Augmented PDBu-mediated contraction of bronchial smooth muscle of mice with antigen-induced airway hyperresponsiveness

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Abstract

To explore the role of protein kinase C (PKC) in the augmented bronchial smooth muscle (BSM) contraction observed in the antigen-induced airway hyperresponsive (AHR) mice, the effects of a PKC activator, phorbol 12,13-dibutylate (PDBu), on BSM contraction were compared between the AHR and control mice. Actively sensitized mice were repeatedly challenged by antigen inhalation. Twenty-four hours after the final antigen challenge the isometrical contractions of the BSMs were measured. The BSM contraction induced by acetylcholine, but not high K+ depolarization, was significantly augmented in the AHR mice. In BSMs of control mice, PDBu caused a significant increase in tension when the tissues were precontracted with high K+, although PDBu itself had no effect on basal tone. The PDBu-mediated contraction was markedly augmented in BSMs of the AHR mice. These findings suggest that an increase in the PKC-mediated signaling is involved in the augmented contraction of BSMs in the antigen-induced AHR mice.

Key words: airway hyperresponsiveness, protein kinase C, bronchial smooth muscle, phorbol 12,13-dibutylate

Introduction

The airway hyperresponsiveness (AHR) associated with heightened airway resistance and inflammation is the asthmatic characteristic feature (Bousquet, 2001). The importance of AHR in the cause of bronchial asthma was suggested by the correlation with the severity of the illness (Lotvall et al., 1998). Therefore, understanding the fundamental mechanism of AHR is important to determine asthmatic medical treatment.

The primary determinant of smooth muscle contraction is phosphorylation of 20-kDa myosin light chain (MLC) (Hartshorne, 1987), which is regulated not only by the Ca2+/calmodulin (CAM)-dependent MLC kinase (MLCK)-mediated pathway but also by a Ca2+ independent mechanism (Ca2+ sensitization) (Somlyo and Somlyo, 1994). Multiple second messengers/signaling pathways,
including the protein kinase C (PKC) pathway which are linked to the Ca\textsuperscript{2+} sensitization mechanisms (Jensen et al., 1996; Walsh et al., 1994), have been reported. The PKC has been proposed to mediate the inhibition of MLC phosphatase (MLCP) in response to various agonists (Somlyo and Somlyo, 2000).

Our previous studies revealed both \textit{in vivo} and \textit{in vitro} hyperresponsiveness to ACh and other spasmogens in rats that were actively sensitized and repeatedly challenged with aerosolized antigen (Chiba and Misawa, 1993; 1995; Misawa and Chiba, 1993). In this animal model of AHR, the muscarinic receptor density of bronchial tissues was within normal level (Chiba and Misawa, 1995). Furthermore, no significant difference in the ACh-induced increase in cytosolic Ca\textsuperscript{2+} concentration of the main BSM was observed between the control and AHR animals (Chiba et al., 1999a). These findings strongly suggest that the mechanisms responsible for the augmented ACh-induced contraction of the main BSM might exist in post-receptor signaling including augmented Ca\textsuperscript{2+} sensitization. Indeed, Ca\textsuperscript{2+} sensitization in bronchial preparation of repeatedly antigen challenged animals was significantly enhanced as compared with that of control animals (Chiba et al., 1999b). Moreover, PKC plays an important role in agonists-induced Ca\textsuperscript{2+} sensitization and contraction in BSM (Sakai et al., 2005; 2007). To explore the role of protein kinase C (PKC) in the augmented bronchial smooth muscle (BSM) contraction observed in the antigen-induced airway hyperresponsive (AHR) mice, the effects of a PKC activator, phorbol 12,13-dibutyrate (PDBu), on BSM contraction were compared between the AHR and control mice.

**Materials and Methods**

**Animals**

Male BALB/c mice (8 weeks of age, specific pathogen-free) were used. All experiments were done according to the Guiding Principles for the Care and Use of Laboratory Animals (Hoshi University, Tokyo Japan) and also with the approval of the ethics committee in Hoshi University.

**Sensitization and antigenic challenge**

Preparation of a murine model of allergic bronchial asthma was performed as described previously (Chiba et al., 2009). In brief, mice were actively sensitized by intraperitoneal injections of 8 \( \mu \)g ovalbumin (OA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockfold, IL, USA) on Days 0 and 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/mL) for 30 min on Days 12, 16 and 20. A control group of mice received the same immunization procedure but inhaled saline aerosol instead of OA challenge.

**Functional studies**

Twenty-four hours after the last OA challenge, mice were killed by exsanguination from the abdominal aorta under urethane anesthesia (1.6 g/kg, \textit{i.p.}), and the airway tissues under the larynx to lungs were immediately removed. About a 3-mm length of the left main bronchus (0.5 mm in diameter) was isolated, and the epithelium was removed by gently rubbing with sharp tweezers (Chiba et al., 2009). The resultant tissue ring preparation was then suspended in a 5-mL organ
bath by two stainless steel wires (0.2 mm in diameter) passed through the lumen. For all tissues, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T; Nihon Kohden, Japan) for the measurement of isometric force. A resting tension of 0.5 g was applied. The buffer solution contained modified Krebs-Henseleit solution with the following composition (in mM): 118.0 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25.0 NaHCO₃, 1.2 KH₂PO₄, and 10.0 glucose. The buffer solution was maintained at 37°C and oxygenated with 95% O₂–5% CO₂. After the equilibration period, the concentration-response curve to ACh (10⁻⁷–10⁻³ M in final concentration) was constructed cumulatively 20 min after 1 mM PDBu or its vehicle treatment. The BSM responsiveness to high K⁺ was also measured by applying isotonic 60 mM K⁺ solution in the presence of atropine and indomethacin (both 10⁻⁶ M). After the contraction was reached plateau, 1 μM phorbol 12,13-dibutyrate (PDBu), a direct PKC activator, was applied.

**Statistical analyses**

All the data were expressed as the mean with S.E. Statistical significance of difference was determined by two-way and one-way ANOVA followed by Bonferroni/Dunn’s test.

**Results**

Figure 1 shows the ACh responsiveness of the BSMs isolated from normal, sensitized-control and repeatedly antigen-challenged mice. ACh elicited a concentration-dependent contractile response in all groups. The contractile responsiveness to ACh of BSM from antigen-challenged mice was significantly augmented as compared with those from sensitized-control and non-
sensitized normal mice [Emax (maximal contraction): normal; 0.36 ± 0.05 g, sensitized-control; 0.39 ± 0.03 g, repeated antigen-challenged mice; 0.58 ± 0.05 g] although –logEC₅₀ was not changed in among the groups (–logEC₅₀: normal; 5.5 ± 0.4, sensitized-control; 4.9 ± 0.2, repeated antigen-challenged mice; 5.4 ± 0.3). However, no significant difference in the high K⁺-induced BSM contraction was observed between sensitized-control and repeatedly antigen-challenged mice (Fig. 2). PDBu itself had no effect on basal tone in any groups (data not shown). However, in the BSMs pre-contracted with high K⁺, PDBu caused a significant increase in tension in both the groups. The contractile responsiveness to PDBu of high K⁺-treated BSM from antigen-challenged mice was significantly augmented as compared with that from sensitized-control mice (Emax: sensitized-control; 306.1 ± 20.72%, repeated antigen-challenged mice; 447.1 ± 41.53%) although –logEC₅₀ was not changed in among the groups (–logEC₅₀: sensitized-control; 7.5 ± 0.2, repeated antigen-challenged mice; 7.3 ± 0.2) (Fig. 3).

Discussion

Our previous study demonstrated an in vivo AHR to inhaled ACh in animals that were sensitized and repeatedly challenged with antigen (Misawa and Chiba, 1993). Even isolated smooth muscle of the bronchus from the AHR animal had a hyperresponsiveness (Chiba and Misawa, 1995; Misawa and Chiba, 1993). The augmented contractile responsiveness of bronchi to ACh obtained from the AHR mice in the present study is consistent with our previous results (Chiba and Misawa, 1995; Misawa and Chiba, 1993; Chiba et al., 1999a; 1999b; Sakai et al., 2005; 2007). The contraction of permeabilized muscle induced by ACh was also enhanced in BSM of the airway hyperresponsive animals, although no significant difference in the increase in [Ca²⁺], was observed (Chiba et al., 1999b). Thus, the increased smooth muscle contractility is proposed to be related to the augmented agonist-induced Ca²⁺ sensitization of myofilaments in AHR model animals. At least two pathways are responsible for the increased Ca²⁺ sensitization: the PKC and

Fig. 2. Contraction induced by 60 mM K⁺ of isolated bronchial rings from sensitized-control (Sensitized) and repeatedly antigen challenged (Challenged) mice. Each column represents the mean with S.E. from 5 independent experiments.
Our previous studies demonstrated that enhancement of the ACh-induced Ca\(^{2+}\) sensitization in hyperresponsive muscle was effectively inhibited by C3 exoenzyme, a RhoA inhibitor, in \(\beta\)-escin-permeabilized BSM. The augmented contraction of the intact (non-permeabilized) BSM to ACh was also inhibited by Y-27632, a ROCK inhibitor (Chiba et al., 2001). The increased smooth muscle contractility was therefore suggested to be related to the augmented agonist-induced, RhoA-mediated Ca\(^{2+}\) sensitization of myofilaments. It is possible that the increased expression of RhoA in the BSM causes an enhancement of RhoA-mediated Ca\(^{2+}\) sensitization, resulting in the augmented contraction at the AHR state. On the other hand, CPI-17, a phosphorylation-dependent inhibitory protein of myosin phosphatase, has been suggested to be the downstream effector of PKC (Kitazawa et al., 1999; Woodsome et al., 2001), because PKC phosphorylates and activates CPI-17. We previously reported that Ca\(^{2+}\) sensitization is mediated not only by RhoA/ROCK but
also by crosstalk of PKC/CPI-17 pathways, and plays important roles in the agonists-induced BSM contraction and phosphorylation of MLC (Sakai et al., 2005; 2007). In the state of antigen-induced AHR, these Ca\(^{2+}\) sensitization pathways may be upregulated much more intensely than in the non-AHR state.

PDBu has been demonstrated to be a potent specific PKC activator, without any alteration in intracellular Ca\(^{2+}\) concentration (Ozaki et al., 2003; Sato et al., 1994), that has been widely used to activate PKC and to study the PKC signaling pathway. PKC isoforms are classified into three groups. The conventional PKCs (cPKC) \(\alpha, \beta I, \beta II, \gamma\) have four conserved regions (C1–C4) and five variable regions. The C1 region contains the binding site for diacylglycerol (DAG) or phorbol esters such as PDBu. The C2 region contains the binding site for Ca\(^{2+}\). The C3 and C4 regions contain the binding site for ATP, some PKC substrates and PKC antagonists. The PKC molecule folds to bring the ATP binding site into proximity with the substrate-binding site. The novel PKCs (nPKC) \(\delta, \epsilon, \eta, \theta\) lack the C2 region and therefore do not require Ca\(^{2+}\) for activation. The atypical PKCs (aPKC) \(\xi, \lambda, \iota\) have only one cysteine-rich zinc finger-like motif and are dependent on phosphatidylserine, but are not affected by DAG, phorbol esters or Ca\(^{2+}\) (Salamanca and Khalil, 2005). The involvement of conventional PKC\(\alpha\) has been reported in activation of arterial smooth muscle by using recombinant CPI-17 protein (Kitazawa et al., 1999).

It is generally known that PDBu itself causes the development of force in vascular smooth muscle. The activation of PKC by addition of PDBu induces a contraction and inhibition of MLC phosphatase (MLCP) in vascular smooth muscles (Ito et al., 1993; Masuo et al., 1994). On the other hands, the contraction was not elicited by PDBu itself in mouse BSM, but PDBu caused a force development in the BSM pre-contracted by high K\(^{+}\) stimulation in both groups. These findings also support that cPKC activated by PDBu may activate CPI-17 under cytosolic Ca\(^{2+}\) increase in a state of BSM contraction of mice.

Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), one of the inflammatory cytokines, has been shown to be involved in the activation of inflammatory processes, which lead to asthma (Gosset et al., 1999). TNF-\(\alpha\) is released in allergic responses from both mast cells and macrophages via IgE-dependent mechanisms, and elevated levels have been demonstrated in the bronchoalveolar fluid (BALF) of asthmatic subjects undergoing allergen challenge (Thomas, 2001). Morin et al. (2008) demonstrated that TNF-\(\alpha\) increased the CPI-17 expression level in human bronchial tissues. Previous studies revealed that CPI-17 expression of bronchial smooth muscle (Sakai et al., 2005) were increased by repeatedly antigen-challenge in rat. Taken together, TNF-\(\alpha\) might play an important role in PKC/CPI-17 signaling in BSM of AHR mice.

In conclusion we suggested that an increase in the PKC-mediated signaling is involved in the augmented contraction of BSMs in the antigen-induced AHR mice.

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