

## Full Paper

## Glucocorticoids Ameliorate Antigen-Induced Bronchial Smooth Muscle Hyperresponsiveness by Inhibiting Upregulation of RhoA in Rats

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**Abstract.** To determine the mechanism(s) of the inhibitory effect of glucocorticoids on airway hyperresponsiveness in allergic bronchial asthma, the effects of systemic treatment with glucocorticoids on bronchial smooth muscle hyperresponsiveness and RhoA upregulation were investigated in rats with allergic bronchial asthma. Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen. Animals were also treated with prednisolone or beclomethasone (each 10 mg/kg, i.p.) once a day during the antigen inhalation period. Repeated antigen inhalation caused a marked bronchial smooth muscle hyperresponsiveness to acetylcholine with an upregulation of RhoA. Augmented acetylcholine-induced activation of RhoA and phosphorylation of myosin light chain were observed in bronchial smooth muscles of the antigen-exposed animals. Systemic treatment with either glucocorticoid used inhibited the bronchial smooth muscle hypercontraction until the level of the sensitized control rats that received saline inhalation instead of antigen challenge. Interestingly, both glucocorticoids also inhibited the upregulation of RhoA and augmented acetylcholine-induced activation of RhoA and phosphorylation of myosin light chain. In conclusion, glucocorticoids ameliorated the augmented bronchial smooth muscle contraction by inhibiting upregulation of RhoA. These effects of glucocorticoids may account for, in part, their beneficial effects in the treatment of asthma.

**Keywords:** airway hyperresponsiveness, bronchial smooth muscle, RhoA, prednisolone, beclomethasone

### Introduction

Increased airway narrowing in response to non-specific stimuli is a characteristic feature of human obstructive diseases, including bronchial asthma. This abnormality is an important symptom 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 (1), increased mucosal secretions (2), and mechanical factors related to remodeling of the airways (3). In addition, it has also been suggested that one of the factors that contribute to the exaggerated airway narrowing in asthmatics is an abnormality of the nature of airway smooth muscle

(4, 5). Rapid relief from airway limitation in asthmatic patients by  $\beta$ -stimulant inhalation may also suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus, it may be important for development of asthma therapy to understand changes in the contractile signaling of airway smooth muscle cells associated with the disease.

Smooth muscle contraction is mainly regulated by an increase in cytosolic  $\text{Ca}^{2+}$  concentration in myocytes. Recently, an additional mechanism, termed  $\text{Ca}^{2+}$  sensitization, has also been suggested in the agonist-induced smooth muscle contraction including airways (6–12). Although the detailed mechanism is not fully understood, there is increasing evidence that a monomeric guanosine 5'-triphosphate (GTP)-binding protein RhoA and its downstream target Rho-kinase are involved in the agonist-induced  $\text{Ca}^{2+}$  sensitization of airway smooth muscle contraction (6, 7, 9, 10). When the RhoA/Rho-kinase system was activated by contractile agonists, the activity of myosin light chain (MLC) phosphatase is

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reduced and the level of phosphorylated MLC is then increased, resulting in an augmentation of smooth muscle contraction. Recent studies revealed an augmentation of the RhoA/Rho-kinase-mediated  $\text{Ca}^{2+}$  sensitization in diseased smooth muscles such as coronary (13–15) and cerebral vasospasms (16–18) and hypertension (19–21). In addition, our previous studies demonstrated that the agonist-induced, RhoA-mediated  $\text{Ca}^{2+}$  sensitization of bronchial smooth muscle contraction is augmented in rats (7) and mice (10) with allergic bronchial asthma. It is thus possible that an augmentation of the RhoA/Rho-kinase system might be one of the causes of the bronchial smooth muscle hyperresponsiveness.

Glucocorticoids are the most effective therapy currently available for the treatment of allergic bronchial asthma. It has been believed that glucocorticoids act primarily as anti-inflammatory agents, that is, inhibition of recruitment of inflammatory cells and inhibition of release of pro-inflammatory mediators such as cytokines, in asthmatics (22). Recent studies have also demonstrated that glucocorticoids may affect diverse functions of airway smooth muscles (23, 24), which also express the glucocorticoid receptors (25). Moreover, clinical studies revealed that glucocorticoids could reduce, at least in part, the AHR (26–29). However, little is known about the action of glucocorticoids on the function of airway smooth muscle and on the pathogenesis of bronchial smooth muscle hyperresponsiveness.

In the present study, to determine whether glucocorticoids can reduce the bronchial smooth muscle hyperresponsiveness associated with AHR, the effects of prednisolone and beclomethasone on the augmented bronchial smooth muscle contraction were investigated in rats with allergic bronchial asthma (7, 11). Furthermore, the effects of both agents on the increased expression of RhoA in bronchial smooth muscles of the AHR rats were also studied.

## Materials and Methods

### *Animals and treatments*

Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g; Charles River Japan, Inc., Kanagawa) were used. All experiments were approved by the Animal Care Committee at the Hoshi University (Tokyo).

Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen (DNP-Asc) by the method described in the previous papers (7, 11, 30). In brief, the rats were sensitized with DNP-Asc together with *Bordetella pertussis* and were boosted 5 days later. Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc for 40 min

under conscious state. The animals were subjected to a total of three antigen challenges, spaced 48-h apart. The sensitized control animals received the same immunization procedure but inhaled saline aerosol instead of antigen challenge. Animals also received intraperitoneal injection with prednisolone (10 mg/kg, dissolved in 16 mM  $\text{Na}_2\text{CO}_3$  - saline; Wako, Osaka), beclomethasone (10 mg/kg, dissolved in 40% dimethyl sulfoxide (DMSO) - 10% Tween 80 - saline; MP Biomedicals, Inc., Solon, OH, USA) or either vehicle daily for 5 days from the day of the first antigen challenge. The treatment was carried out 1 h before the antigen inhalation on each day of antigen challenge.

### *Functional study on bronchial smooth muscles*

To determine the change in bronchial smooth muscle contractility, the isometric contraction of the circular smooth muscle of the main bronchus, defined as bronchial smooth muscle, was measured as described previously (7, 11, 12, 30). In brief, 24 h after the last antigen challenge, the rats were sacrificed by exsanguinations from abdominal aorta under chloral hydrate (400 mg/kg, i.p.) anesthesia. Then the airway tissues below the larynx to lungs were immediately removed. About 4-mm length (3-mm diameter) of the left main bronchus was isolated (8–9 cartilages) and the resultant tissue ring preparation was then suspended in an organ bath at a resting tension of 1 g. The organ bath contained modified Krebs-Henseleit solution with the following composition: 118.0 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 25.0 mM  $\text{NaHCO}_3$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , and 10.0 mM glucose (pH 7.4). The isometric contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T; Nihon Kohden, Tokyo). During an equilibration period, the tissues were washed three or four times at 15–20-min intervals and were equilibrated slowly to a baseline tension of 1 g. After the equilibration period, the concentration-response curve to acetylcholine (ACh,  $10^{-7}$ – $10^{-3}$  M in final concentration) was constructed cumulatively. A higher concentration of ACh was successively added after attainment of a plateau response to the previous concentration. In another series of experiments, isotonic  $\text{K}^+$  solution (10–90 mM in final concentration) was cumulatively administered in the presence of atropine and indomethacin (both  $10^{-6}$  M) to determine the bronchial smooth muscle responsiveness to high  $\text{K}^+$ -depolarization.

### *Western blot analyses*

Protein samples of bronchial tissues were prepared as previously described (7, 11, 12, 30). In brief, the airway tissues below the main bronchi to lungs were removed

and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The airways were carefully cleaned of adhering connective tissues, blood vessels and lung parenchyma under a stereomicroscopy. The epithelium was removed as much as possible by gently rubbing with keen-edged tweezers: histological examinations revealed that almost all the cells other than bronchial smooth muscle cells and cartilages were removed by this process (7, 11, 12, 30). The resultant bronchial tissues were defined as "bronchial smooth muscles" in this manuscript. Then the bronchial tissue (containing the main and intrapulmonary bronchi) segments were quickly frozen with liquid nitrogen, and the tissue was crushed to pieces by a Cryopress<sup>TM</sup> (15 s × 3) (CP-100W; Microtec, Co., Ltd., Chiba). The tissue powder was homogenized in ice-cold T-PER<sup>TM</sup> Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) containing 5  $\mu$ M 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1.5 nM aprotinin, 10 nM (2S,3S)-3-(N-{(S)-1-[N-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl) oxirane-2-carboxylic acid (E-64), 5  $\mu$ M ethylenediaminetetracetic acid (EDTA), and 10 nM leupeptin. The tissue homogenate was then centrifuged (3,000 × g, 4°C, for 15 min) and the resultant supernatant was stored at -85°C until use.

To determine the levels of RhoA, MLC, phosphorylated MLC (phospho-MLC), smooth muscle  $\alpha$ -actin, MLC kinase (MLCK), Rho-associated coiled-coil forming protein kinase-I (ROCK-I) and ROCK-II proteins in bronchial smooth muscles, the samples (10  $\mu$ g of total protein per lane) were subjected to 7.5% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then electrophoretically transferred to a poly(vinylidene fluoride) (PVDF) membrane. After blocking with 3% gelatin, the PVDF membrane was incubated with polyclonal rabbit anti-RhoA (1:2,500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-MLC (1:2,500 dilution; Santa Cruz Biotechnology, Inc.), anti-phospho-MLC (1:1,000 dilution; Santa Cruz Biotechnology, Inc.), anti-MLCK (1:1,000 dilution; Santa Cruz Biotechnology, Inc.), goat anti-ROCK-I (1:1,000 dilution; Santa Cruz Biotechnology, Inc.), anti-ROCK-II (1:1,000 dilution; Santa Cruz Biotechnology, Inc.), or monoclonal mouse anti- $\alpha$ -actin (1:5,000 dilution; Sigma, St. Louis, MO, USA) antibody. Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG), donkey anti-goat IgG, or sheep anti-mouse IgG (1:2,500 dilution; Amersham Biosciences, Co., Piscataway, NJ, USA); detected by an enhanced chemiluminescent system (Amersham Biosciences, Co.); and analyzed by a densitometry system. Detection of house-keeping gene

was also performed on the same membrane by using monoclonal mouse anti- $\beta$ -actin antibody (1:5,000 dilution; Santa Cruz Biotechnology, Inc.) to confirm that the same amount of each protein was loaded.

#### *Reverse transcription-polymerase chain reactions (RT-PCR)*

The semi-quantitative analyses of mRNA levels of RhoA were examined by RT-PCR. Briefly, total RNA was isolated from an aliquot of the tissue powder as described above with a one-step guanidium-phenol-chloroform extraction procedure using TRI Reagent<sup>TM</sup> (Sigma). cDNAs were prepared from the total RNA (1.0  $\mu$ g) by using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Inc., Hanover, MD, USA) in a total volume of 50  $\mu$ l reaction buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl<sub>2</sub>, 1 mM dNTP mixture, 1 U/ $\mu$ l RNase inhibitor, 10 ng/ $\mu$ l random 6mers, and 10 U/ $\mu$ l M-MuLV reverse transcriptase. The reaction mixture was incubated for 10 min at 25°C followed by 60 min at 42°C to initiate the synthesis of cDNAs. Reverse transcriptase was inactivated at 70°C for 5 min. Then the RT reaction mixture (4  $\mu$ l) was subjected to PCR (0.1  $\mu$ M forward and reverse primers, 0.025 U/l Taq DNA polymerase, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs) in a final volume of 20  $\mu$ l. The PCR primer sets used were as follows: 5'-TGGTGATTGT TGGTGATGGAGC-3' (sense) and 5'-CAGGATGA TGGGCACATTTGG-3' (antisense) for RhoA, 5'-CCATCACTGCCACTCAGAAGAC-3' (sense) and 5'-TACTCCTTGGAGGCCATGTAGG-3' (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were designed from published sequences (Accession No. NM\_057132 and NM\_017008, respectively). The thermal cycle profile used was 1) denaturing for 30 s at 95°C, 2) annealing primers for 30 s at 60°C, and 3) extending the primers for 60 s at 72°C. The PCR amplification was performed at 30 cycles according to the preliminary cycle dependence experiment. The PCR products were subjected to electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining. The band intensity was quantified by a densitometer (Atto Densitograph; Atto Co., Tokyo). The ratio of the RhoA/GAPDH was calculated for the relative expression of RhoA mRNA.

#### *Determination of active form of RhoA*

The active form of RhoA, GTP-bound RhoA, in bronchial smooth muscles was measured by RhoA pull down assay as described previously (10). In brief, bronchial tissues containing the main and intrapulmonary bronchi were isolated as described above. The isolated bronchial tissues were equilibrated in oxy-

generated Krebs-Henseleit solution at 37°C for 1 h. After the equilibration period, the tissues were stimulated by ACh ( $10^{-3}$  M for 10 min) and were quickly frozen with liquid nitrogen. The tissues were then lysed in lysis buffer with the following composition: 25.0 mM 2-[4-(2-hydroxyethyl)-1-piperidinyl] ethanesulfonic acid (HEPES) (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630, 10.0 mM  $MgCl_2$ , 1.0 mM EDTA, 10% glycerol, 25.0 mM NaF, 1.0 mM sodium orthovanadate, and peptidase inhibitors. Active RhoA in tissue lysates (200  $\mu$ g protein) was precipitated with 25  $\mu$ g glutathione *S*-transferase (GST)-tagged Rho binding domain (amino acids residues 7–89 of mouse rhotekin; Upstate, Lake Placid, NY, USA), which was expressed in *Escherichia coli* and bound to glutathione-agarose beads. The precipitates were washed three times in lysis buffer; and after adding the SDS loading buffer and boiling for 5 min, the bound proteins were resolved in 15% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-RhoA antibody as described above.

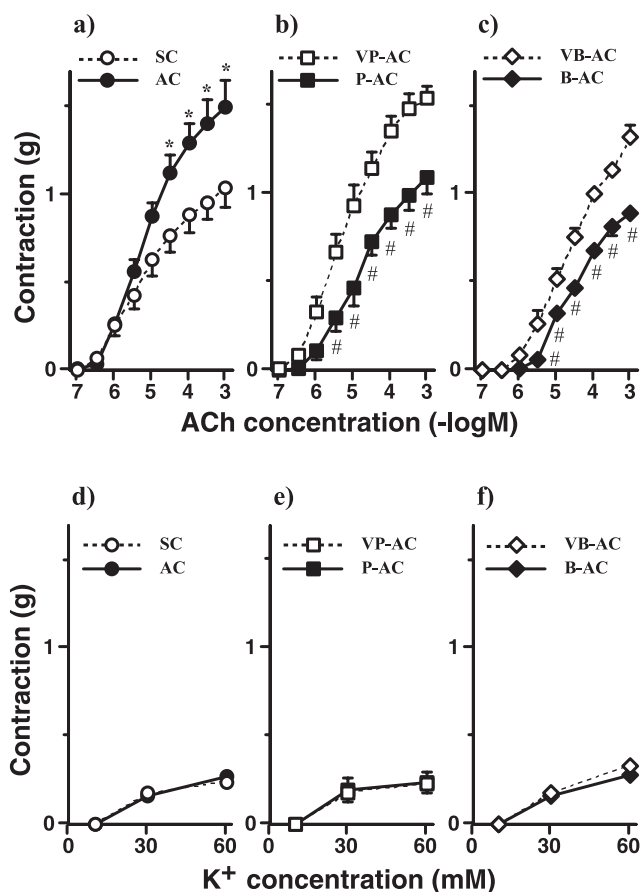
#### Statistical analyses

All the data were expressed as the mean with S.E.M. Statistical significance of difference was determined by the unpaired Student's *t*-test or two-way analysis of variance (ANOVA) with *post hoc* Bonferroni/Dunn test (StatView for Macintosh ver. 5.0; SAS Institute, Inc., Cary, NC, USA). A value of  $P < 0.05$  was considered significant.

## Results

#### Bronchial smooth muscle hyperresponsiveness after repeated antigen exposure

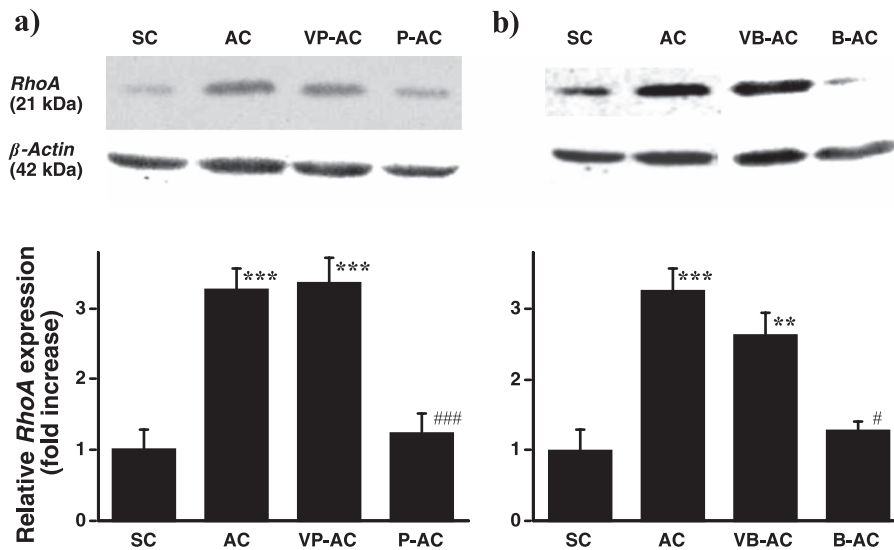
Figure 1a shows the ACh responsiveness of bronchial smooth muscles isolated from sensitized control and repeatedly antigen-challenged rats. Application of ACh ( $10^{-7}$ – $10^{-3}$  M) to isolated bronchial smooth muscles elicited a concentration-dependent contraction in all animals used. The contractile response to ACh of bronchial smooth muscles from the repeatedly antigen-challenged rats was markedly augmented as compared to that from the sensitized control animals: the ACh concentration-response curve was significantly shifted upward by the antigen exposure ( $P < 0.05$ ). On the other hand, no significant difference in the contractile response induced by high  $K^+$ -depolarization was observed between groups (Fig. 1d). These findings are consistent with our previous reports (7, 11, 31) that demonstrate an augmented agonist-induced  $Ca^{2+}$  sensitization of contraction in the repeatedly antigen-challenged rats.



**Fig. 1.** Inhibitory effects of glucocorticoids on bronchial smooth muscle hyperresponsiveness induced by repeated antigen exposure. The ACh (a–c)- and high  $K^+$  depolarization (d–f)-induced contractions of bronchial smooth muscles isolated from sensitized control (SC) and repeatedly antigen-challenged (AC) rats were measured isometrically. The glucocorticoids, prednisolone (10 mg/kg) and beclomethasone (10 mg/kg), and vehicles for prednisolone (VP: 16 mM  $Na_2CO_3$  in saline) and beclomethasone (VB: 40% DMSO–10% Tween 80 in saline) were also pretreated intraperitoneally as described in the Methods section. Each point represents the mean  $\pm$  S.E.M. from 5–6 animals. Note that the bronchial smooth muscle responsiveness to ACh (a), but not to high  $K^+$ -depolarization (d), was significantly augmented in the AC group ( $P < 0.05$ , by two-way ANOVA). The augmented ACh responsiveness induced by antigen challenge was significantly inhibited by pretreatment with prednisolone (b: P-AC) or beclomethasone (c: B-AC) when compared with respective vehicle-treated groups (VP-AC and VB-AC;  $P < 0.05$  by two-way ANOVA, respectively). \* $P < 0.05$  vs SC and # $P < 0.05$  vs VP-AC or VB-AC by unpaired Student's *t*-test.

#### Effects of glucocorticoids on the bronchial smooth muscle hyperresponsiveness induced by repeated antigen exposure

To determine the effect of systemic treatment with glucocorticoid on the augmented bronchial smooth muscle responsiveness observed in the repeatedly antigen-challenged rats, animals also received intraperitoneal injection with prednisolone, beclomethasone (each 10 mg/kg), or the respective vehicles during the period



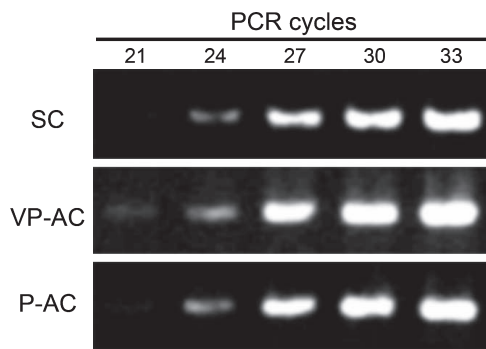
**Fig. 2.** Inhibitory effect of glucocorticoids, prednisolone (a) and beclomethasone (b), on the antigen-induced upregulation of RhoA protein in bronchial smooth muscles of rats. The RhoA protein levels in bronchial smooth muscles of sensitized control (SC) and repeatedly antigen-challenged (AC) animals were determined by Western blot analyses. Prednisolone (10 mg/kg) or beclomethasone (10 mg/kg), or with vehicle for prednisolone (VP: 16 mM Na<sub>2</sub>CO<sub>3</sub> in saline) or beclomethasone (VB: 40% DMSO – 10% Tween 80 in saline) were also pretreated intraperitoneally as described in the Methods section. Upper photos: Typical bands for RhoA and  $\beta$ -actin. The expression levels of RhoA were calculated as ratios of the intensities of RhoA to the corresponding  $\beta$ -actin bands and summarized in the lower panel. Data are expressed as the fold increase from the SC group. Each column represents the mean  $\pm$  S.E.M. from 6 animals. Note that the RhoA protein expression in bronchial smooth muscle of the AC group was significantly increased as compared with that of the SC one. The increased expression of RhoA induced by antigen challenge was significantly inhibited by pretreatment with prednisolone (a: P-AC) or beclomethasone (b: B-AC) when compared with the respective vehicle-treated groups, VP-AC (a) and VB-AC (b). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs SC, #### $P < 0.001$  vs VP-AC (a), and # $P < 0.05$  vs VB-AC (b) by two-way ANOVA with *post hoc* Bonferroni/Dunn test.

of repeated antigen exposure. As shown in Fig. 1b, the bronchial smooth muscle hyperresponsiveness to ACh induced by antigen challenge was markedly attenuated by the treatment with prednisolone: the ACh concentration-response curve of the prednisolone-treated group (P-AC) was significantly shifted downward as compared with that of the vehicle-treated one (VP-AC,  $P < 0.05$ ). In addition, the bronchial smooth muscle responsiveness to ACh of the antigen-challenged rats returned to normal level by the treatment with prednisolone: no significant difference was observed when compared with the sensitized control group (P-AC in Fig. 1b vs SC in Fig. 1a). In the case of the K<sup>+</sup> responsiveness, however, no significant change was observed by the treatment with prednisolone (Fig. 1e). Similar results were also observed when beclomethasone was used instead of prednisolone (Fig. 1: c and f). Both prednisolone and beclomethasone had no effect on the bronchial smooth muscle responsiveness of the sensitized control animals (data not shown).

#### *Effects of glucocorticoids on the upregulation of RhoA in bronchial smooth muscles induced by repeated antigen exposure*

The expressions of RhoA protein and mRNA were

assessed in the main and intrapulmonary bronchial smooth muscles without epithelia and lung parenchyma (7, 11, 12, 30). As reported previously, the expression of RhoA protein in bronchial smooth muscle of the repeatedly antigen-challenged rats was markedly and significantly increased as compared with that of the sensitized control group (Fig. 2a; SC vs AC,  $P < 0.001$ ). The increased expression of RhoA protein induced by antigen exposure was significantly inhibited by the treatment with prednisolone when compared with the group treated with its vehicle (Fig. 2a; VP-AC vs P-AC,  $P < 0.001$ ), which had no effect on the RhoA protein expression (Fig. 2a, AC vs VP-AC). The antigen-induced upregulation of RhoA protein was abolished by the treatment with prednisolone: no significant difference in the RhoA protein level was observed between the sensitized control and the prednisolone-treated, antigen-challenged groups (Fig. 2a, SC vs P-AC). Similar results were also obtained when beclomethasone was used instead of prednisolone (Fig. 2b). Furthermore, semi-quantitative analyses using RT-PCR revealed that both prednisolone and beclomethasone inhibited the increased mRNA expression of RhoA induced by repeated antigen exposure (Figs. 3 and 4). The antigen-challenged group was not subjected to the



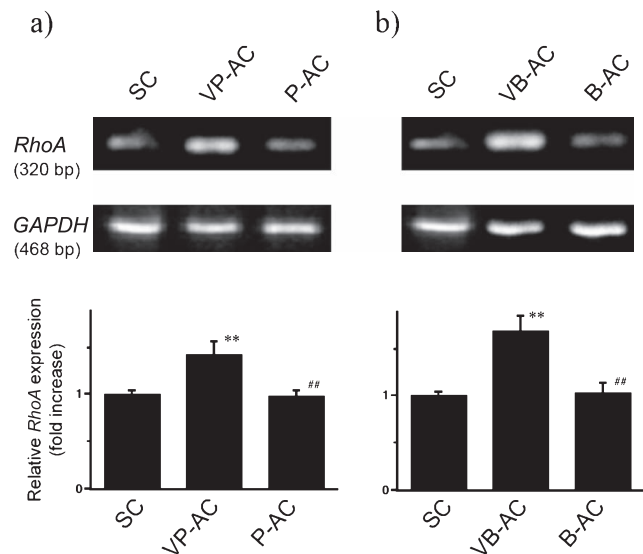
**Fig. 3.** RT-PCR analysis of RhoA mRNA levels in bronchial smooth muscles of rats. The repeatedly antigen-challenged (AC) animals were pretreated intraperitoneally either with prednisolone (10 mg/kg) or its vehicle (VP: 16 mM Na<sub>2</sub>CO<sub>3</sub> in saline) as described in the Methods section. The sensitized control (SC) animals were also used as a control. The RhoA mRNA levels were determined by RT-PCR using total RNA from bronchial smooth muscles. Typical PCR products for RhoA (320 bp) from 21, 24, 27, 30, and 33 PCR cycles of SC (upper), VP-AC (middle), and P-AC (lower) are shown.

RT-PCR analyses because the RhoA protein expression was not affected by vehicle for prednisolone and vehicle for beclomethasone (Fig. 2).

#### *Effects of glucocorticoids on the augmented ACh-induced activation of RhoA and phosphorylation of MLC in bronchial smooth muscles of airway hyperresponsive rats*

Figure 5a shows the results of RhoA pull down analyses. Representative blots for the GTP-bound active form of RhoA (GTP-RhoA) and inputted total RhoA protein are shown. ACh stimulation ( $10^{-3}$  M, for 10 min) elicited an increase in GTP-RhoA in bronchial smooth muscle of the sensitized control rats (Fig. 5a, SC group): about 2-fold increase in GTP-RhoA from the basal level (no ACh stimulation) was observed. In the repeatedly antigen-challenged animals that also received treatments with vehicle for prednisolone (VP-AC group), a marked increase in GTP-RhoA was observed when smooth muscles were stimulated with ACh. The increased levels of GTP-RhoA in ACh-stimulated bronchial smooth muscles of the antigen-exposed animals were distinctly inhibited by the treatments with prednisolone (P-AC group).

Figure 5b shows the representative immunoblots for MLC and phosphorylated MLC (phospho-MLC) in bronchial smooth muscles. ACh stimulation ( $10^{-3}$  M, for 10 min) also elicited increases in phospho-MLC in all bronchial smooth muscle tissues used. A marked increase in the level of phospho-MLC was observed in the vehicle-treated, antigen-challenged group (VP-AC) when smooth muscles were stimulated with ACh. The increment of phospho-MLC was obviously attenuated



**Fig. 4.** Inhibitory effects of glucocorticoids, prednisolone (a) and beclomethasone (b), on the antigen-induced upregulation of RhoA mRNA in bronchial smooth muscles of rats. The repeatedly antigen-challenged (AC) animals were pretreated intraperitoneally either with glucocorticoid, prednisolone (10 mg/kg) or beclomethasone (10 mg/kg), or with vehicle for prednisolone (VP: 16 mM Na<sub>2</sub>CO<sub>3</sub> in saline) or beclomethasone (VB: 40% DMSO - 10% Tween 80 in saline) as described in the Methods section. The sensitized control (SC) animals were also used as a control. The RhoA mRNA levels were determined by semi-quantitative RT-PCR using total RNA from bronchial smooth muscles. Upper photos: Typical PCR products for RhoA (320 bp) and GAPDH (468 bp). The mRNA expression levels of RhoA were calculated as ratios of the intensities of RhoA to the corresponding GAPDH bands and summarized in the lower panels. Data are expressed as the fold increase from the SC group. Each column represents the mean  $\pm$  S.E.M. from 5–6 animals. Note that the increased mRNA expression of RhoA induced by antigen challenge was significantly inhibited by pretreatment with prednisolone (a: P-AC) and beclomethasone (b: B-AC) when compared with the respective vehicle-treated groups, VP-AC (a) and VB-AC (b). \*\* $P < 0.01$  vs SC and ## $P < 0.01$  vs VP-AC (a) or VB-AC (b) by two-way ANOVA with *post hoc* Bonferroni/Dunn test.

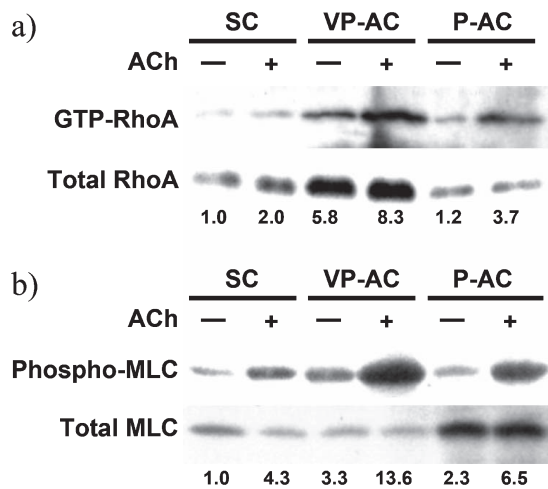
by the treatments with prednisolone (P-AC group).

Similar results of changes in the levels of GTP-RhoA and phospho-MLC were also obtained when beclomethasone was used instead of prednisolone (Fig. 6). Surprisingly, the expression of MLC (total MLC) was markedly increased by the treatments with glucocorticoids, prednisolone (Fig. 5, P-AC) and beclomethasone (Fig. 6, B-AC).

#### *Effects of repeated antigen exposure and glucocorticoids on the expression of contractile proteins in rat bronchial smooth muscle*

Figure 7 shows the representative immunoblots for ROCK-I, ROCK-II, MLCK, MLC, and  $\alpha$ -smooth muscle actin ( $\alpha$ -actin) in bronchial smooth muscles of rats. The relative expression of these proteins were



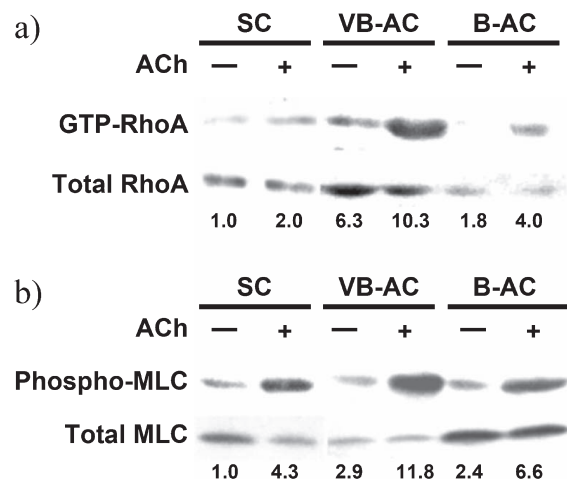


**Fig. 5.** Inhibitory effects of prednisolone on the augmented ACh-induced activation of RhoA (a) and phosphorylation of MLC (b) in bronchial smooth muscles of antigen-induced airway hyper-responsive rats. The repeatedly antigen-challenged (AC) animals were pretreated intraperitoneally either with prednisolone (10 mg/kg) or with vehicle for prednisolone (VP: 16 mM Na<sub>2</sub>CO<sub>3</sub> in saline) as described in the Methods section. The sensitized control (SC) animals were also used as a control. Isolated bronchial smooth muscles were incubated for 10 min in the absence (–) or presence (+) of 10<sup>–3</sup> M ACh before homogenization. Protein samples were then subjected to the RhoA pull down assay (for GTP-RhoA) and immunoblottings (for total RhoA, phospho-MLC, and total MLC). The ratios of the densities of GTP-RhoA/total RhoA (a) and phospho-MLC/total MLC (b) are indicated by the numbers reported on each lane representing the fold increase over the SC in the absence of ACh. The blots shown are representative of 3 independent experiments, respectively. Note that the increased GTP-bound active form of RhoA (a: GTP-RhoA) and phosphorylated MLC (b: phospho-MLC) in ACh-stimulated bronchial smooth muscles of AC animals (VP-AC) were markedly inhibited by the pretreatment with (P-AC).

calculated and summarized in Table 1. The repeated antigen challenge had no effect on the expression of these proteins studied: in each protein, no significant difference was observed between the sensitized control and the repeatedly antigen-challenged groups. Likewise, the prednisolone treatments (P-AC group) also had no effect on the expression of these proteins except for MLC when compared with its vehicle-treated (VP-AC) group. As mentioned above, the protein expression of MLC was markedly and significantly increased by the treatments with prednisolone ( $P < 0.05$  vs vehicle-treated group). Similar results were also obtained when beclomethasone was used instead of prednisolone (data not shown).

## Discussion

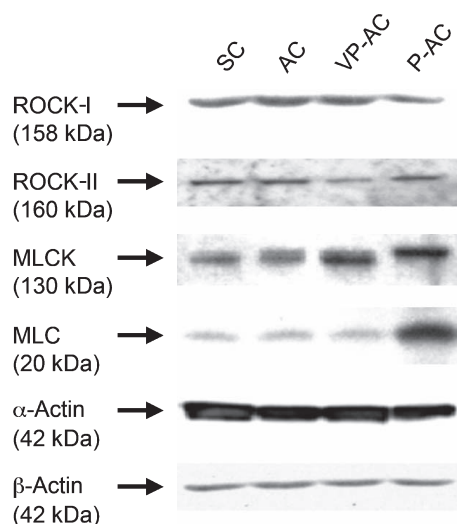
Glucocorticoids, either inhaled or systemically delivered, are the most effective medication currently



**Fig. 6.** Inhibitory effects of beclomethasone on the augmented ACh-induced activation of RhoA (a) and phosphorylation of MLC (b) in bronchial smooth muscles of antigen-induced airway hyper-responsive rats. The repeatedly antigen-challenged (AC) animals were pretreated intraperitoneally either with beclomethasone (10 mg/kg) or with vehicle for beclomethasone (VB: 16 mM Na<sub>2</sub>CO<sub>3</sub> in saline) as described in the Methods section. The sensitized control (SC) animals were also used as a control. Isolated bronchial smooth muscles were incubated for 10 min in the absence (–) or presence (+) of 10<sup>–3</sup> M ACh before homogenization. Protein samples were then subjected to the RhoA pull down assay (for GTP-RhoA) and immunoblottings (for total RhoA, phospho-MLC, and total MLC). The ratios of the densities of GTP-RhoA/total RhoA (a) and phospho-MLC/total MLC (b) are indicated by the numbers reported on each lane representing the fold increase over the SC in the absence of ACh. The blots shown are each representative of 2 independent experiments. Note that the increased GTP-bound active form of RhoA (a: GTP-RhoA) and phosphorylated MLC (b: phospho-MLC) in ACh-stimulated bronchial smooth muscles of AC animals (VB-AC) were markedly inhibited by the pretreatment with beclomethasone (B-AC).

available for the control of asthma and are widely recommended as the first-line treatment for this disease (32, 33). A regular use of glucocorticoids has been shown to markedly reduce the mortality and morbidity of asthma (34, 35). It has been believed that the anti-asthmatic effect of glucocorticoids is primarily based on their anti-inflammatory effects. In addition, glucocorticoids can reduce AHR in asthmatics (26–29), although little is known about the mechanism(s) of their inhibitory effects on the AHR. Here, we propose a new insight that glucocorticoids might inhibit the AHR by reducing the protein expression and activation of a monomeric GTP-binding protein, RhoA, which is involved in agonist-induced Ca<sup>2+</sup> sensitization of bronchial smooth muscle contraction.

The AHR is a common feature and a critical component in allergic bronchial asthma. The importance of AHR in the pathogenesis of bronchial asthma has been suggested by its correlation with the severity of this



**Fig. 7.** Typical immunoblots for contractile proteins, ROCK-I, ROCK-II, MLCK, MLC, and  $\alpha$ -actin, in bronchial smooth muscles of rats. The expression of these proteins in bronchial smooth muscles isolated from sensitized control (SC) and repeatedly antigen-challenged (AC) animals were determined by Western blot analyses. Prednisolone (10 mg/kg) and vehicle for prednisolone (VP: 16 mM  $\text{Na}_2\text{CO}_3$  in saline) were also pretreated intraperitoneally as described in the Methods section. The blots shown are each representative of 6 independent experiments. The expression levels of these proteins relative to  $\beta$ -actin were calculated and summarized in Table 1. Note that, except for MLC protein, no marked change in the expression of these proteins was observed among the groups. A marked increase in MLC protein expression was observed in the prednisolone-pretreated, AC rats (P-AC group). Similar results were also obtained when beclomethasone was used instead of prednisolone.

disease (36–38). In addition, the airways of asthmatics are characterized by an inflammation with marked eosinophilia. An importance of eosinophils in the pathogenesis of the AHR has also been suggested since the increase in number of eosinophils correlates well with an increase in airway responsiveness (38–40). Interleukin-5 (IL-5) is one of the central mediators in the regulation of eosinophilic airway inflammation (41). However, the recent clinical trials of application of

humanized monoclonal anti-IL-5 antibody to patients with asthma revealed a reduction in eosinophilia but not in AHR (42, 43). In contrast, clinical studies showed that inhaled or oral glucocorticoid treatment reduced both the numbers of airway eosinophils and AHR (26–29, 44, 45). Similar results have also been reported in murine models of allergic bronchial asthma (46, 47). These findings suggest the dissociation between eosinophilic airway inflammation and the AHR induced by allergen and that the inhibitory effect of glucocorticoids on the AHR does not appear to be explained simply by their inhibitory effect on eosinophilic inflammation.

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 (4, 5). 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. Currently, an increased responsiveness of the isolated bronchial smooth muscle to ACh was observed in rats that were sensitized and repeatedly challenged with antigen (Fig. 1a). This observation is consistent with our previous reports (7, 11, 31, 48, 49), indicating that reproducible bronchial smooth muscle hyperresponsiveness has occurred by the procedure used. In this rat with allergic bronchial asthma, an augmented agonist-induced, RhoA-mediated  $\text{Ca}^{2+}$  sensitization of bronchial smooth muscle contraction accompanied with an upregulation of RhoA protein has been demonstrated (7). The importance of RhoA and its downstream Rho-kinases (ROCK-I and ROCK-II) in contraction of human bronchial smooth muscle was also demonstrated (6), and the RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (50).

In the present study, effects of systemic treatment with glucocorticoids, prednisolone and beclomethasone, on the bronchial smooth muscle hyperresponsiveness

**Table 1.** Effects of repeated antigen exposure and prednisolone on the expression of contractile proteins, ROCK-I, ROCK-II, MLCK, MLC, and  $\alpha$ -actin, in bronchial smooth muscles of rats

Group	n	Relative expression (fold increase)				
		ROCK-I	ROCK-II	MLCK	MLC	$\alpha$ -Actin
SC	6	1.01 $\pm$ 0.12	1.00 $\pm$ 0.18	1.00 $\pm$ 0.27	1.00 $\pm$ 0.28	1.00 $\pm$ 0.07
AC	6	1.26 $\pm$ 0.18	0.81 $\pm$ 0.07	0.91 $\pm$ 0.17	1.11 $\pm$ 0.26	1.01 $\pm$ 0.08
VP-AC	6	1.13 $\pm$ 0.14	0.81 $\pm$ 0.26	0.74 $\pm$ 0.33	1.00 $\pm$ 0.33	0.90 $\pm$ 0.06
P-AC	6	1.54 $\pm$ 0.23	1.21 $\pm$ 0.13	1.46 $\pm$ 0.20	9.43 $\pm$ 1.21*	1.02 $\pm$ 0.27

SC: sensitized control group, AC: repeatedly antigen challenged group. Some of the AC animals were also pretreated intraperitoneally either with prednisolone (P: 10 mg/kg) or with vehicle for prednisolone (VP: 16 mM  $\text{Na}_2\text{CO}_3$  in saline), i.e., P-AC and VP-AC groups, as described in the Methods section. Data are expressed as the fold increase from the SC group. Each value represents a mean  $\pm$  S.E.M. \* $P < 0.05$  vs VP-AC by unpaired Student's *t*-test.



and an upregulation of RhoA protein induced by repeated antigen exposure were determined. The dosages of glucocorticoids used were chosen based on the report that prednisolone completely inhibited IL-5-induced airway eosinophilia in rats (51). Our preliminary histological studies also revealed that both prednisolone and beclomethasone, given by the same protocol as the present study, inhibited almost completely the lung eosinophilia induced by repeated antigen exposure (data not shown). Under these conditions, both glucocorticoids abolished the exaggerated bronchial smooth muscle contraction to ACh (Fig. 1) and the upregulation of RhoA protein observed in the repeatedly antigen-challenged rats (Fig. 2). Although the mechanism of action of glucocorticoids on the RhoA expression is not known here, the results of RT-PCR analyses (Figs. 3 and 4) also suggest their inhibitory effect on the transcription level at least in part. To date, the transcription factor(s) involved in the RhoA expression in bronchial smooth muscle is unclear now, but it has been suggested that proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induce airway smooth muscle cell hyperresponsiveness to contractile agonists directly (52). In addition, our previous studies revealed that TNF- $\alpha$  has an ability to increase RhoA expression in bronchial smooth muscle (53, 54). TNF- $\alpha$  is known to increase the expression of several genes via activation of transcription factors, nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1), in airway smooth muscle (55). Both NF- $\kappa$ B and AP-1 are activated in the airways of asthmatic patients and animals (56, 57). Although the exact RhoA promoter and/or enhancer regions have not yet been identified, the transcriptional start site of rat RhoA was identified by 5'-RACE (our personal communication). The DNA sequence analysis for the directly upstream region of the rat RhoA transcriptional start site (from -1 to -3901) using the TFSEARCH program (<http://mbs.cbrc.jp/research/db/TFSEARCH.html>) revealed 5 and 10 putative binding sites for NF- $\kappa$ B and AP-1 with 80.0 threshold scores, respectively. It is thus possible that antigen challenge causes the activation of NF- $\kappa$ B and AP-1 in bronchial smooth muscle, resulting in an increased RhoA transcription: glucocorticoids might exert their inhibitory effect on RhoA expression through inhibition of NF- $\kappa$ B and AP-1 (58, 59). In addition, the TFSEARCH analysis also revealed one putative glucocorticoid response element (GRE): the binding of glucocorticoid and its receptor complex to GRE may also affect the RhoA transcription. Alternatively, the inhibitory effect of glucocorticoids may result from their inhibitory effect on TNF- $\alpha$  production (60). Further detailed studies are needed to clarify the mechanism of action of gluco-

corticoids on RhoA transcription in bronchial smooth muscle. Nonetheless, the results of the current study suggest that glucocorticoids can inhibit the upregulation of RhoA in bronchial smooth muscles induced by antigen exposure.

There is increasing evidence that RhoA and its downstream target Rho-kinases (ROCK-I and ROCK-II) are involved in the agonist-induced  $\text{Ca}^{2+}$  sensitization of smooth muscle contraction including airways (6, 7, 9, 10). When the RhoA/Rho-kinase pathway was activated by contractile agonists, the activity of MLC phosphatase is reduced and the level of phosphorylated MLC is then increased, resulting in an augmentation of smooth muscle contraction. Currently, ACh stimulation of isolated bronchial smooth muscle caused both an increase in the GTP-bound form of RhoA and phosphorylation of MLC (Figs. 5 and 6). These observations suggest that stimulation of muscarinic receptors on the bronchial smooth muscle induces an activation of RhoA, which, in part, is involved in the contraction through an increase in MLC phosphorylation. Interestingly, both the activation of RhoA and the phosphorylation of MLC induced by ACh were markedly augmented in bronchial smooth muscles of the repeatedly antigen-challenged rats as compared with those of the sensitized control animals (Figs. 5 and 6). In contrast, the protein expression of MLCK, a  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase that specifically phosphorylates MLC, and MLC itself were not changed in the antigen-challenged group (Fig. 7, Table 1), suggesting that the augmented ACh-induced phosphorylation of MLC is not mediated by a  $\text{Ca}^{2+}$ -dependent but by a  $\text{Ca}^{2+}$ -independent,  $\text{Ca}^{2+}$ -sensitizing mechanism(s) such as the RhoA/Rho-kinase pathway, probably via the upregulated RhoA proteins. In the present study, both prednisolone and beclomethasone also reduced the augmented ACh-induced activation of RhoA and phosphorylation of MLC observed in the antigen-challenged group (Figs. 5 and 6). These effects may be due to their inhibitory effect on RhoA protein expression: the reduced expression of RhoA decreases the relative quantity of the active form of RhoA, resulting in a reduction of MLC phosphorylation and contraction. Surprisingly, the current study also revealed an upregulation of MLC protein by the systemic treatment with glucocorticoids (Figs. 5b, 6b, and 7 and Table 1). However, it is unlikely that the increased MLC protein is involved in the level of phosphorylated MLC and the contraction: the MLC phosphorylation induced by ACh (Figs. 5b and 6b) and the contraction induced by ACh and high  $\text{K}^+$  depolarization (Fig. 1) were the same as the control levels even in bronchial smooth muscles of the animals treated with glucocorticoids. These discrepancies should be solved in the future.

In conclusion, the current study suggests that glucocorticoids inhibit the upregulation of RhoA and the agonist-induced activation of RhoA and phosphorylation of MLC in bronchial smooth muscles, resulting in a reduction of the augmented contraction induced by antigen exposure. These inhibitory effects of glucocorticoids may account for, in part, their beneficial effects in the treatment of allergic bronchial asthma.

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