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Short communication

Possible involvement of G_{i3} protein in augmented contraction of bronchial smooth muscle from antigen-induced airway hyperresponsive rats

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Abstract

To investigate a possible involvement of pertussis toxin (PTX)-sensitive heterotrimeric G proteins in the pathogenesis of airway hyperresponsiveness, the effect of PTX treatment on the augmented contractile response to acetylcholine (ACh) in bronchial smooth muscle of antigen-induced airway hyperresponsive rats was determined. In bronchial smooth muscle of airway hyperresponsive rats that were actively sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen, ACh-induced contractions were markedly augmented. The augmented contractile responses in the airway hyperresponsive group were significantly inhibited after treatment with PTX (1 μg/mL for 6 hr, 37°), whereas only a slight attenuation was observed in the normal control group. The level of $G\alpha_{i3}$ (measured by immunoblotting), but not other α-subunits of $G_{i/o}$ family proteins, in bronchial smooth muscle of the airway hyperresponsive rats was significantly increased as compared with that of control animals. It is concluded that PTX-sensitive muscarinic contractile responses of bronchial smooth muscle might be augmented upon antigen-induced airway hyperresponsiveness in rats, probably due to an up-regulation of $G\alpha_{i3}$ protein of bronchial smooth muscle.

Keywords: Asthma; Airway hyperresponsiveness; Bronchial smooth muscle; Muscarinic contractile response; Pertussis toxin-sensitive G proteins; Acetylcholine

1. Introduction

Asthmatic patients have an increased contractility of airway smooth muscle [1], which might be a major cause of airway hyperresponsiveness. Similarly, an increased responsiveness of bronchial smooth muscle has been demonstrated in a rat model of airway hyperresponsiveness induced by repeated antigen inhalation [2–4]. In this animal model of airway hyperresponsiveness, bronchial smooth muscle contraction induced by receptor agonists such as ACh, but not by high K^+ depolarization, was markedly augmented [2–4], although the level of muscarinic receptors was within normal [3]. Moreover, it has also been demonstrated that ACh-induced, monomeric G protein (RhoA)-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction is markedly augmented in airway hyperresponsive rats [5]. It is therefore possible that increased agonist-induced, RhoA-mediated Ca^{2+} sensitization might be one of the essential mechanisms of the exaggerated bronchial smooth muscle contraction upon airway hyperresponsiveness, although the activation pathway of RhoA in airway smooth muscle is not clear.

ACh-induced airway smooth muscle contraction has been thought to be mediated by an activation of a PTX-insensitive heterotrimeric G protein, G_q. However, recent studies have suggested an involvement of PTX-sensitive G proteins, $G_{i/o}$, in muscarinic receptor-mediated airway smooth muscle contraction [6] and Ca^{2+} sensitization [7]. Furthermore, treatment of airway smooth muscle with inflammatory cytokine induced an up-regulation of G_i protein concurrent with ACh hyperresponsiveness [8]. In the present study, the effect of PTX treatment on the augmented contractile response to ACh in bronchial smooth muscle of airway hyperresponsive rats was determined to confirm the involvement of PTX-sensitive G proteins in the pathogenesis of airway hyperresponsiveness. The expression levels

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Abbreviations: ACh, acetylcholine; and PTX, pertussis toxin.

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of α-subunits of the $G_{i/o}$ protein subfamily were also measured in the animal model of airway hyperresponsiveness.

2. Materials and methods

2.1. Sensitization and antigenic challenge

Male Wistar rats (6 weeks of age, specific pathogen-free; Charles River Japan, Inc.) were sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen by the method described in our previous papers [2–5].

2.2. Functional study

Animals were killed by exsanguination from abdominal aorta under anesthetization by chloral hydrate (400 mg/kg, i.p.). An approximate 4-mm length of the left main bronchus was isolated, and epithelium was removed by gently rubbing with keen-edged tweezers [5]. The resultant tissue ring preparation was then suspended in an organ bath at a resting tension of 1.0 g. 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 isometrical contraction of the circular smooth muscle was measured as described previously [3,4]. PTX treatment was performed by incubation with 1 μg/mL of PTX for 6 hr at 37° in the organ bath [6].

2.3. Western blotting

The plasma membrane preparations of bronchial smooth muscles (containing the main and intrapulmonary bronchi that were dissected free from lung parenchyma and epithelium) were prepared as described previously [5]. The samples (10 μg of total protein per lane) were subjected to 10% SDS–PAGE, and the proteins were then electrophoretically transferred to a nitrocellulose membrane. After blocking with 3% gelatin, the nitrocellulose membrane was incubated with primary antibody (polyclonal rabbit anti-$G\alpha_{i1}$, anti-$G\alpha_{i2}$, anti-$G\alpha_{i3}$, or anti-$G\alpha_o$; 1:2500 dilution; Santa Cruz Biotechnology). Then, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:2500 dilution; Amersham), detected by an enhanced chemiluminescent system (Amersham), and analyzed by a densitometry system. Thereafter, the primary and secondary antibodies were stripped and the membrane was reprobed by using monoclonal mouse anti-β-actin (1:5000 dilution; Sigma) and goat anti-mouse IgG (1:2500 dilution; Amersham). Under the above conditions, a linear relationship between the band density of β-actin and the amounts of loaded proteins was found for protein concentrations ranging between 5 and 25 μg [9]. Similar results were obtained for Gα proteins (data not shown). The ratios of corresponding Gα protein/β-actin in each lane were calculated as indices of Gα protein levels.

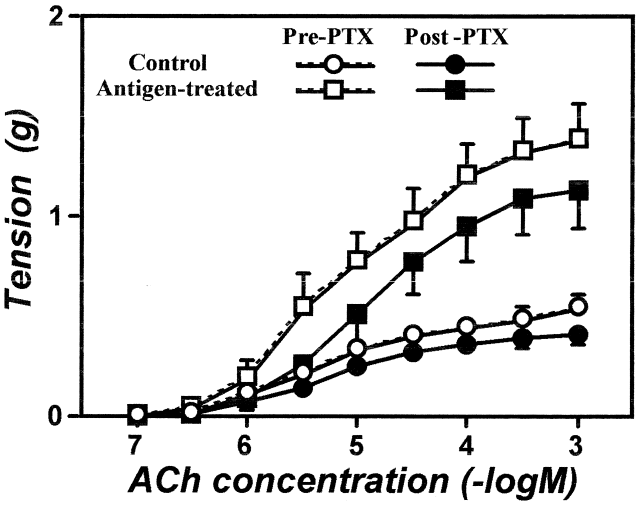

Fig. 1. Effect of treatment with PTX on ACh-induced contractile responses of bronchial smooth muscle from normal control (Control) and repeatedly antigen-challenged rats (Antigen-treated). ACh responsiveness was measured before (Pre-PTX) and after treatment with PTX (1 μg/mL for 6 hr, 37°; Post-PTX). The values are means ± SE from 7 experiments.

2.4. Data and statistical analysis

In the functional study, the $-\log EC_{50}$ (M), Hill coefficient (n), and maximum response (E_{max}; g) values were obtained from individual concentration–response curves. Hill plots were constructed by plotting $\log[E/(E_{max}-E)]$ versus $-\log([ACh])$, where E = response. The n was calculated as the slope of the least square regression line fit to points. The $-\log EC_{50}$ was calculated as the mean abscissal intercept for each Hill plot.

All the data are presented as mean values with standard error of the mean (SEM). Statistical evaluation of data was performed with the StatView-J 5.0 software program (SAS) using one- or two-way ANOVA, where appropriate, and Dunnett's multiple analysis.

3. Results and discussion

As shown in Fig. 1, cumulatively administered ACh elicited a concentration–dependent contraction of bronchial rings in all groups. Without PTX treatment, the concentration–response curve to ACh of antigen-treated rats was markedly and significantly shifted upward as compared with that of control animals (Fig. 1, Table 1), indicating that reproducible airway hyperresponsiveness occurs at the level of bronchial smooth muscle after repeated antigen inhalation [2–5]. After treatment with PTX (1 μg/mL for 6 hr at 37°), the ACh-induced bronchial contractions in antigen-treated rats were significantly inhibited, whereas only a

Table 1
Effect of PTX on ACh-induced contractile responses in bronchial smooth muscles of normal control (Control) and repeatedly antigen-challenged (Antigen-treated) rats

Group	PTX	$-\log EC_{50}$ (M)	n	E_{max} (g)
Control	−	4.78 ± 0.19	0.73 ± 0.09	0.55 ± 0.06
	+	4.97 ± 0.12	1.02 ± 0.10***	0.41 ± 0.05
Antigen-treated	−	5.39 ± 0.11*	0.75 ± 0.04	1.42 ± 0.12**
	+	5.41 ± 0.17	0.96 ± 0.05***	1.10 ± 0.16**,***

Values represent means ± SEM of seven experiments. The $-\log EC_{50}$ (M), Hill coefficient (n) and maximum response (E_{max}; g) values were obtained from individual concentration–response curves as described in section 2.
* $P < 0.05$ and ** $P < 0.01$ vs corresponding control groups;
*** $P < 0.05$ versus respective PTX (−) groups by Dunnett's multiple analysis.

slight attenuation was observed in control animals (Fig. 1, Table 1). In both groups, sham incubation with vehicle for PTX had no effect on the ACh-induced contractile responses (data not shown). PTX is known to inhibit $G_{i/o}$-mediated functions by ADP-ribosylation of α-subunits of these G proteins. Kume *et al.* [6] also reported that treatment with PTX (1 μg/mL for 6 hr at 37°, i.e. the same conditions used in the present study) reduced the contractile response to methacholine in guinea pig tracheal smooth muscle. It is thus possible that the $G_{i/o}$-mediated pathway might be, at least in part, involved in muscarinic contractile responses of airway smooth muscle in rodents. Moreover, the PTX-sensitive component of the ACh-induced contraction was much greater in the airway hyperresponsive group (Fig. 1, Table 1), indicating that $G_{i/o}$-mediated contractile responses might be augmented in the hyperresponsive bronchial smooth muscle. However, because the antigen-induced enhancement was not completely reversed by PTX treatment (Fig. 1), other mechanism(s) might also be involved in this enhanced responsiveness. For example, an increased expression of $G\alpha_q$, a PTX-insensitive G protein, has also been demonstrated in this rat model of airway hyperresponsiveness [9].

Representative immunoblots of α-subunits of G_i family proteins are shown in Fig. 2. Immunoblottings with the antibody against $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$ showed a single 41–42 kD band, indicating the existence of these α-subunits in rat bronchial smooth muscle. As compared with the control group, the level of $G\alpha_{i3}$ was significantly increased in bronchial smooth muscles of antigen-treated rats, whereas the other α-subunits of $G_{i/o}$ proteins remained at the same levels or decreased (in the case of $G\alpha_{i1}$) (Fig. 3). Therefore, it is possible that augmented PTX-sensitive bronchial contractile responses in airway hyperresponsive rats (Fig. 1) might be, at least in part, due to the up-regulation of $G\alpha_{i3}$ protein in bronchial smooth muscle. On the other hand, the down-regulation of $G\alpha_{i1}$ protein irrespective of the augmented PTX-sensitive contractions in airway hyperresponsive rats may indicate no functional linkage between $G\alpha_{i1}$ and muscarinic contractile responses.

It has been reported that treatment of airway smooth muscle with interleukin-1β, which is increased in the airways of asthmatics [10] and airway hyperresponsive rats,[1] induced an up-regulation of $G\alpha_i$ proteins concurrent with ACh hyperresponsiveness [8]. Although the detailed mechanism(s) of $G\alpha_i$-mediated muscarinic contractile responses is not clear now, an involvement of G_i protein in ACh-induced, Rho-mediated Ca^{2+} sensitization of airway smooth muscle contraction has been suggested [7]. Furthermore, we recently demonstrated that ACh-induced, RhoA-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction is markedly augmented in airway hyperresponsive rats [5]. It is thus possible that the up-regulated $G\alpha_i$ protein, especially $G\alpha_{i3}$, might cause an enhancement of ACh-induced Ca^{2+} sensitization, resulting in an augmented contractile response of bronchial smooth muscle upon airway hyperresponsiveness.

[1]Chiba Y, Sakai H, Misawa M, unpublished observation.

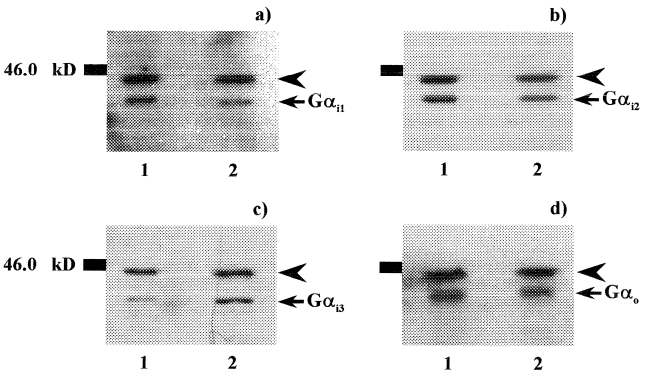

Fig. 2. Typical immunoblots of $G\alpha_{i1}$ (*a*), $G\alpha_{i2}$ (*b*), $G\alpha_{i3}$ (*c*), and $G\alpha_o$ (*d*) in membrane preparations of bronchial smooth muscles from normal control (*lane 1*) and repeatedly antigen-challenged rats (*lane 2*). The membrane preparations were subjected to 10% SDS–PAGE and transferred to nitrocellulose membranes, which were incubated with specific antibody against α-subunits of the respective G proteins. Detection was performed by incubation with horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescent system. Then, the nitrocellulose membranes were stripped and reprobed by specific antibody against β-actin (*arrow heads*). Note that each panel shows a superimposition of two films obtained from $G\alpha$ and reprobed β-actin immunoblottings of the same gel. The left bars show a protein molecular weight marker (biotin-labeled ovalbumin; 46.0 kD).

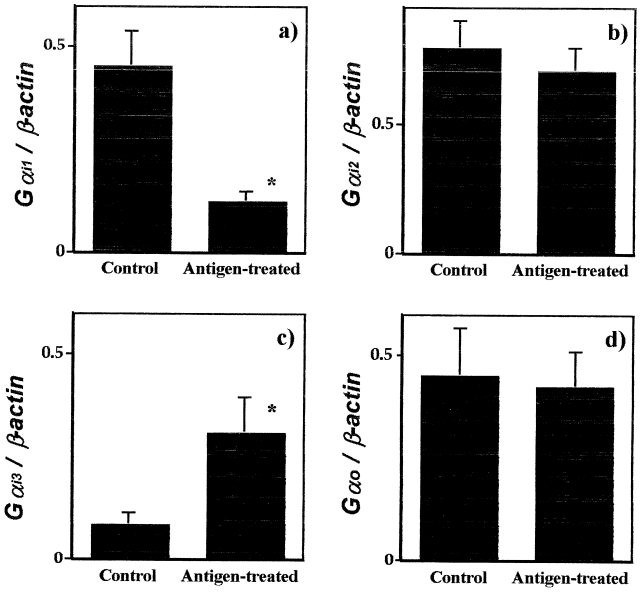

Fig. 3. Levels of $G\alpha_{i1}$ (*a*), $G\alpha_{i2}$ (*b*), $G\alpha_{i3}$ (*c*), and $G\alpha_{o}$ (*d*) in membrane preparations of bronchial smooth muscles from normal control (Control) and repeatedly antigen-challenged rats (Antigen-treated). The band densities obtained from $G\alpha$ and reprobed β-actin immunoblottings of the same lane were measured, and the ratios of corresponding $G\alpha$ protein/β-actin in the respective lanes were calculated as indices of these $G\alpha$ protein levels. The values are means ± SE from 4–6 independent experiments. $*P < 0.05$ versus control by Dunnett's multiple analysis.

In conclusion, PTX-sensitive muscarinic contractile responses of bronchial smooth muscle might be augmented at antigen-induced airway hyperresponsiveness in rats, probably due to an up-regulation of $G\alpha_{i3}$ protein of bronchial smooth muscle.

Acknowledgment

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