Acetylcholine-Induced Smooth Muscle Contraction of Intrapulmonary Small Bronchi Is Augmented in Antigen-Induced Airway Hyperresponsive Rats

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ABSTRACT—Smooth muscle responsiveness of intrapulmonary small bronchi obtained from repeatedly antigen-challenged rats was compared with that from control animals to determine whether smooth muscle contractility of peripheral airways is augmented by such repeated challenge. In intact (non-permeabilized) smooth muscles of intrapulmonary bronchi, the acetylcholine (ACH)-induced contractile response was significantly augmented in the repeated challenge group, although 60-mM K⁺-induced contraction was within the normal level. In β-escin-permeabilized muscles, no significant difference between groups was observed in the Ca²⁺-induced contractile responses. Thus, augmented ACh-induced contraction of intact intrapulmonary small bronchial smooth muscle might be, at least in part, due to an enhanced ACh-mediated Ca²⁺-sensitizing signal.

Keywords: Airway hyperresponsiveness, Permeabilized intrapulmonary bronchial smooth muscle, Ca²⁺ sensitization

Increased airway narrowing in response to nonspecific stimuli is a characteristic feature of human obstructive diseases, including asthma. This abnormality is an important morbid state of the disease, although the pathophysiological variations leading to the hyperresponsiveness are unclear now. Several mechanisms have been suggested to explain airway hyperresponsiveness (AHR) such as alterations in the neural control of airway smooth muscle (1), increased sensitivity of airway smooth muscle (2–5), increased mucosal secretions (6) and mechanical factors related to remodeling of the airways (7).

We previously reported that repeated antigen inhalation to actively sensitized rats causes airway inflammation and marked AHR in vivo to inhaled contractile agonists (3). In this animal model of AHR, the isolated smooth muscle of the main bronchus had also hyperresponsiveness (3–5), although the smooth muscle responsiveness of the upper trachea was within the normal level (3). It is thus possible that a regional difference in the induction of augmented contractility exists in airway smooth muscles of the hyperresponsive animals. Moreover, it has been suggested that obstructive airways disease is associated with an increase in the ability of the peripheral airway smooth muscle to generate force (8). In the present study, to characterize the contractile response of peripheral airways subjected to repeated challenge, smooth muscle responsiveness of intrapulmonary small bronchi (ring strips; about 200-μm width, <500-μm diameter) obtained from AHR rats was compared with that from control animals.

Male Wistar rats (170–190 g, specific pathogen-free) were used. The induction of AHR was performed as described previously (4, 5). Briefly, the rats were sensitized with 2,4-dinitrophenylated Ascaris suum extract (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 20 min under the conscious state. Then the animals were subjected to a total of 3 times repeated antigen challenge every 48 h with the same inhalational challenge method.

At 24 h after the last antigen challenge, the third branch of intrapulmonary bronchus was isolated, carefully cleared of lung parenchyma and adhering connective tissue, and then cut into ring strips (about 200-μm width, <500-μm diameter). The epithelium was removed by gently rubbing with keen-edged tweezers. Then the intact (non-permeabilized) muscle strip was suspended in a 400-μl organ bath containing oxygenated Krebs-Henseleit solution (118.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 1.2 mM KH₂PO₄, 10.0 mM glucose, pH

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7.4) at room temperature. The contractile force developed was measured by an isometric transducer under a resting tension of 50 mg.

In the case of permeabilized smooth muscle experiments, the muscle strips were permeabilized by a 30-min treatment with 10 μM β-escin (Sigma, St. Louis, MO, USA) in relaxing solution at room temperature. Relaxing solution contained: 20 mM PIPES, 7.1 mM Mg$^{2+}$-dimethanesulfonate, 108 mM K$^+$-methanesulfonate, 2 mM EGTA, 5.875 mM Na$_2$ATP, 2 mM creatine phosphate, 4 U/ml creatine phosphokinase, 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and 1 μg/ml E-64 (pH 6.8) containing 10 μM A23187. Free Ca$^{2+}$ concentration was changed by adding an appropriate amount of CaCl$_2$. The apparent binding constant of EGTA for Ca$^{2+}$ was considered to be 10$^6$ M$^{-1}$ (9). The permeabilized muscle strip was then suspended in a 400-μl organ bath at room temperature under a resting tension of 50 mg.

All the data were expressed as the mean with S.E.M. Statistical significance of difference was determined by Dunnett's multiple or two way analysis of variance (ANOVA). A value of P<0.05 was considered significant.

Figure 1 shows the acetylcholine (ACh) responsiveness of intact (non-permeabilized) intrapulmonary bronchial smooth muscles isolated from nonsensitized normal control and repeatedly antigen challenged rats. ACh (10$^{-7}$ - 10$^{-3}$ M) elicited concentration-dependent contractile responses in both the groups. However, the concentration-response curve to ACh was significantly shifted upward in the antigen-treated group (P<0.01 by ANOVA); the contractile response induced by 10$^{-5}$ M ACh was significantly greater in the antigen-treated group (P<0.01, Fig. 1). On the other hand, no significant difference in the 60 mM K$^+$-induced contraction (in the presence of 10$^{-6}$ M atropine and 10$^{-6}$ M indomethacin) of intact smooth muscle of intrapulmonary bronchi was observed between groups (Fig. 2). The 60 mM K$^+$-induced contraction was completely inhibited by a voltage-dependent Ca$^{2+}$ channel blocker, nicardipine (10$^{-6}$ M, data not shown).

In β-escin-permeabilized smooth muscle of intrapulmonary

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**Fig. 1.** Acetylcholine (ACh) concentration-response curves for contractile responses of intact (non-permeabilized) smooth muscles of intrapulmonary small bronchi from nonsensitized normal control (Control) and repeatedly antigen challenged rats (Antigen-treated). Values are means ± S.E.M. from 6 experiments. *P<0.05 and **P<0.01 vs Control group. The concentration-response curve to ACh was significantly shifted upward in the antigen-treated group (P<0.01 by ANOVA).

**Fig. 2.** Depolarization (60 mM K$^+$)-induced contraction of intact (non-permeabilized) smooth muscle of intrapulmonary small bronchi from nonsensitized normal control (Control) and repeatedly antigen challenged rats (Antigen-treated). Values are means ± S.E.M. from 6 experiments. No significant difference was observed between groups.

**Fig. 3.** Ca$^{2+}$ concentration-response curves of β-escin-permeabilized smooth muscles of intrapulmonary small bronchi from nonsensitized normal control (Control) and repeatedly antigen challenged rats (Antigen-treated). Values are means ± S.E.M. from 6 experiments. No significant difference was observed between groups.
bronchi, the application of free Ca$^{2+}$ (pCa = 6.5, 6.0, 5.5 and 5.0) induced a concentration-dependent reproducible contractile response (data not shown), indicating successful permeabilization. In the β-escin-permeabilized strips, no significant difference between groups was observed in the Ca$^{2+}$ responsiveness and the maximal contractile response induced by pCa = 5.0 (Fig. 3).

We previously demonstrated the in vivo AHR to inhaled Ach in rats that were treated with antigen by the same method described in the present study (Materials and Methods section) (3). Furthermore, the isolated smooth muscle itself of the main bronchus, but not of the upper trachea, from the AHR rat had a hyperresponsiveness (3–5). In the present study, smooth muscle responsiveness of intrapulmonary small bronchi obtained from AHR rats was compared with that from control animals to determine whether smooth muscle contractility of peripheral airways is also augmented by repeated antigen challenge. In our previous study, no histological change of the smooth muscles was observed in this animal model of AHR (3).

In general, smooth muscle contraction is thought to be induced by an increase in cytosolic Ca$^{2+}$ concentration via the activation of plasma membrane Ca$^{2+}$ channels and/or Ca$^{2+}$ release from sarcoplasmic reticulum. The increased cytosolic Ca$^{2+}$ forms a 4-Ca$^{2+}$-calmodulin-myosin light chain kinase (MLCK) complex and activates MLCK. The activated MLCK phosphorylates the 20-kD myosin light chain leading to smooth muscle contraction (10). In the β-escin-permeabilized strips of the present study, the Ca$^{2+}$ responsiveness of intrapulmonary bronchial smooth muscles from repeatedly antigen challenged rats was similar to that from the control group (Fig. 3). Therefore, in the absence of contractile agonist, the basal Ca$^{2+}$ sensitivity of the contractile apparatus (e.g., the activities of MLCK and actomyosin-ATPase) in the peripheral airway smooth muscle of AHR rats might be within the normal level. Furthermore, the function of voltage-dependent Ca$^{2+}$ channels might also be normal because no change of the contractile response induced by 60 mM K$^+$ depolarization was observed in the intact (non-permeabilized) muscle strips (Fig. 2). These findings may be consistent with our previous results obtained by using main bronchial smooth muscles of the AHR rats (5).

In contrast to the Ca$^{2+}$-induced (in permeabilized strips) and high K$^+$-induced (in intact strips) contraction, Ach-induced contraction of intact (non-permeabilized) intrapulmonary bronchial smooth muscles of repeatedly antigen challenged rats was markedly augmented as compared to that of control animals (Fig. 1). A significant enhancement of maximal contraction (induced by 10$^{-7}$ M Ach, Fig. 1) suggests to our mind that muscarinic receptor density may be increased in peripheral airway smooth muscle of AHR rats. However, this hypothesis appears unlikely because we previously demonstrated that muscarinic receptor density and antagonist affinity of airway homogenates including peripheral airway smooth muscles are normal in this animal model of AHR (4). Similarly, no change in the level of muscarinic receptors has been reported in murine (11) and guinea pig models of AHR (12). On the other hand, we recently reported the existence of Ach-mediated, RhoA-mediated Ca$^{2+}$ sensitization in smooth muscle contraction of β-escin-permeabilized intrapulmonary bronchial strips and an augmentation of this Ca$^{2+}$ sensitizing effect in the AHR rats (13). Furthermore, simultaneous measurement of Ach-induced increase in cytosolic Ca$^{2+}$ and tension developed by using Fura-2 loaded intact (non-permeabilized) bronchial smooth muscles showed that the level of increase in cytosolic Ca$^{2+}$ in AHR rats was similar to that in control rats, although the tension developed was significantly augmented (14). It is thus possible that the augmented Ach-induced contraction of intact intrapulmonary bronchial smooth muscle might be, at least in part, due to an enhanced Ca$^{2+}$ sensitizing signal, especially a RhoA-mediated pathway, in antigen-induced AHR rats. Nevertheless, further studies are necessary to reveal the involvement of enhanced Ach-induced Ca$^{2+}$ sensitization in the augmented Ach-induced contraction of intact small bronchial smooth muscle at the AHR.

In conclusion, Ach-induced smooth muscle contraction of intrapulmonary small bronchi was augmented in antigen-induced AHR rats, although Ca$^{2+}$-induced contraction was within the normal level. We postulate that Ach released from cholinergic nerve endings in airways might induce hyper-contraction of peripheral airways at AHR and the augmented contractile response of these small airway smooth muscles may cause an in vivo hyperresponsive airway obstruction in allergic asthma.

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REFERENCES

5. Chiba Y and Misawa M: Alteration in Ca$^{2+}$ availability involved


