Functional importance of the cytoskeletal protein in acetylcholine-induced contraction of rat bronchial smooth muscle

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Abstract

The contractile capacity of smooth muscle cells depends on the cytoskeletal framework of the cell. The aim of this study was to determine the functional importance of the microtubule, actin filament and intermediate filament components of the cytoskeleton in acetylcholine (ACh)-induced contractile responses of the rat isolated bronchial smooth muscle. The expressions of α-actin, β-actin, α-tubulin, desmin and vimentin were observed by immunoblotting in rat bronchial tissues. α-Actin and desmin were immunohistochemically observed in smooth muscle layer. Cytochalasin D, latrunculin A (inhibitors of the actin cytoskeleton) and acrylamide (an inhibitor of the intermediate filament) significantly decreased the contractions induced by ACh in concentration-dependent manners. On the other hand, colchicine or nocodazole (inhibitors of the microtubule cytoskeleton) had no effect on the ACh-induced contraction. These findings suggest that the contraction induced by ACh is highly dependent on polymerization of actin and intermediate filament, such as desmin, but not on the polymerization of microtubule in rat bronchial smooth muscle.

Key words: microtubule, actin, intermediate filament, bronchial smooth muscle contraction, acetylcholine

Introduction

The bronchial smooth muscle is a fundamental component of the bronchus. The parasympathetic division of the autonomic nervous system exerts a critical role in the regulation of the contractile status of the bronchus, since acetylcholine (ACh) is the primary parasympathetic neurotransmitter in the airways and is traditionally associated with inducing airway smooth muscle contraction and mucus secretion (Gosens et al., 2006).

In general, three components of the cytoskeleton (i.e., actin filament, microtubule such as tubulin, and intermediate filaments such as desmin and vimentin) have been described in
smooth muscle. Generation of smooth muscle contraction requires not only contraction of actin-myosin filaments but also physical linkage of these filaments to plasma membrane and extracellular matrix. Most investigators have assumed that these linkages are relatively static and play no role in contractile responses in smooth muscle. However, Hirshman and Emala (Hirshman and Emala, 1999) indicated that stimulation with contractile agonists induced an increase in the ratio of filamentous actin (F-actin) to globular actin (G-actin), i.e., actin polymerization in canine tracheal smooth muscle. In addition, latrunculin A inhibits contraction and alters cell stiffness of tracheal smooth muscle cells in dogs (Mehta and Gunst, 1999). Several recent studies have demonstrated that alterations in microtubule polymerization can significantly affect smooth muscle contractile tone. In larger conduit arteries, microtubule depolymerization has been shown to enhance contractile responsiveness to agonists (Chitaley and Webb, 2002; Platts et al., 2002). Similarly, it is reported that colchicine, an inhibitor of microtubule polymerization, enhanced the phenylephrine-induced vascular smooth muscle contraction in the thoracic aorta (Zhang et al., 2001). However, the role of components of the cytoskeleton in ACh-induced bronchial smooth muscle contraction is not to be revealed. In the present study, to determine the functional importance of the actin filament, microtubule and intermediate filament components of the cytoskeleton in contractile responses of the rat bronchial smooth muscle, we investigated the effects of cytochalasin D and latrunculin A, inhibitors of actin polymerization (Mehta and Gunst, 1999; Tang and Tan, 2003), colchicine and nocodazole, inhibitors of the polymerization of microtubule (Oka et al., 2005), and acrylamide, a disturber of intermediate filaments (Eckert, 1985; Moy et al., 2004; Wang and Stamenovic, 2000) on the ACh-induced bronchial smooth muscle contraction.

Materials and Methods

Animals

Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g, Charles River Japan, Inc.) housed for appropriate time intervals in the animal center of Hoshi University after their arrival. Constant temperature and humidity (22 ± 1°C, 55 ± 10%) were maintained with a fixed 12-hr light-dark cycle, and free access to food and water was available. Experiments were done according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

Immunoblot analysis

Homogenate of the main bronchial tissue (about 3 mm length of the left main bronchus) was prepared by the method described previously (Sakai et al., 2006) with minor modifications. In brief, the airway tissues below the main bronchi were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit (K-H) solution. They were carefully cleaned of adhering connective tissues and blood vessels under stereomicroscopy. The tissue was then homogenized in 1.5 ml of ice-cold homogenization buffer with the following composition: 10 mmol/l Tris-HCl (pH 7.5), 5 mmol/l MgCl₂, 2 mmol/l EDTA, 250 mmol/l sucrose, 1 mmol/l dithiothreitol, 1 mmol/l 1,4-(2-aminoethyl) benzenesulphonyl fluoride, 20 µg/ml leupeptin and
20 µg/ml aprotinin. The tissue homogenate was centrifuged (3,000 × g, 4°C for 30 min). The supernatants were collected and stored at −80°C until use.

In the α-actin, β-actin, α-tubulin, desmin and vimentin expression studies, the samples were subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were then electrophoretically transferred to PVDF membranes. After blocking, the membranes were then incubated with the primary antibodies. As the primary antibodies, mouse anti-α-smooth muscle actin (1:1000; Sigma), mouse anti-β-actin (1:3000), mouse anti-desmin (1:1000; Santa Cruz Biotechnology, Inc), mouse anti-vimentin (1:1000; Santa Cruz Biotechnology, Inc), mouse anti-α-tubulin or mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (1:3000 dilution; Chemicon) were used. Then the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G (1:5000 dilution; Amersham) or goat anti-mouse IgG (1:5000 dilution; Amersham), detected by an ECL system. GAPDH was used as an internal control, because this protein is also constitutively expressed in most tissues and is the most widely accepted internal control in the molecular biology literature.

**Histochemistry and immunohistochemistry**

The bronchial rings were fixed in 10% formaldehyde and embedded in Paraplast X-TRATM paraffin (Fisher Healthcare, Houston, TX, U.S.A.). Four-micrometers sections were obtained from blocks and mounted on silane-coated glass slides, deparaffinized with xylene and graded ethanol. For the histochemical examination, the sections were stained with hematoxylin and eosin (HE) by a standard technique. For the immunohistochemical examination, the sections were incubated with each antibody as the primary antibody by using a streptavidin-biotin immunoperoxidase method as described previously (Chiba et al., 2006) with a minor modification. In brief, before immunostaining, rehydrated sections were pretreated by incubation in 0.5% Triton-X 100 in PBS for 10 min for permeabilization and were immersed in 0.3% hydrogen peroxide in 100% methanol for 30 min to remove endogenous peroxidase activity. The pretreated sections were washed with PBS and incubated sequentially at room temperature in the following solutions: (1) primary antibody (diluted 1:100 in PBS containing 1% skim milk) overnight, (2) biotinylated goat anti-mouse IgG (diluted 1:200 in PBS containing skim milk) for 30 min, and (3) avidin-biotinylated peroxidase complexes (Vector Laboratory, Inc., Burlingame, CA, CA) in PBS for 30 min. The bound peroxidase activity was visualized by incubation with 0.7 mg/ml 3,3-diaminobenzidine-0.02% H₂O₂ in 60 mmol/l Tris buffer (pH 7.5). Sections were rinsed in PBS after each step of immunostaining procedure. Finally, the sections were counterstained with Hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA, U.S.A.), dehydrated, and mounted in permanent mounting medium.

**Functional study**

The animals were killed by exsanguination from abdominal aorta under anesthesia with chloral hydrate (400 mg/kg, i.p.). About 3 mm length of the left main bronchus was isolated by the method as described previously (Sakai et al., 2006; 2007). Fat and connective tissues were removed, and epithelial cells were removed by gentle rubbing the intimal surface with a thin stainless rod moistened with Krebs-Henseleit (K-H) solution. The bronchial rings were
mounted on two L-shaped stainless prongs in a 5 ml organ bath. The organ bath contained modified K-H solution with the following composition (mmol/l): NaCl 118.0, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25.0, KH₂PO₄ 1.2 and glucose 10.0 (pH 7.4). The buffer solution was maintained at 37°C and oxygenated with 95% O₂-5% CO₂. One prong was connected to a force displacement transducer (TB-612T, Nihon Kohden, Japan) for continuous recording of isometric tension. The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs. The resting tension was set up by transducer equipped with manipulator. In brief, just after setting the tissue in an organ bath, a tension (around 1.5 g) was loaded to the tissue. During an equilibration period in the organ bath, the tissue was washed three to four times at 15 to 20 min intervals and equilibrated gradually to a baseline tension of 1.0 g. In some case, further equilibration period was required for tissue stabilization. After treatment with cytochalasin D and latrunculin A (inhibitors of actin polymerization), colchicine and nocodazole (inhibitors of the microtubule cytoskeleton), acrylamide (an inhibitor of the intermediate filament), 0.1% dimethyl sulfoxide (DMSO; vehicle for cytochalasin D, latrunculin A and nocodazole) or K-H solution (vehicle for colchicine and acrylamide) for 45 min, higher concentrations of ACh were successively added after attainment of a plateau response to the previous concentration. In the case of another experiments, each agent was cumulatively applied to the strip at intervals of about 45 min after the 10⁻⁴ M ACh-induced contraction reached a plateau.

Statistical analyses

All the data were expressed as the mean with S.E. Statistical significance of difference was determined by two-way analysis of variance (ANOVA) or Bonferroni/Dunn’s post-test. A value of $P<0.05$ was considered significant.

Results

To determine the expression of cytoskeleton protein in bronchial tissue of rat, immunoblottings were performed in the homogenates of the main bronchi. As shown in Fig. 1, immunoblotting with α-actin, β-actin, α-tubulin, desmin and vimentin gave clear bands, indicating the existence of these proteins in the rat bronchial ring strips. Immunohistochemical studies revealed that the desmin and α-actin were clearly detected in smooth muscle layer of bronchus (Fig. 2C and D). However, β-actin, α-tubulin and vimentin were slightly detected overall in bronchial sections (data not shown).

Figure 3 shows the effects of pretreatment with latrunculin A and cytochalasin D (inhibitors of actin polymerization, Fig. 3) on the ACh-induced contractile responses of bronchial muscle strips. These agents were pretreated 45 min before administration of the first concentration (10⁻⁷ M) of cumulatively added ACh and then present throughout the experiment. ACh (10⁻⁷–10⁻³ M) elicited a concentration-dependent contractile response of bronchial smooth muscles. The ACh-induced contraction was not affected by pretreatment with DMSO, vehicle for these compounds (data not shown). The ACh-induced contraction was significantly attenuated in the presence of latrunculin A (maximal contraction: 10⁻⁴ M Lat-A; 84.0 ± 7.8%, 10⁻⁷ M Lat-A; 18.7 ±
9.2%, 10^{-6} M Lat-A; 2.2 ± 0.6%) or cytochalasin D (maximal contraction: 10^{-7} M Cyt-D; 61.8 ± 4.0%, 10^{-6} M Cyt-D; 22.0 ± 3.5%, 10^{-5} M Cyt-D; 0.5 ± 0.3%) in a concentration-dependent manner. Neither latrunculin A nor cytochalasin D had any effect on the resting tension (data not shown).

Figure 4 shows the effects of pretreatment with colchicine and nocodazole, microtubule disrupters, on the ACh-induced contraction in bronchial muscle strips. Neither colchicine (maximal contraction: 10^{-5} M; 102.3 ± 0.2%, 10^{-4} M; 102.4 ± 0.2%) nor nocodazole (maximal

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**Fig. 1.** Immunoblotting of α-actin, β-actin, α-tubulin, desmin, vimentin and GAPDH in the rat main bronchial tissue. The expression of each protein was detected by Western blots using total protein extracted from bronchial ring.

**Fig. 2.** Immunohistochemical localization of α-actin (C) and desmin (D) in the rat main bronchi ring section. Four-µm sections of paraformaldehyde-fixed tissue were stained with each antibody before examination by light microscopy. A and B represent control (no 1st antibody treatment). Original magnification: A: ×40; B, C, D and E: ×160.
contraction: $10^{-5}$ M; $92.0 \pm 5.2\%$, $10^{-4}$ M; $91.7 \pm 5.9\%$) had any effect on the ACh-induced bronchial smooth muscle contraction.

Pretreatment with $5 \times 10^{-3}$ M acrylamide, a concentration previously reported to disturb intermediate filament stability (Moy et al., 2004; Strege et al., 2003), significantly attenuated the ACh-induced contraction (maximal contraction: $5 \times 10^{-4}$ M; $92.5 \pm 3.1\%$, $5 \times 10^{-3}$ M; $60.0 \pm 4.5\%$, Fig. 5). The acrylamide did not also have any effect on the resting tension (data not shown).

Figures 6 and 7 show posttreatment with latrunculin A, cytochalasin D, colchicines, nocodazole and acrylamide on the submaximal bronchial smooth muscle contraction induced by $10^{-4}$ M ACh. Although nocodazole and their vehicle (DMSO) slightly increased the ACh-induced contraction, the ACh-induced contraction was attenuated by treatment with latrunculin A and cytochalasin D. Acrylamide ($5 \times 10^{-3}$ M) also decreased the ACh-induced contraction.

**Discussion**

The contractions to ACh were elicited in the presence and absence of cytochalasin D and latrunculin A (inhibitors of the actin cytoskeleton), colchicines and nocodazole (inhibitors of the microtubule cytoskeleton) and acrylamide (inhibitor of the intermediate filament). Cytochalasin D and latrunculin A significantly decreased the contractions induced by ACh. Acrylamide also significantly decreased the contraction induced by ACh. On the other hand, the contractile...
response to ACh was not affected in the presence of colchicines or nocodazole.

Smooth muscle cells possess a contractile apparatus of actin (thin) and myosin (thick) filaments which produce contraction (Horowitz et al., 1996; Somlyo and Somlyo, 2003) following...
phosphorylation of myosin light chain (MLC), and in association with a number of effector proteins. Smooth muscle internal scaffolding cytoskeleton consists of three classes of filamentous assemblies: actin microfilaments (70 Å diameter), intermediate filaments (70–110 Å diameter), and microtubules (300 Å diameter). In addition, there exists a well-developed membrane skeleton, which provides the interface among the extracellular matrix, plasma membrane, and the contractile structure within the cells (Carpenter, 2000; Small and Gimona, 1998). Because of the apparent lack of high structural order of smooth muscle cells (as compared with skeletal or cardiac muscle), the architecture of the contractile units, the nature of coupling of the contractile apparatus to the cytoskeleton, the exact role of the cytoskeleton in the mechanical properties of the cell and its acute regulation, and the organization of the

Fig. 6. Effects of post-treatment with latrunculin A (10^{-8}–10^{-6} M, A), cytochalasin D (10^{-7}–10^{-5} M, C) and nocodazole (10^{-4}–10^{-1} M) on the acetylcholine (ACh)-induced contraction in rat bronchial ring. The traces of ACh-induced contraction are the representatives of three independent experiments, respectively. A: DMSO was treated as vehicle-control.
contractile apparatus per se, are poorly understood. In addition, the changes in the cytoskeleton and associated proteins during the course of agonists-induced force development in bronchial smooth muscle have not been described.

In smooth muscle, the ‘thin’ filament α-actin interacts with the ‘thick’ filament myosin to produce a contraction. In the present studies, we show for the first time that ACh-induced contraction was significantly inhibited when actin polymerization was blocked in rat bronchial smooth muscle. Previous studies on smooth muscle, however, have indicated that an additional determination of the mechanical response may be remodeling actin filaments during contractile activation (Mehta and Gunst, 1999). The smooth muscle actin network is compromised of contractile and cytoskeletal elements. Actin filaments anchor to the plasma membrane at periodically occurring electron-dense plaques enriched in integrin-associated proteins (Small and Gimona, 1998; Somlyo and Somlyo, 2000). The 25–30% of smooth muscle actins in resting condition are in the monomeric form (Tseng et al., 1997). These also appear to be a dynamic equilibrium between filamentous F-actin and monomeric G-actin such that receptor stimulation results in an increase in the F/G-actin ratio (Togashi et al., 1998). The presently used agents are known to inhibit actin polymerization by different mechanisms. Cytochalasins bind to free barbed ends of F-actin, and thus prevent filament extension. Latrunculins sequester G-actin,

![Diagram](image.png)

**Fig. 7.** Effects of post-treatment with colchicine (10⁻⁶–10⁻⁴ M, B) and acrylamide (5 × 10⁻⁵–5 × 10⁻³ M, C) on the acetylcholine (ACh)-induced contraction in rat bronchial ring. The traces of ACh-induced contraction are the representatives of three independent experiments, respectively. A: Krebs-Henseleit (K-H) was treated as vehicle-control.
and therefore prevent monomer addition to filament ends (Mauss et al., 1989; Saito et al., 1996; Battistella-Patterson et al., 1997). The agents have been reported to also attenuate the receptor-coupled smooth muscle contractility in guinea pig ileum, rat aorta, chicken gizzard, and canine tracheal smooth muscles (Mauss et al., 1989; Mehta and Gunst, 1999; Saito et al., 1996). There is evidence to suggest that force inhibition is accompanied by and thereby dependent upon a lowering of \([\text{Ca}^{2+}]\) in canine tracheal smooth muscle (Mehta and Gunst, 1999). In many other cell types, disruption of the actin cytoskeleton does alter patterns of \([\text{Ca}^{2+}]\) homeostasis (Shaw et al., 2003; Tseng et al., 1997; Wang et al., 2002). In addition, noradrenalin-induced carotid smooth muscle contraction was abolished by latrunculin A treatment, although MLC phosphorylation was not modulated by the latrunculin A (Tang and Tan, 2003). Mehta and Gunst (Mehta and Gunst, 1999) showed that not only agonist- but also \(\text{Ca}^{2+}\) itself-induced contractions were inhibited by latrunculin A in \(\alpha\)-toxin permeabilized smooth muscle. Therefore disruption of the actin cytoskeleton may alter the \(\text{Ca}^{2+}\)-induced contraction rather than the patterns of \([\text{Ca}^{2+}]\) homeostasis. In the present studies, we have shown for the first time that following latrunculin A and cytochalasin D-induced inhibition of actin polymerization, the ACh-induced contraction in rat bronchial smooth muscle decreased significantly. This suggests that actin polymerization plays an important role in regulating the agonist-induced contraction in bronchial smooth muscle. Moreover, the post-treatment with latrunculin A and cytochalasin D attenuated the ACh-induced contraction, suggesting that actin polymerization was caused in not only phasic phase but also tonic phase of the ACh-induced contraction.

Microtubules have hollow and cylindrical structures, and are formed by two similar, but alternating, 55 kDa subunits, \(\alpha\)- and \(\beta\)-tubulin, arranged in a helical array. Importantly, microtubules form a network that participates in the movement of vesicles and organelles within the cell (Yildiz et al., 2004). In the present study, the inhibition of microtubule polymerization by colchicine and nocodazone had no significant effect on the ACh-induced contraction. This finding suggests that microtubules appear not to play a major role in regulating the agonist-induced contraction in rat bronchial smooth muscle.

The intermediate filaments are a large family of cytoskeletal structures composed of several classes of proteins (Lazarides, 1982). Five types of structurally related intermediate filament proteins have been identified and their expression is cell-type specific (Herrmann and Aebi, 2000). In smooth muscle, the intermediate filaments are mainly composed of two major proteins, desmin and vimentin (Berner et al., 1981; Osborn et al., 1981). Vimentin and desmin are major intermediate filament proteins in airway and vascular smooth muscle (Halayko et al., 1996; Johansson et al., 1997; Tang et al., 2005). In the present study, the inhibition of intermediate filament by acrylamide significantly attenuated the ACh-induced contraction. The intermediate network has been proposed to interact with the actin filament in certain cell types including macrophages, epithelial cells, and fibroblasts (Correia et al., 1999; Green et al., 1987). Treatment of epithelial cells with cytochalasin D disrupts the organization of the keratin, one of the intermediate filaments, network.

In conclusion, ACh-induced contraction is highly dependent, from a functional point of view, on actin and intermediate filament polymerization and not, to any important degree, on the polymerization of microtubule in rat bronchial smooth muscle.
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