Involvement of M₃ Muscarinic Receptors in ACh-Induced Increase in Membrane-Associated RhoA of Rat Bronchial Smooth Muscle

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It is known that RhoA is translocated from cytoplasm to cell membrane in bronchial smooth muscle when activated by acetylcholine (ACh) stimulation. In the present study, the effects of selective muscarinic receptor antagonist methoctramine, AF-DX116 (for M₂) and 4-diphenylacetoxy *N*-methylpiperidine (4-DAMP; for M₃) on the ACh-induced rat bronchial smooth muscle contraction and increase in membrane-associated RhoA were investigated to elucidate the muscarinic receptor subtype participating in these responses. To evaluate ACh-induced contraction of bronchial smooth muscle, bronchial ring of rat was prepared, suspended in an organ bath and the tension was measured isometrically. To quantify the ACh-induced increase in membrane-associated RhoA protein, western blot analysis was performed by using homogenates of membrane and cytosolic fractions of the rat bronchi. The muscarinic M₂ and M₃ receptors were detected by using RT-PCR in rat bronchial smooth muscle. Both the ACh-induced smooth muscle contraction and increase in membrane-associated RhoA were markedly inhibited by 4-DAMP, but not by methoctramine or AF-DX116. In conclusion, these results indicated contraction for the first time that the activation of RhoA occurs *via* M₃ receptor in rat bronchial smooth muscle.

Key words bronchial smooth muscle; acetylcholine; muscarinic receptor; RhoA

Excitatory agonists initially increase intracellular Ca²⁺ levels, leading to activation of myosin light chain kinase (MLCK) in smooth muscle. This action is followed immediately by a decrease in MLC phosphatase (MLCP) activity and an increase in MLC₂₀ phosphorylation, which induces a further contraction, that is, Ca²⁺ sensitization.¹⁾ Second messengers/signaling pathways, including the RhoA/ROCK (Rho associated coiled-coil forming protein kinase)^{2–4)} pathways have been linked to Ca²⁺ sensitization. ROCK has been proposed to inhibit MLCP in smooth muscle response to various agonists.⁵⁾ It has been demonstrated that agonist stimulation increases myofilament Ca²⁺ sensitivity in various types of smooth muscle which were permeabilized by β -escin or α -toxin.^{6–8)}

Airway hyperresponsiveness (AHR) connected with increased airway resistance and inflammation is the asthmatic characteristic features.⁹⁾ The importance of AHR in an asthmatic cause of disease was suggested by the correlation with the severity of this illness.¹⁰⁾ Therefore, the fundamental mechanism of AHR is important for determining for asthmatic medical treatment. We previously reported the occurrence of both *in vivo* and *in vitro* hyperresponsiveness to ACh in rats that were actively sensitized and repeatedly challenged with aerosolized antigen.^{11–13)} Recently, we also demonstrated an existence of ACh-induced RhoA-mediated Ca²⁺ sensitization in smooth muscle contraction of permeabilized intrapulmonary bronchial strips and, furthermore, a strong augmentation of this Ca²⁺ sensitizing effect in the bronchial smooth muscle from repeatedly antigen-challenged rats.¹⁴⁾ It is thus possible that the augmented ACh-induced contraction of intact bronchial smooth muscle might be due to an enhanced Ca²⁺ sensitizing signal in antigen-induced AHR rats. Therefore, it is very important to solve the mechanism of Ca²⁺ sensitization for understanding the underlying mechanism of AHR. A participation of RhoA in the agonist-induced Ca²⁺ sensitization has been suggested by many investigators.^{7,15,16)} Although the activation pathway of RhoA *via* membrane receptors is not yet clear in smooth muscle, it

is known that translocation of RhoA from cytosol to plasma membrane occurs when RhoA is activated.^{14,16–18)} Indeed, we also demonstrated an ACh-induced translocation of RhoA, which means an increase in membrane-associated RhoA.¹⁹⁾ The ACh-induced translocation of RhoA was markedly increased in the bronchial smooth muscle of AHR rats, indicating that the RhoA activation induced by ACh might be augmented in hyperresponsive bronchial smooth muscle. However, the muscarinic receptor subtype(s) participating in the RhoA activation is still unclear. Thus, the aim of this study is to identify the receptor subtype which participates in an ACh-induced increase in membrane-associated RhoA in rat bronchial smooth muscle. For this purpose, the effects of selective muscarinic receptor antagonists, AF-DX116 and methoctramine (for M₂) and 4-diphenylacetoxy *N*-methylpiperidine (4-DAMP; for M₃) on the ACh-induced increase in membrane-associated RhoA were investigated.

MATERIALS AND METHODS

Animals Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g, Charles River Japan, Inc.) were housed for appropriate time intervals in Animal Center of Hoshi University after their arrival. Constant temperature and humidity (22±1 °C, 55±10%) were maintained with a fixed 12 h light–dark cycle and free access to food and water. The experiments were performed under the guiding principles for the care and use of laboratory animals approved by the local Animal Care Committee of Hoshi University (Tokyo, Japan).

RT-PCR Analysis The main and intrapulmonary bronchial tissues were quickly frozen with liquid nitrogen, and the tissues were crushed to pieces by CryopressTM (CP-100W; Niti-on, Co. Ltd., Japan: 15 s×3). Total RNA was isolated from each frozen tissue powder by using TRI[®] REAGENT (Sigma) and stored at –85 °C until use. cDNAs were prepared from the total RNA (1 µg) by reverse transcriptase reaction in a total volume of 50 µl reaction buffer containing 10 mM Tris–HCl, pH 8.3, 50 mM MgCl₂, 1 mM

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dNTP mixture, 1 U/ml RNase inhibitor, 2.5 μM random 9 mers, and 0.25 U/ml avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated for 5 min at 25 °C followed by 60 min at 42 °C to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at 70 °C for 5 min. Then the RT reaction mixture (1 μl) was added by 1 μl of 1 μM forward primer, 1 μl of 1 μM reverse primer, 3 μl of 2 X PCR Master Mix (0.05 units μl^{-1} Taq DNA polymerase, 4 mM MgCl_2 and 0.4 mM of each dNTPs; Fermentas Life Science). The PCR primers for rat muscarinic M_2 receptor used were 5'-GGGTCCTTTCCTTCATCCTCTG-3' (sense) and 5'-AACACAGGTTTCAGTCACGCCG-3' (antisense). The PCR primers for rat muscarinic M_3 receptor used were 5'-GAAAGAAGGAGAGGCATACCGC-3' (sense) and 5'-CAGTTCAGTGTTCCCCATCACG-3' (antisense), which were designed from published sequences (NM_031016 and NM_012527). The thermal cycle profile used in the present study was 1) denaturing for 3 min at 95 °C, 2) annealing primers for 15 s at 55 °C, 3) extending the primers for 1 min at 72 °C. The PCR amplifications were performed for 30 cycles. The PCR mixtures were subjected to electrophoresis on 2% agarose gel with DNA MW standard marker 100 bp DNA ladder (Takara Bio Inc.) and visualized by densitometer (Atto Densitograph; Atto Co., Tokyo, Japan).

Functional Study of Intact Smooth Muscle The animals were killed by exsanguination from abdominal aorta under anesthesia with chloral hydrate (400 mg/kg, i.p.). About 4 mm length of the left main bronchus was isolated by the method described previously.¹⁹ The resultant tissue ring preparations were then suspended in a 5 ml organ bath at a resting tension of 1.0 g. The isometrical contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T, Nihon Kohden, Japan). The organ bath contained modified Krebs–Henseleit solution with the following composition (mM): NaCl 118.0, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, NaHCO_3 25.0, KH_2PO_4 1.2 and glucose 10.0 (pH 7.4). The buffer solution was maintained at 37 °C and oxygenated with 95% O_2 –5% CO_2 . During an equilibration period in the organ bath, the tissues were washed four times at 15 min intervals and equilibrated slowly to a baseline tension of 1.0 g. After the equilibration period, a concentration–response curve to ACh (10^{-7} – 10^{-3} M) was constructed cumulatively. AF-DX116, methoctramine (a selective muscarinic M_2 receptor antagonist) and 4-diphenylacetoxy *N*-methylpiperidine (4-DAMP, a selective muscarinic M_3 receptor antagonist) were pretreated 10 min before administration of the first concentration of ACh (10^{-7} M) and was then present throughout the experiment.

Preparation of Membrane and Cytosolic Fractions Membrane and cytosolic fractions of bronchial tissue were prepared by the method described previously¹⁴ with minor modifications. In brief, the airway tissues below the main bronchi 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 stereomicroscopy. Then the bronchial tissue was equilibrated in oxygenated Krebs–Henseleit solution (37 °C) for 60 min with 10 min washout intervals. After the equilibration period, the tissue segments were stimulated by an indicated concentration of ACh (10^{-3} M) for 20 min. The reaction was stopped by quickly

freezing with liquid nitrogen and the tissue was then homogenized in 1 ml of ice-cold homogenization buffer with the following composition (mM): Tris–HCl (pH 7.5) 10, MgCl_2 5, EDTA 2, sucrose 250, dithiothreitol 1,4-(2-aminoethyl) benzenesulphonyl fluoride 1, 20 $\mu\text{g/ml}$ leupeptin and 20 $\mu\text{g/ml}$ aprotinin. This homogenate was used to quantify the expression of RhoA. The tissue homogenate was centrifuged ($105000\times g$, 4 °C for 30 min) and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 3 ml homogenization buffer and recentrifuged ($105000\times g$, 4 °C for 30 min). The resultant pellet was resuspended in 2 ml ice-cold homogenization buffer containing 1% (v/v) Triton X-100 and 1% (w/v) sodium cholate and used as the membrane fraction. These preparations were stored at –80 °C until use.

Western Blots To quantify the ACh-induced increase in membrane-associated RhoA, immunoblotting was performed as described previously.¹⁹ Briefly, the samples (10 μg protein per lane) were subjected to 15% SDS-PAGE. Proteins were then electrophoretically transferred for 4 h onto PVDF membranes (Hybond–ECL, Amersham, Little Chalfont, U.K.) in cold transfer buffer (20% methanol containing Tris 25 mM and glycine 192 mM). After repeated washing with Tris buffer (Tris 20 mM, NaCl 500 mM, pH 7.5) containing 0.1% (v/v) Tween 20 (TTBS), the PVDF membranes were incubated with blocking buffer (3% gelatine in TTBS) for 1.5 h at room temperature. The PVDF membranes were then incubated with primary antibody [polyclonal rabbit anti-RhoA (amino acids 119–132; 1: 3000 dilution, Santa Cruz Biotechnology) in antibody buffer (1% gelatine in TTBS) for 12 h at room temperature. The PVDF membranes were then washed five times (each for 15 min) with TTBS. They were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham) for 1.5 h at room temperature, and then washed five times with TTBS. The blots were detected with an enhanced chemiluminescent method (ECL System; Amersham) and quantified by densitometry (Atto Densitograph Software ver. 4.0; Atto Co., Japan). To normalize the RhoA contents to an internal control protein, β -actin, immunoblotting was also performed on the same gel by using monoclonal mouse anti- β -actin N-terminal (Sigma, St. Louis, Missouri, U.S.A.) and goat anti-mouse IgG (Amersham). Under the above conditions, a linear relationship between the band density of RhoA and amounts of loaded proteins was found for protein quantities ranging between 5 and 25 μg .¹⁹ Similar results were obtained for β -actin. The ratio of corresponding RhoA/ β -actin in each lane was calculated as indices of RhoA protein levels. The membrane/total RhoA in each animal sample was calculated according to the formula (membrane RhoA/ β -actin)/[(membrane RhoA/ β -actin)+(cytosolic RhoA/ β -actin)].

Statistical Analyses All the data were expressed as the mean with S.E. Statistical analyses were performed by one or two-way ANOVA with *post-hoc* tests performed using a Bonferroni/Dunn's test.

RESULTS

Figure 1 shows the expression of muscarinic M_2 and M_3 receptor mRNAs in rat bronchial smooth muscle, as determined by RT-PCR using total RNA. The PCR amplifications

Fig. 1. Expression of M_2 and M_3 Receptor mRNA in the Rat Bronchial Smooth Muscle

The PCR amplifications were performed for 30 cycles. The lane 1 was represented 100 bp DNA ladder marker. The predicted sizes of the bands for M_2 (lanes 2 and 3; 383 bp) and M_3 (lanes 4 and 5, 511 bp) receptors were detected.

Fig. 2. The Effects of Pretreatment with Methoctramine (Selective M_2 receptor Antagonist, 10^{-7} M), AF-DX116 (Selective M_2 Receptor Antagonist, 10^{-7} M) and 4-DAMP (Selective M_3 Receptor Antagonist, 10^{-7} M) on the ACh-Induced Bronchial Smooth Muscle Contraction

Each point represents the mean $\pm$ S.E. from 6 experiments. ACh-induced bronchial smooth muscle contraction was significantly inhibited by pretreatment with 4-DAMP as compared with vehicle ($p < 0.001$, two-way ANOVA).

were performed for 30 cycles. The expected sizes of the bands for M_2 (383 bp) and M_3 (511 bp) were clearly detected in rat bronchial smooth muscle.

Figure 2 shows the effects of pretreatment with selective M_2 or M_3 receptor antagonist on ACh-induced contractile responses of intact muscle bronchial smooth muscle. ACh (10^{-7} – 10^{-3} M) elicited a concentration-dependent contractile response in bronchial smooth muscle. The ACh-induced contraction was attenuated by treatment with 4-DAMP (10^{-7} M, a selective M_3 receptor antagonist). However, pretreatment with the M_2 antagonists, methoctramine (10^{-7} M) and AF-DX116 (10^{-7} M), had no effect on the ACh-induced contractile responses.

To determine the ACh-induced increase in membrane-associated RhoA in rat bronchial smooth muscle, immunoblottings were performed in the homogenates of main and intrapulmonary bronchi which were dissected free from lung parenchyma. As shown in Fig. 3, immunoblotting with RhoA antibody of membrane fraction gave a single band with 21 kD molecular weight, indicating existence of RhoA protein in the rat bronchial smooth muscle (Fig. 3A). The level of membrane RhoA was significantly increased by ACh (10^{-3} M) stimulation (Fig. 3B). No significant difference in the ACh-induced increase in membrane-associated RhoA in bronchial smooth muscle was observed between the control and methoctramine- or AF-DX116-treated groups. However, the ACh-induced increase in membrane-associated RhoA

Fig. 3. The Effects of Selective Muscarinic Receptor Antagonists, Methoctramine (Met, Selective M_2 Receptor Antagonist, 10^{-7} M), AF-DX116 (AF-DX, Selective M_2 Receptor Antagonist, 10^{-7} M) and 4-DAMP (DAMP, Selective M_3 Receptor Antagonist, 10^{-7} M) on the ACh-Induced Increase in Membrane-Associated RhoA in Rat Bronchial Smooth Muscle

(A) Typical photograph of bands for RhoA (upper) and β -actin (lower) in membrane fraction by immunoblots. The bronchi were pretreated with muscarinic receptor antagonists for 10 min before stimulation by ACh (10^{-3} M). Ten minutes after the ACh stimulation, the reaction was stopped by liquid nitrogen, and tissues were homogenized to prepare cytosolic and membrane fractions. Western blot was performed by using cytosolic and membrane fractions both on RhoA and β -actin in the identical transferred membrane, and the ratios of RhoA to the corresponding β -actin band densities were calculated and summarized in (B) (membrane RhoA/total RhoA). Values are the means $\pm$ S.E. from 4–5 experiments. ACh-induced increase in membrane-associated RhoA was significantly inhibited by pretreatment with 4-DAMP, but not with methoctramine and AF-DX116. * $p < 0.05$ and *** $p < 0.001$ vs. None. # $p < 0.05$ vs. vehicle (ACh alone).

was significantly attenuated by treatment with 4-DAMP.

DISCUSSION

ACh interacts with two muscarinic receptors expressed in airway smooth muscles,²⁰ M_3 receptors coupled *via* $G\alpha_q$ with phosphoinositide hydrolysis and inositol 1,4,5-trisphosphate-dependent Ca^{2+} release and M_2 receptors coupled *via* $G\alpha_i$ with inhibition of adenylate cyclase.^{20–22} Indeed, in the present study, M_2 and M_3 receptor mRNAs were detected in rat bronchial smooth muscle.

In both the ACh-induced smooth muscle contraction and increase in membrane-associated RhoA were markedly inhibited by M_3 receptor selective antagonist, 4-DAMP, but not by M_2 receptor selective antagonists, methoctramine or AF-DX116.

Studies of Ca^{2+} sensitization demonstrated G-protein-dependent augmentation of contractile activity at fixed Ca^{2+} concentrations.^{4,23–26} The pathway is initiated by receptor-mediated activation of a heterotrimeric G-protein coupled with activation of the monomeric G-protein RhoA *via* dissociation of the Rho guanine nucleotide dissociation inhibitor (RhoGDI). When activated by agonists acting on surface membrane receptors in permeabilized smooth muscle, RhoA dissociates from its cytosolic complex with GDI and translocates to surface membrane.^{16,27} Activated RhoA is translocated to the plasma membrane where it stimulates a specific Rho kinase.¹⁶

In the present study, ACh-induced contraction was significantly attenuated by 4-DAMP (a selective M_3 antagonist), suggesting that M_3 receptor is mainly involved in the ACh-induced rat bronchial smooth muscle contraction. This finding is identical to our previous report.²⁸⁾ Furthermore, the ACh-induced increase in membrane-associated RhoA in rat bronchial smooth muscle was inhibited by 4-DAMP, suggesting that M_3 receptor, but not M_2 receptor, has an important role in ACh-induced RhoA activation. Lately, Murthy *et al.*²⁰⁾ demonstrated that ACh-induced activation of Rho kinase and myosin phosphatase (MYPT) phosphorylation were inhibited by pretreatment with 4-DAMP in rabbit intestine smooth muscle cell. Activation of transfected human M_3 receptor induced cytoplasmic myosin reorganization by Rho-dependent mechanism in Chinese hamster ovary (CHO-m3) cells.³¹⁾ This result might be consistent with the current study. We previously reported the existence of ACh-induced Ca^{2+} sensitization in β -escin-permeabilized bronchial smooth muscle, which was inhibited by a RhoA inhibitor, C3 exoenzyme¹⁴⁾ and Y-27632.²⁹⁾ Moreover, distinct expression of ROCK-I (p160 ROCK) and ROCK-II (Rho-kinase α) was also demonstrated in airway smooth muscle.^{29,30)} Thus, the RhoA/ROCK pathway seems to be considered as a major signaling pathway in upstream of ACh-induced Ca^{2+} sensitization in rat bronchial smooth muscle. Although the signal transduction between M_3 and RhoA is not solved currently, further research is required about these mechanisms.

In conclusion, the current findings clearly demonstrated for the first time that ACh induces an increase in membrane-associated RhoA protein *via* activation of muscarinic M_3 receptors in rat bronchial smooth muscle.

Acknowledgements We thank Ms. Miho Aiba and Ms. Akiko Okada for their help in technical assistance.

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