G_s protein dysfunction in allergen-challenged human isolated passively sensitized bronchi

PINGFANG SONG, MANLIO MILANESCE, EMANUELE CRIMI, SANTINA BRUZZONE, ELENA ZOCCHI, KAI REHDER, AND VITO BRUSASCO

Cattedra di Fisiopatologia Respiratoria, Dipartimento di Scienze Motorie e Riabilitative, and Cattedra di Biochimica, Dipartimento di Medicina Sperimentale, Università di Genova, 16132 Genoa, Italy

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Am J Physiol Lung Cell Mol Physiol 279: L209–L215, 2000.—We studied the intracellular mechanisms of allergen-induced β2-adrenoceptor dysfunction in human isolated passively sensitized bronchi. Sensitization was obtained by overnight incubation of bronchial rings with serum containing a high specific IgE level to Dermatophagoides but a low total IgE level. Allergen challenge was done by incubation with a Dermatophagoides mix. The G_s protein stimulant cholera toxin (2 μg/ml) displaced the concentration-response curves to CCh of control, sensitized, and challenged rings to the right. Cholera toxin (10 μg/ml) displaced the concentration-response curves to CCh of control, sensitized, and challenged rings to the right, but this effect was less in challenged rings. The effects of the G_s protein inhibitor pertussis toxin (250 ng/ml or 1 μg/ml) on salbutamol concentration-relaxation curves did not differ significantly between challenged and sensitized rings. The adenylyl cyclase activator forskolin and the Ca^{2+}-activated K^+ channel opener NS-1619 relaxed CCh-contracted bronchial rings without significant differences between control, sensitized, and challenged rings. Neither G_s nor G_i α-subunit expression differed between control, sensitized, and challenged tissues. We conclude that G_s protein dysfunction may be a mechanism of allergen-induced β2-adrenoceptor dysfunction in human isolated passively sensitized bronchi.

DECREASED AIRWAY RELAXATION to β-adrenoceptor stimulation has been proposed as a potential mechanism of airway hyperresponsiveness in asthma (6, 19). This hypothesis is supported by the demonstration of impaired β-adrenoceptor function in isolated bronchi from patients with fatal asthma (1) or from antigen-sensitized animals (20, 22). Recently, it has been shown that β2-adrenoceptor dysfunction can also be induced by allergen challenge of human passively sensitized bronchi (18). The dysfunction seems to be related to the release of peptido-leukotrienes (17), but the intracellular mechanisms involved have not been elucidated.

The relaxant effect of β-adrenoceptor stimulation is mediated through a series of intracellular events, including receptor-effector coupling by activation of the stimulating guanine nucleotide regulating protein (G_s protein), increase in cAMP by activation of adenylyl cyclase, and cell membrane hyperpolarization by opening of Ca^{2+}-activated K^+ (K_{Ca}) channels. Moreover, the response to β-adrenoceptor stimulation is negatively affected by M_2 receptor or G_i protein signaling.

The aim of the study was to investigate which mechanisms are affected by allergen challenge of human isolated passively sensitized bronchial rings. The functions of G_s protein, G_i protein, K_{Ca} channel, and adenylyl cyclase were compared between control, sensitized, and allergen-challenged rings. The expression of G_s protein and G_i protein in the three conditions was determined by Western blot analysis.

METHODS

Tissue Preparation

Materials. Bronchi were obtained from 35 patients who underwent lobectomies for resection of lung cancer. After tissue for microscopic examination had been removed, the bronchi were immersed in chilled (4°C) and aerated (95% O_2-5% CO_2) physiological salt solution (PSS) and transported to the laboratory. The PSS had the following composition (in mM): 110.5 NaCl, 3.4 KCl, 2.4 CaCl_2, 0.8 MgSO_4, 1.2 KH_2PO_4, 25.7 NaHCO_3, and 5.6 dextrose. Within 4 h after resection, bronchi (3- to 5-mm ID) were dissected from the surrounding tissue and cut into rings. Sera from asthmatic patients with concentrations of specific IgE > 17.5 Phadebas radioallergosorbent test (RAST) units/ml (4th RAST class; Pharmacia, Uppsala, Sweden) to Dermatophagoides pteronyssinus and D. farinae were obtained; the concentration of total IgE in the serum was 220 IU/ml. The serum was diluted 1:10 with PSS before use as a sensitizing serum.

Passive sensitization. Aerated bronchial rings were incubated with the diluted sensitizing serum for 18 h at room temperature. These rings are referred to as sensitized rings.

Address for reprint requests and other correspondence: V. Brusasco, Dipartimento di Scienze Motorie e Riabilitative, Università di Genova, Largo R. Benzi, 10, 16132 Genoa, Italy (E-mail: brusasco@dism.unige.it).
Paired rings from each patient were treated identically with sera from nonallergic subjects (paper radioimmunosorbent test < 20 IU/ml and negative RAST for *D. pteronyssinus* and *D. farinae*). These rings are referred to as control rings.

**Allergen challenge.** Sensitized rings from each patient were washed for 15 min with PSS and then incubated for 60 min with 5 ml of aerated (37°C) PSS containing 200 arbitrary units/ml of *Dermatophagoides* mix (Laboratorio Farmaceutico Lofarma, Milan, Italy). These rings are referred to as challenged rings.

**Verification of passive sensitization and exclusion of natural sensitization.** Passive sensitization and absence of natural sensitization was verified by incubating sensitized and control rings with 1,000 arbitrary units of *Dermatophagoides* mix in 25 ml of the tissue bath for 1 h. Successful passive sensitization was assumed if the allergen challenge resulted in a contractile response > 0.5 g. Absence of natural sensitization was assumed if control rings did not contract after allergen challenge.

**Functional Studies**

Bronchial rings were suspended between two stirrups in water-jacketed 25-ml tissue baths containing aerated PSS at 37°C. The lower stirrup was connected via a silk string to a fixed hook at the bottom of the tissue bath. The upper stirrup was connected via a silk string to a force transducer (model FT 03D, Grass Medical Instruments, Quincy, MA) mounted on a micromanipulator. Isometric forces were continuously recorded (model TA 4000, Gould, Valley View, OH). All rings were equilibrated for 2 h with PSS and washed every 20 min with PSS. During this time, the rings were gradually stretched to a resting force of 1 g. The length of the rings was not altered during the experiments.

**Effect of cholera toxin on carbachol concentration-response curves.** One control, one sensitized, and one challenged ring from each of nine patients were incubated with 10⁻⁸ M carbachol (CCh), and the contractile responses were recorded. After a steady contractile response was achieved, the CCh concentration was cumulatively increased in half-log increments to 10⁻⁴ M, and the contractile responses were recorded. After completion of the CCh concentration-response curves, the rings were washed with PSS until the resting forces had been reestablished. The rings were then incubated with 2 (n = 6 patients) or 10 (n = 3 patients) μg/ml of cholera toxin (CTX) for 6 h and then washed with PSS. Complete sets of CCh concentration-response curves were then obtained.

**Effect of pertussis toxin on relaxant salbutamol concentration-relaxation curves.** Two sensitized and two challenged rings from each of 10 patients were incubated with 250 ng/ml (n = 4 patients) or 1 μg/ml (n = 6 patients) of pertussis toxin (PTX) for 4 h. The rings were then half-maximally contracted with 10⁻⁶ M CCh (18). After the contractile responses had become stable, salbutamol was added cumulatively (10⁻⁴ to 10⁻⁴ M in half-log increments), and the relaxant responses were recorded.

**Relaxant effect of forskolin.** One control, one sensitized, and one challenged ring from each of six patients were incubated with 10⁻⁶ M CCh. After the contractile responses had become stable, forskolin was added cumulatively (10⁻⁹ to 10⁻⁴ M in half-log increments), and the relaxant responses were recorded.

**Effect of CTX on CCh concentration-response curves.** Expression of Gᵢ and Gₛ subunits were assayed by Western blot analysis of control, sensitized, and bronchial tissues from four separate patients. Samples were harvested 8 h after completion of the challenge, cut into small pieces, and homogenized in ice for 3 min in 0.5 ml of a buffer solution containing 50 mM Tris-HCl (pH 6.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 5 μg/ml each of leupeptin, aprotonin, and trypsin inhibitor. After a 2-min centrifugation in an Eppendorf centrifuge at 13,000 rpm, the supernatants were recovered, and the protein concentration was measured according to Bradford (4). Ten micrograms of each sample were diluted in Laemmli sample buffer (11), heated to 100°C, and subjected to SDS-PAGE on a 10% acrylamide gel. Proteins were blotted on nitrocellulose membranes as described by Towbin et al. (21). Saturation of the membranes with milk powder, incubation with the first antibody (rabbit polyclonal IgG anti-Gᵢα subtypes 1, 2, and 3 or anti-Gₛα, Santa Cruz Biotechnology, Santa Cruz, CA) with the second antibody (anti-rabbit IgG, Amersham, Milan, Italy), and immunodetection were performed following the instructions of the Amersham ECL kit. Successful passive sensitization and absence of natural sensitization were demonstrated in bronchial rings from the same subjects.

**Data Analysis**

The force developed by muscles used for relaxation studies is expressed as a percentage of the contractile response to 10⁻⁶ M CCh. The force developed by muscles used for CTX studies is expressed as a percentage of the contractile response to 10⁻⁴ M CCh in the absence of CTX.

Concentration-response curves were analyzed by two- or three-factor repeated-measures ANOVA with Newman-Keuls post hoc test. Bronchial ring characteristics were compared by a between-within groups mixed ANOVA. Differences were considered significant if P values were < 0.05. Data are presented as means ± SD.

**RESULTS**

Passive sensitization was demonstrated in all patients and the absence of natural sensitization in 30 of the 35 patients.

**Functional Studies**

The mean weight of the 27 rings was 107 ± 53 mg, the mean resting force was 1.1 ± 0.4 g, and the maximal response to 10⁻⁴ M CCh was 28 ± 15 g/g (force/wet weight of tissue). Mean weight, resting force, and maximal response were not significantly different between control, sensitized, and challenged rings.
Incubation of control (Fig. 1A) and sensitized (Fig. 1B) rings with 2 μg/ml of CTX displaced the CCh concentration-response curves significantly to the right \((P < 0.01)\) and reduced maximal force \((P < 0.01)\). In contrast, in challenged rings (Fig. 1C), the CCh concentration-response curve \((P > 0.2)\) and maximal force \((P > 0.6)\) were not significantly altered by incubation with CTX.

Incubation of control (Fig. 2A) and sensitized (Fig. 2B) rings with 10 μg/ml of CTX displaced the whole CCh concentration-response curves significantly to the right \((P < 0.001)\) and reduced maximal force \((P < 0.001)\). In contrast, in challenged rings (Fig. 2C), CTX reduced the response to \(10^{-6}\) and \(3 \times 10^{-7}\) M CCh only, without affecting maximal response.

**Effect of PTX on salbutamol concentration-relaxation curves.** The mean weight of the 38 rings used (a pair of sensitized rings was not included because one of the two rings did not contract in response to CCh) was \(136 \pm 49\) mg, the mean resting force was \(1.0 \pm 0.3\) g, and the response to \(10^{-6}\) M CCh was \(13 \pm 7\) g/g (force/wet weight of tissue). Mean weight and resting force were not significantly different between sensitized and challenged rings. Salbutamol relaxed CCh-contracted sensitized and challenged rings in a concentration-related manner \((P < 0.001;\) Fig. 3). The presence of 250 ng/ml of PTX did not alter the salbutamol concentration-relaxation curves of either sensitized \((P > 0.95)\) or challenged \((P > 0.8)\) rings (Fig. 3, A
and B, respectively). PTX (1 μg/ml) significantly displaced the salbutamol concentration-relaxation curves of both sensitized (Fig. 3C) and challenged (Fig. 3D) rings to the left (P < 0.001) without a significant difference between the 2 conditions (P > 0.95). *Significant difference between salbutamol concentration-relaxation curves of both sensitized and challenged rings, P < 0.05.

Relaxant effect of forskolin. The mean weight of the 18 rings was 110 ± 36 mg, the mean resting force was 0.9 ± 0.3 g, and the response to 10^{-6} M CCh was 17 ± 5 g/g (force/wet weight of tissue). Mean weight, resting force, and response to CCh were not significantly different between control, sensitized, and challenged rings.

Forskolin relaxed CCh-contracted control, sensitized, and challenged rings in a concentration-related manner (P < 0.001; Fig. 4). There were no significant differences between control, sensitized, and challenged rings (P > 0.6).

Relaxant effect of NS-1619. The mean weight of the 18 rings was 78 ± 28 mg, the mean resting force was 0.9 ± 0.1 g, and the response to 10^{-6} M CCh was 15 ± 5 g/g (force/wet weight of tissue). Mean weight, resting force, and response to CCh were not significantly different between control, sensitized, and challenged rings.

NS-1619 relaxed CCh-contracted control, sensitized, and challenged rings in a concentration-related manner (P < 0.001; Fig. 5). There were no significant differences between control, sensitized, and challenged rings (P > 0.8).

G Protein Expression

Western blot analysis showed comparable expression of either G_iα or G_sα (Fig. 6, Table 1) in control, sensitized, and challenged rings.
DISCUSSION

The major findings of this study are that allergen challenge of human isolated passively sensitized bronchi did not reduce the relaxant effects of either the KCa channel opener NS-1619 or the adenylyl cyclase activator forskolin. An inhibitory effect of the Gs protein stimulator CTX on CCh-induced contractile response was observed in control and sensitized rings, but this inhibitory effect was reduced in allergen-challenged rings.

Before the underlying mechanisms for β2-adrenoceptor dysfunction are considered, the current understanding of β2-adrenoceptor activation and signal transduction is briefly discussed. Agonist binding to β2-adrenoceptors causes conformational changes of the receptor, allowing activation of the α-subunit of the Gs protein. The activated Gsα stimulates adenylyl cyclase, which converts ATP into cAMP. The latter, in turn, activates protein kinase (PK) A and PKG, which induce several intracellular events, resulting in the relaxation of the smooth muscle. The activated Gsα can also directly activate the KCa channel independent of an increase in cAMP (8). Dysfunction of the β2-adrenoceptor can occur at any level in this cascade of events. In addition “cross talk” between muscarinic or inflammatory mediator receptors and β2-adrenoceptors may result in phosphorylation of both the β2-adrenoceptor itself and the Gs protein, thus inducing β2-adrenoceptor dysfunction (5, 9, 15, 16).

In the current study, dysfunction of β2-adrenoceptors in airway smooth muscle was induced by allergen challenge of human passively sensitized isolated bronchial rings (17, 18). Control, sensitized, and challenged rings were then incubated with compounds with specific actions on the function of either the Gs protein, the KCa channel, or adenylyl cyclase. The effects of these compounds on muscle contractions and relaxation were compared in the three conditions.

The function of the KCa channel was assessed by incubating the muscles with the KCa channel opener NS-1619 (13) and comparing the relaxation before and after incubation with NS-1619. The relaxant effect of NS-1619 on the control, sensitized, and allergen-challenged rings was not significantly different, suggesting that KCa transport through the KCa channel was not affected by allergen challenge of human isolated bronchial rings.

The effect of allergen challenge on adenylyl cyclase activity was inferred from its effect on the relaxant effect of forskolin in control, sensitized, and allergen-challenged rings. If the activity of adenylyl cyclase were reduced by allergen challenge, muscle relaxation should be less after incubation with forskolin. Allergen challenge did not reduce the relaxation of the bronchial rings by forskolin, suggesting that the activity of the adenylyl cyclase and the function of the relaxation machinery were not affected. This conclusion is similar to that by Kume and Tagaki (10). These authors reported that homologous desensitization of guinea pig trachealis did not alter adenylyl cyclase activity.

Incubation of control and sensitized (but not of challenged) rings with CTX resulted in a rightward displacement of the CCh concentration-response curves and a reduction in the maximal force. Incubation with CTX irreversibly stimulates the α-subunit of the Gs protein, increasing the intracellular concentration of
cAMP and thereby inhibiting smooth muscle contraction. Allergen challenge greatly reduced the inhibitory effects of CTX on CCh-induced contractions, suggesting that the Gs protein was dysfunctional. Western blot analysis showed no difference in Gs protein expression between control, sensitized, and challenged tissues. Therefore, the reduced response to CTX seems to be the result of reduced Gs protein activity rather than expression. Homologous β2-adrenoceptor desensitization of isolated guinea pig trachealis can also be prevented by incubation with CTX, suggesting that the α-subunit of the Gs protein is dysfunctional (10).

The present study cannot rule out that desensitization through phosphorylation of β2-adrenoceptors may contribute to the reduced response to β2-agonists. Iralukast, a peptido-leukotriene receptor antagonist prevents β2-adrenoceptor dysfunction induced by allergen challenge (17). But the underlying mechanisms for this effect are still unclear. Allergen challenge may phosphorylate β2-adrenoceptors, Gs protein, or both (5, 16). A cross talk between β2-adrenoceptors and inflammatory mediator receptors via PKC may occur (3, 7). The PKC may phosphorylate both β2-adrenoceptors and Gs protein.

β2-Adrenoceptor hyporesponsiveness may also be induced by treatment of human isolated smooth muscle cells with interleukin-1β (12). This effect has been suggested to be mediated by the release of prostanoids from the smooth muscle cells as a result of cyclooxygenase-2 activation because it is prevented by indomethacin. In human passively sensitized bronchi, indomethacin does not prevent β2-adrenoceptor dysfunction induced by allergen challenge (17).

As in a previous study (18) on human isolated bronchi passively sensitized with sera containing a low level of IgE, neither sensitization nor allergen challenge altered the contractile responses to CCh, suggesting that the capacity to generate force was similar between the two conditions and the control condition. Therefore, differences in response to CTX between allergen-challenged and control or sensitized rings are not due to a difference in force generation capacity. Sensitization with sera containing high levels of total IgE caused β2-adrenoceptor dysfunction (6) and variable effects on airway smooth muscle response to contractile agonists as the response to histamine was increased (2), whereas the response to cholinergic agonists was either decreased (2) or increased (6). Whether Gs protein, adenylyl cyclase, or Kcach channel function can be altered by passive sensitization with high total IgE levels without allergen challenge has not been determined.

The nonselective muscarinic agonist CCh was used to contract airway smooth muscle in functional studies. Activation of M2 receptors or Gi protein signaling pathway may inhibit adenylyl cyclase, thus causing a reduced response to β2-adrenoceptor or Gs protein stimulation. Increased expression or increased activity of Gi protein would, therefore, have negatively affected the response to CTX. In rabbit bronchi sensitized with human serum containing a high level of total IgE, Hakonarson et al. (6) found an increased expression of Gi protein and an enhanced relaxant response to isoproterenol with PTX, suggesting an increased Gi protein function. In the present study, with human bronchi sensitized with human serum containing a relatively low level of total IgE, neither sensitization nor allergen challenge increased Gi protein expression. Furthermore, the effect of PTX on salbutamol concentration-relaxation curves was not different between sensitized and challenged rings. It is therefore unlikely that the present data are accounted for by increased M2 receptor or Gi protein signaling. The difference between the present findings and those of Hakonarson et al. may be either species related or sensitizing serum related.

In conclusion, exposure to sensitizing allergens may cause Gs protein dysfunction in human isolated passively sensitized bronchi, an effect that may be due to phosphorylation of the Gs protein by PKC. The Gs protein dysfunction may be a contributing mechanism underlying β2-adrenoceptor dysfunction in bronchial asthma.

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