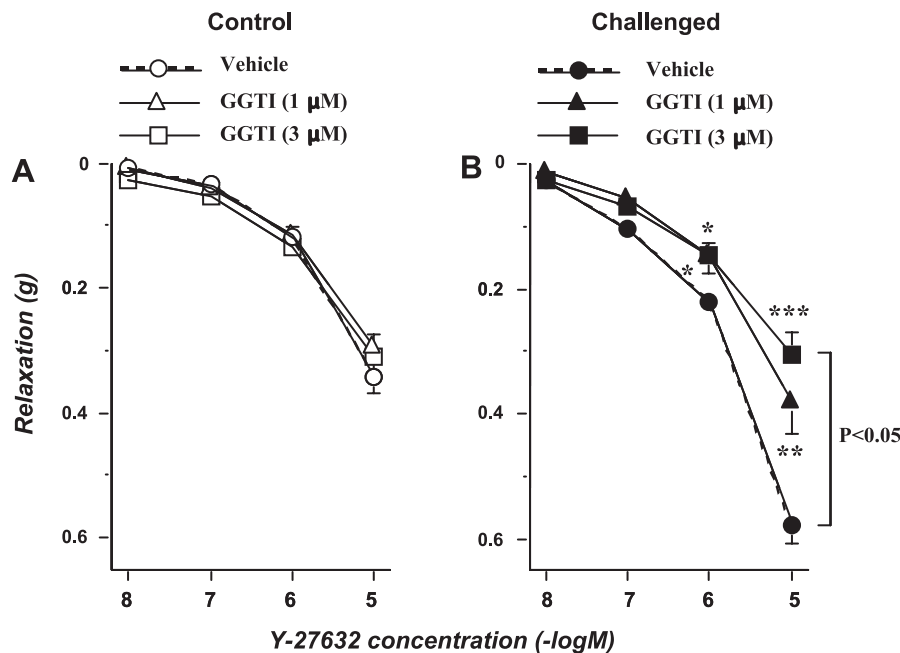


Fig. 5. Effects of in vitro incubation with GGTI on BSM relaxation induced by a Rho-kinase inhibitor, Y-27632. The BSMs isolated from control (A) and repeatedly antigen-challenged mice (B) were incubated in the absence (vehicle only) or presence (1 or 3  $\mu$ M) of GGTI for 48 h, and then isometric tension was measured. Y-27632 was cumulatively applied when the contraction induced by  $10^{-3}$  M ACh reached a plateau. Each point represents the mean  $\pm$  SE from 4–6 different animals. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. vehicle-treated group (by Bonferroni/Dunn's test).

0.05 by 2-way ANOVA). The in vitro incubation with GGTI-2133 caused a significant inhibition of the Y-27632-induced relaxation in BSMs of the repeatedly antigen-challenged mice (Fig. 5B), whereas no effect was observed in the case of the control group (Fig. 5A).

## DISCUSSION

In addition to an upregulation of RhoA, the present study revealed that the anti-farnesyl-positive 21-kDa protein, probably GG-RhoA, is increased in BSMs isolated from the repeatedly antigen-challenged mice (Fig. 1). The findings remind us that inhibition of RhoA geranylgeranylation might improve the augmented BSM contractility, one of the causes of AHR in asthmatics, since geranylgeranylation is required for the activation of RhoA (32, 33), a key protein for BSM contraction. Indeed, statins such as lovastatin, which inhibits RhoA geranylgeranylation indirectly, can ameliorate AHR in experimental asthma (3, 6). Currently, we have shown for the first time that systemic treatment with GGTI-2133, a direct inhibitor of GGTase, ameliorates the BSM hyperresponsiveness to ACh induced by antigen exposure (Figs. 2 and 4).

It has been suggested that one of the factors that contribute to the AHR in asthmatics is an abnormality of the nature of airway smooth muscle (21, 29). The airway smooth muscle is the main structure of airway walls and plays a major role in the contraction of the airways. The excessive contraction of airway smooth muscles may be one of the crucial factors that directly cause the asthma symptoms such as the AHR. Consistent with our previous reports (5, 6, 9, 10), an increased responsiveness of the isolated BSM to ACh was observed in mice that were sensitized and repeatedly challenged with OA antigen (see RESULTS), indicating that reproducible BSM hyperresponsiveness has occurred with the antigen sensitization and challenge procedure used. In this mouse model of allergic bronchial asthma, an augmented ACh-induced, RhoA-mediated  $\text{Ca}^{2+}$  sensitization of BSM contraction accompanied by an upregulation of RhoA protein has been demonstrated (10). Similar

observations, including BSM hyperresponsiveness and augmented RhoA-mediated  $\text{Ca}^{2+}$  sensitization of contraction, also have been reported in a rat model of bronchial asthma induced by DNP-*Ascaris* antigen (3, 7). An importance of RhoA and its downstream Rho-kinases in contraction of human airway smooth muscle also has been demonstrated (40), and the RhoA/Rho-kinase pathway is now considered as a therapeutic target of asthma (11, 17, 26, 27).

It has been believed that posttranslational geranylgeranylation is required for the activation of monomeric GTP-binding proteins, including RhoA (32, 33). In the resting state, the GDP-bound inactive form of RhoA is located in the cell cytosol with a GDP dissociation inhibitor, called RhoGDI, which buries the geranylgeranylated, hydrophobic tail of RhoA. Activation of RhoA is initiated by guanine nucleotide exchange factors (RhoGEFs) through activation of plasma membrane receptors coupled to certain heterotrimeric GTP-binding proteins. The active RhoGEFs exchange GDP for GTP on RhoA, and the RhoGDI dissociates from RhoA, resulting in the association of GTP-bound RhoA with the plasma membrane and its activation (32). Indeed, receptor stimulation by contractile agonist caused translocation of RhoA correlated with induction of the BSM contraction (3). In addition, Patel and Rattan (25) demonstrated that RhoA geranylgeranylation is essential for downstream signal transduction of RhoA. It is thus possible that inhibition of geranylgeranylation of RhoA could reduce the BSM contractility by inhibiting the RhoA-mediated  $\text{Ca}^{2+}$  sensitization of contraction. On the other hand, monomeric GTP-binding proteins other than RhoA, such as Ras, Rac, and Cdc42, also have been suggested to be farnesylated/geranylgeranylated (28, 34), although their contribution to BSM contraction is not clear now.

In the present study, systemic treatments with GGTI-2133 significantly inhibited the BSM hyperresponsiveness induced by the repeatedly antigen exposure (Fig. 2B). On the other hand, however, only a lesser inhibitory effect of GGTI-2133 was observed in the control animals (Fig. 2A). These findings

are consistent with the previous reports that used lovastatin instead of GGTI-2133 (3, 6). It is thus suggested that the contribution of RhoA/Rho-kinase signaling to ACh-induced contraction is relatively small in BSMs of the normal mice. The idea might also be supported by the observations that in the chemically skinned BSM fibers, only a small ACh-induced  $\text{Ca}^{2+}$  sensitization was induced in normal rats (7) and mice (10). An augmented RhoA-mediated  $\text{Ca}^{2+}$  sensitization has been suggested in the diseased smooth muscles of allergic asthma models (4, 9, 11, 17, 26, 27), including mice (10). The current finding that the augmented BSM contraction in the repeatedly antigen-challenged mice was almost completely blocked by the systemic treatment with GGTI-2133 (see RESULTS) also indicates that the enhanced component of contraction is mediated by an activation of RhoA protein. It is thus possible that functional inhibition of RhoA protein, such as by the inhibition of its geranylgeranylation using a selective inhibitor of GGTTases, might effectively improve the AHR in asthmatics.

Currently, therapeutic potency of GGTI-2133 in BSM hyperresponsiveness was also determined using BSM tissues isolated from the repeatedly antigen-challenged mice. It is unlikely that the in vivo AHR induced by antigen exposure is a sustained phenomenon in animal models of asthma. For instance, although the peak of AHR was observed at 24 h after the last antigen challenge, the AHR recovered naturally within 72 h (23). Thus, in the present study, the effect of GGTI-2133 was tested using the organ-cultured BSM tissues that were isolated at 24 h after the last antigen challenge. In the BSM tissues of the repeatedly antigen-challenged mice, the BSM hyperresponsiveness (Fig. 4B, vehicle) and the increased expression of RhoA (data not shown) and 21-kDa GG-protein (Fig. 3, 0  $\mu\text{M}$  GGTI) were maintained for at least 48 h under the culture conditions used. However, as shown in Fig. 4B, the BSM hyperresponsiveness was inhibited when the tissues were cultured in the presence of GGTI-2133. The inhibition is probably caused by the impairment of activation of RhoA due to the reduction of its geranylgeranylation (Fig. 3). The finding that the GGTI-2133 incubation reduced the Y-27632-induced relaxation of BSMs in the antigen-challenged mice (Fig. 5B) might also support the idea. Thus the GGTTase inhibitors might have a therapeutic potency for the AHR by inhibiting the activation of RhoA even in the BSMs that already have hyperresponsiveness.

As previously reported, a 24-h incubation of isolated internal anal sphincter smooth muscle tissues with another GGTTase inhibitor, GGTI-297, caused a reduction of prenylated RhoA without affecting the expression level of RhoA protein itself (25). Consistently, the current 48-h incubation with GGTI-2133 also reduced the putative geranylgeranylated RhoA in the BSM tissues isolated from both the control and antigen-challenged mice in concentration-dependent fashions (Fig. 3). Although the exact mechanism of the reduction of prenylated RhoA by GGTTase inhibitors is still unclear, it is possible that the RhoA protein might be variable between its geranylgeranylated and degeranylgeranylated forms. The following observations reported by Ohkawara et al. (24) may support our idea: rapid geranylgeranylation of RhoA was induced when cells were stimulated, and the event was inhibited by treatment with a GGTTase inhibitor, GGTI-286.

In the ACh-precontracted BSM tissues, Y-27632 caused a concentration-dependent relaxation (Fig. 5). The relaxation is

mediated by an inhibition of Rho-kinase, because the Y-27632 concentrations used were selective to Rho-kinase (8). The relaxation induced by Y-27632 was greater in the antigen-challenged group (Fig. 5B, vehicle) than in the control group (Fig. 5A, vehicle). The finding that the augmented Y-27632-induced relaxation was attenuated by the in vitro treatment with GGTI-2133 in BSMs of the repeatedly antigen-challenged mice (Fig. 5B) also indicates that the RhoA/Rho-kinase signaling is augmented in the diseased smooth muscles. However, contrary to our expectation, the inhibitory effect of GGTI-2133 was only partial (Fig. 5B), although the agent inhibited the putative geranylgeranylation almost completely (Fig. 3). Rho-kinase also may be activated by unknown mechanism(s) other than the geranylgeranylated RhoA in BSMs of mice.

Because the prevalence of asthma is recently rising (36), there is an increased need for the development of new drugs for its treatment, especially for patients who respond poorly to conventional therapy such as glucocorticoids. Recently, statins have been proposed as a novel treatment of respiratory diseases, including asthma (12, 13, 35, 38). Although their effectiveness to asthma is still controversial (12, 22), statins could improve the AHR by inhibiting GGTTases indirectly (3, 6). The contradictory effects of statins may be explained by the inhibition of downstream pathways of HMG-CoA reductase other than GGTTase. The current findings suggest that selective inhibition of GGTTases is effective for asthma by improving the AHR. Further studies are necessary to assess whether the GGTTase inhibitors have therapeutic potential in allergic bronchial asthma.

In conclusion, the present study demonstrated that a GGTTase inhibitor, GGTI-2133, ameliorates the BSM hyperresponsiveness induced by repeated antigen exposure in mice. Selective GGTTase inhibitors such as GGTI-2133 may be beneficial for the treatment of AHR, one of the characteristic features of allergic bronchial asthma.

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