

# Possible Involvement of CPI-17 in Augmented Bronchial Smooth Muscle Contraction in Antigen-Induced Airway Hyper-Responsive Rats

Hiroyasu Sakai, Yoshihiko Chiba, Tomona Hirano, and Miwa Misawa

*Department of Pharmacology, School of Pharmacy, Hoshi University, Tokyo, Japan*

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## ABSTRACT

Airway hyper-responsiveness (AHR) associated with heightened airway resistance and inflammation is a characteristic feature of asthma. It has been demonstrated that contractile responsiveness and  $\text{Ca}^{2+}$  sensitization to acetylcholine (ACh) in repeated antigen challenge-induced airway hyper-responsive bronchial preparation were significantly increased. The CPI-17 (PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa) is activated by protein kinase C and acts on a myosin light-chain phosphatase-specific target. The aim of the present study was to explore the role of CPI-17 in hyper-responsiveness of bronchial smooth muscle

in antigen-induced AHR rats. In immunoblotting, the levels of expression of CPI-17 mRNA and protein were significantly increased in bronchus from rats that were repeatedly challenged with antigen. ACh-induced CPI-17 phosphorylation and translocation to membrane fraction were also significantly increased in bronchus from antigen-challenged rats. In conclusion, we suggest that augmented expression and activation of CPI-17 observed in the hyper-responsive bronchial smooth muscle might be responsible for the enhanced ACh-induced  $\text{Ca}^{2+}$  sensitization of bronchial smooth muscle contraction associated with AHR.

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  $\text{Ca}^{2+}$ /calmodulin-dependent MLC kinase-mediated pathway but also by a  $\text{Ca}^{2+}$ -independent mechanism ( $\text{Ca}^{2+}$  sensitization) (Somlyo and Somlyo, 1994). Multiple second messengers/signaling pathways, including the RhoA/ROCK (Rho-associated coiled-coil-forming protein kinase) (Kimura et al., 1996; Kureishi et al., 1997; Uehata et al., 1997) and protein kinase C (PKC) (Walsh et al., 1994; Jensen et al., 1996) pathways, have reportedly been linked to the  $\text{Ca}^{2+}$  sensitization mechanisms. ROCK and PKC have been proposed to mediate the inhibition of myosin light-chain phosphatase (MLCP) in response to various agonists (Somlyo and Somlyo, 2000).

CPI-17 (named PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa), which is activated by PKC and acts on an MLCP-specific target, was isolated from pig aorta smooth muscle extracts (Eto et al., 1995). Expression of

CPI-17 is highly restricted to smooth muscle tissues (Woodsome et al., 2001). Phosphorylation of Thr<sup>38</sup> in CPI-17 converts it to a potent MLCP inhibitor with an  $\text{IC}_{50}$  of ~5 nM (Eto et al., 1995, 1997). Phospho-CPI-17 enhances myosin phosphorylation and contraction of permeabilized arterial smooth muscle (Li et al., 1998). Permeabilization of femoral artery strips using Triton X-100 depletes endogenous CPI-17 with loss of the contractile response to phorbol ester. The PKC-induced contraction of permeabilized artery was reconstituted by addition of recombinant CPI-17 (Kitazawa et al., 1999). Furthermore, the expression pattern of CPI-17 among six different smooth muscle tissues correlates with their extent of PKC-induced contraction, implying that CPI-17 is key to the PKC-mediated  $\text{Ca}^{2+}$  sensitization (Woodsome et al., 2001). Assays with purified kinases showed that Thr<sup>38</sup> of CPI-17 can be phosphorylated by multiple kinases such as PKC, ROCK, protein kinase N, and Zip-like kinase (Eto et al., 1995; Hamaguchi et al., 2000; Koyama et al., 2000; MacDonald et al., 2001).

Airway hyper-responsiveness (AHR) associated with heightened airway resistance and inflammation is the char-

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**ABBREVIATIONS:** MLC, myosin light chain; PKC, protein kinase C; ROCK, Rho-associated coiled-coil-forming protein kinase; MLCP, myosin light chain phosphatase; CPI-17, PKC-potentiated inhibitory protein for heterotrimeric MLCP phosphatase of 17 kDa; AHR, airway hyper-responsiveness; PCR, polymerase chain reaction; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Y-27632, N-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride; TTBS, Tris buffer containing Tween 20; PVDF, polyvinylidene difluoride; ACh, acetylcholine.

acteristic feature of asthma (Bousquet, 2000). 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 of the fundamental mechanism of AHR is important to determine medical treatment for asthma.

Our previous studies found both in vivo and in vitro hyper-responsiveness to ACh and other spasmogens in rats that were actively sensitized and repeatedly challenged with aerosolized antigen (Chiba and Misawa, 1993; Misawa and Chiba, 1993, 1995). In this animal model of AHR, the muscarinic receptor density of bronchial tissues was within the normal range (Chiba and Misawa, 1995). Furthermore, no significant difference in the ACh-induced increase in cytosolic  $\text{Ca}^{2+}$  concentration of the main bronchial smooth muscle was observed between the control and AHR rats (Chiba et al., 1999a). These findings strongly suggest that the mechanisms responsible for the augmented ACh-induced contraction of the main bronchial smooth muscle might exist in after receptor signaling, including augmented  $\text{Ca}^{2+}$  sensitization. Indeed,  $\text{Ca}^{2+}$  sensitization in bronchial preparation of rats repeatedly challenged with antigen was significantly enhanced compared with that of control rats (Chiba et al., 1999b). However, the mechanism of augmented  $\text{Ca}^{2+}$  sensitization to contractile agonists in bronchial smooth muscle from AHR rats remains to be solved in detail. The aim of the present study was to explore the role of CPI-17 in hyper-responsiveness of bronchial smooth muscle in antigen-induced AHR rats.

## Materials and Methods

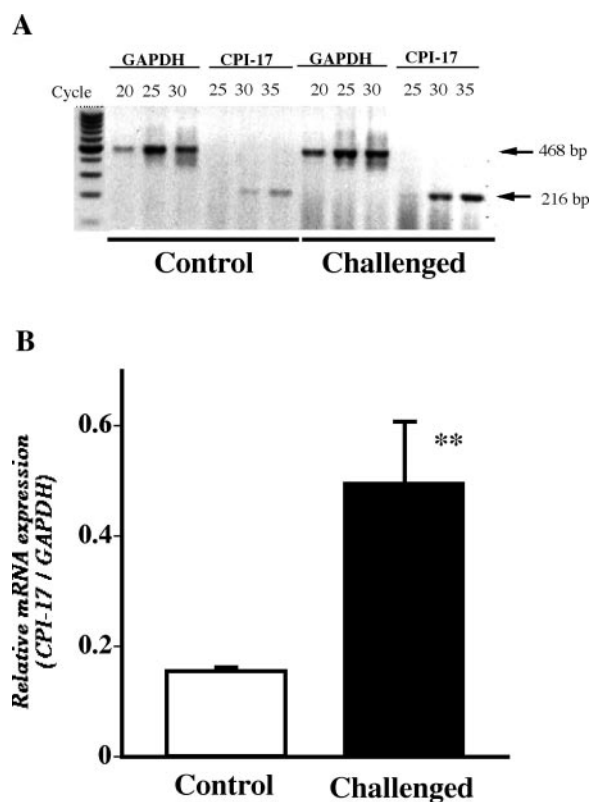
**Animals.** Male Wistar rats (6 weeks of age, specific pathogen-free, 170 to 190 g; Charles River Japan, Inc., Yokohama, Japan) were housed for appropriate time intervals in the animal center of Hoshi University after their arrival. Constant temperature and humidity ( $22 \pm 1^\circ\text{C}$ ,  $55 \pm 10\%$  humidity) were maintained with a fixed 12-h light/dark cycle and free access to food and water. The experiments were performed under the guiding principles for the care and use of laboratory animals approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

**Sensitization and Antigenic Challenge.** Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen by a method described previously (Chiba et al., 1999a,b). Our previous and current studies (Fig. 1) revealed that the sensitization procedure alone had no effect on the ACh responsiveness of the bronchial muscle and muscarinic receptors property in rats (Chiba and Misawa, 1995). Therefore, in the present study, age-matched nonsensitized normal rats were used as control.

**RT-PCR analyses.** The main and intrapulmonary bronchial tissues were quickly frozen with liquid nitrogen, and the tissues were crushed to pieces by Cryopress (15 s  $\times$  3; CP-100W; NITI-ON Medical Supply Co. Ltd., Chiba, Japan). Total RNA was isolated from each frozen tissue powder by the acid guanidinium thiocyanate/phenol/chloroform extraction method (Mullis et al., 1989) and stored at  $-85^\circ\text{C}$  until use. cDNAs were prepared from the total RNA (0.5  $\mu\text{g}$ ) by using a Takara RNA PCR kit (ver. 2.1; Takara, Tokyo, Japan) in a total volume of 20  $\mu\text{l}$  of reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM  $\text{MgCl}_2$ , 1 mM dNTP mixture, 1 U/ml RNase inhibitor, 2.5  $\mu\text{M}$  concentration of random 9-mers, and 0.25 U/ml avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated for 10 min at  $30^\circ\text{C}$  followed by 60 min at  $42^\circ\text{C}$  to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at  $99^\circ\text{C}$  for 5 min. Then, the RT reaction mixture (10  $\mu\text{l}$ ) was added to 0.5  $\mu\text{l}$  of 0.1 mM forward primer, 0.5  $\mu\text{l}$  of 0.1 mM reverse

primer, 4  $\mu\text{l}$  of  $10\times$  amplification buffer (100 mM Tris-HCl, pH 8.3, and 0.5 M KCl), 3  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$ , 31.8  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , and 0.25  $\mu\text{l}$  of 5 U/ml *Taq* polymerase. The PCR primers for rat CPI-17 used were 5'-GCGAGTCACCGTCAAATACGAC-3' (sense) and 5'-TCCTCTGTGGGATTCAGGCAAGC-3' (antisense). The PCR primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used were 5'-CCATCACTGCCACTCAGAAGAC-3' (sense) and 5'-TACTCCTTGAGGCCATGTAGG-3' (antisense), which were designed from published sequences (NM\_110403 and XM\_342145). The thermal cycle profile used in the present study was 1) denaturing for 30 s at  $95^\circ\text{C}$ , 2) annealing primers for 30 s at  $60^\circ\text{C}$ , and 3) extending the primers for 60 s at  $72^\circ\text{C}$ . The PCR amplifications were performed for 25, 30, and 35 cycles for CPI-17 and 20, 25, and 30 cycles for GAPDH. A portion (10  $\mu\text{l}$ ) of the PCR mixture was subjected to electrophoresis on 2% agarose gel and visualized by densitometer (Atto Densitograph; Atto Co., Tokyo, Japan). The ratios of the corresponding CPI-17 (30 cycles)/GAPDH (20 cycles) were calculated as indices of CPI-17 mRNA levels.

**Protein Extraction.** Membrane and cytosolic fractions of bronchial tissue were prepared by a method described previously (Chiba et al., 1999b) with minor modifications. In brief, the airway tissues below the main bronchi were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. They were carefully cleaned of adhering connective tissues, blood vessels, and lung parenchyma under stereomicroscopy. Then, the bronchial tissue was equilibrated in oxygenated Krebs-Henseleit solution ( $37^\circ\text{C}$ ) for 60 min with 10-min washout intervals. After the equilibration period, the tissue segments were stimulated by an indicated concentration of

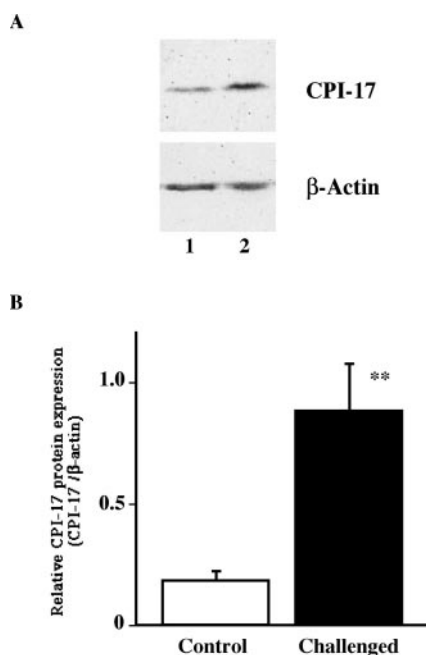


**Fig. 1.** The levels of expression of CPI-17 mRNA levels in bronchial smooth muscles from the control rats (Control) and rats repeatedly challenged with antigen (Challenged). A, typical photographs of RT-PCR product bands for CPI-17 and GAPDH. The PCR amplifications were performed for 25 to 35 cycles (CPI-17) and 20 to 30 cycles (GAPDH). The bands were scanned, and the level of CPI-17 mRNA was expressed as density ratio of the CPI-17 to the GAPDH bands. The data are summarized in B. Values are the means with S.E. from four to five experiments. The levels of CPI-17 mRNA were augmented in the repeated antigen challenge group. \*\*,  $p < 0.05$  versus Control.

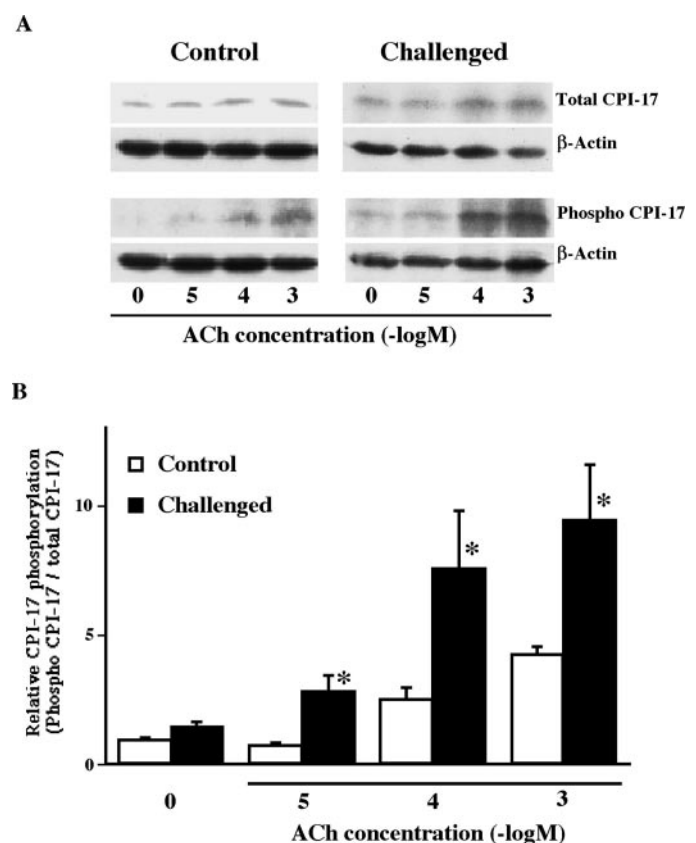
ACh ( $10^{-5}$ – $10^{-3}$  M) for 20 min. In some experiments, the bronchial preparations were pretreated with Y-27632 (ROCK inhibitor;  $10^{-6}$  M) or calphostin C (PKC inhibitor;  $10^{-6}$  M) to determine the role(s) of ROCK and/or PKC on the ACh-induced phosphorylation and translocation of CPI-17 and MLC phosphorylation. The concentration of Y-27632 ( $10^{-6}$  M) and calphostin C ( $10^{-6}$  M) used had no effect on  $\text{Ca}^{2+}$ -induced contraction of bronchial smooth muscle (Chiba et al., 2001; Sakai et al., 2005). Thus,  $10^{-6}$  M concentrations of Y-27632 and calphostin C were used in the present study. The reaction was stopped by quickly freezing with liquid nitrogen, and the tissue was then homogenized in 1 ml of ice-cold homogenization buffer with the following composition: 10 mM Tris-HCl, pH 7.5, 5 mM  $\text{MgCl}_2$ , 2 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20  $\mu\text{g}/\text{ml}$  leupeptin, and 20  $\mu\text{g}/\text{ml}$  aprotinin. The homogenate was used to quantify the expression of CPI-17. The tissue homogenate was centrifuged (105,000g, 4°C for 30 min), and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 3 ml of homogenization buffer and recentrifuged (105,000g, 4°C for 30 min). The resultant pellet was resuspended in 2 ml of ice-cold homogenization buffer and used as the membrane fraction. These preparations were stored at  $-80^\circ\text{C}$  until use.

**Western Blot Analyses.** To quantify the expression, translocation and phosphorylation of CPI-17 proteins, immunoblotting was performed as described previously (Chiba et al., 1999b). In brief, the samples (10  $\mu\text{g}$  of protein per lane) were subjected to 15% SDS-PAGE. Proteins were then electrophoretically transferred for 4 h onto PVDF membranes (Hybond-ECL; Amersham Biosciences, Little Chalfont, UK) in ice-cold transfer buffer (20% methanol containing 25 mM Tris and 192 mM glycine). After repeated washing with Tris buffer (20 mM Tris and 500 mM NaCl, pH 7.5) containing 0.1% (v/v) Tween 20 (TTBS), the PVDF membranes were incubated with blocking buffer (3% gelatin in TTBS) for 1.5 h at room temperature. The PVDF membranes were then incubated with primary antibody, polyclonal goat anti-CPI-17 (1:1500 dilution; Santa Cruz Biotechnology)

or polyclonal goat anti-Thr<sup>38</sup> phospho-CPI-17 (1:5000 dilution; Santa Cruz Biotechnology) in antibody buffer (1% gelatin in TTBS) for 12 h at room temperature. The PVDF membranes were then washed five times (each for 15 min) with TTBS. They were incubated with horseradish peroxidase-conjugated anti-goat IgG (Amersham) for 1.5 h at room temperature, and then washed five times with TTBS. The blots were detected with an enhanced chemiluminescent method (ECL System; Amersham) and quantified by densitometry (Atto Densitograph Software ver. 4.0). To normalize the CPI-17 contents to an internal control protein,  $\beta$ -actin, immunoblotting was also performed on the same gel by using monoclonal mouse anti- $\beta$ -actin N-terminal (Sigma-Aldrich, St. Louis, MO) and goat anti-mouse IgG (Amersham Biosciences). The ratios of corresponding phosphorylated CPI-17/ $\beta$ -actin and CPI-17/ $\beta$ -actin in each lane were calculated as indices of phosphorylated and total CPI-17 protein levels. The membrane/total CPI-17 in each animal sample was calculated according to the formula (membrane CPI-17/ $\beta$ -actin)/(membrane CPI-17/ $\beta$ -actin + (cytosolic CPI-17/ $\beta$ -actin)). In the ACh-induced MLC phosphorylation study, The bronchial preparation were stimulated by ACh  $10^{-3}$  M for 10 min. Then, the samples were homogenized with T-PER tissue protein extraction reagent (Pierce, Rockford, IL). After the samples (20  $\mu\text{g}$ ) were subjected to 15% SDS-polyacrylamide gel electrophoresis, Western blot was performed. The membranes were incubated with the primary antibodies. The primary antibodies used were goat anti-p-MLC (Thr18/Ser19; 1:250 dilution; Santa Cruz Biotechnology, Inc.) or rabbit anti-myosin light chain (1:1000; Santa Cruz Biotechnology, Inc.). Then, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (1:5000 dilution; Santa



**Fig. 2.** The levels of CPI-17 protein in the bronchial smooth muscles from the control (1 or Normal) and rats repeatedly challenged with antigen (2 or Challenged). A, typical photographs of bands for CPI-17 and  $\beta$ -actin. The expression levels of CPI-17 were calculated as ratios of the intensities of CPI-17 to  $\beta$ -actin proteins and summarized in B. Values are the means with S.E. from five to six experiments. The levels of CPI-17 protein were augmented in the repeated antigen challenge group. \*\*,  $p < 0.01$  versus control.



**Fig. 3.** ACh-induced phosphorylation of CPI-17 in rat bronchial smooth muscle from control rats and rats repeatedly challenged with antigen (Challenged). A, typical immunoblots for phosphorylated CPI-17 (p[Thr<sup>38</sup> CPI-17]) and total CPI-17. B, the phosphorylation levels of CPI-17 were calculated as the ratios of the intensities of phosphorylated CPI-17 (p[Thr<sup>38</sup> CPI-17]) to total CPI-17 protein. Values are the means with S.E. from five experiments. ACh-induced phosphorylation was augmented in the repeated antigen challenge group. \*,  $p < 0.05$  versus Control.



Cruz Biotechnology, Inc.) and goat anti-rabbit IgG (1:5000 dilution; Amersham Biosciences), detected by an ECL system. The ratio of corresponding p-MLC/MLC was calculated as an index of p-MLC.

**Statistical Analyses.** All data were expressed as the mean with S.E. Statistical significance of difference was determined by Bonferroni/Dunn's test.

## Results

**RT-PCR Analyses.** Figure 1 shows the expression of CPI-17 mRNAs in rat bronchial smooth muscle, as determined by RT-PCR using total RNA. The PCR amplifications were performed for 25 to 35 cycles for CPI-17 and for 20 to 30 cycles for GAPDH (Fig. 1A). The expected sizes of the bands for CPI-17 (216 bp) and GAPDH (468 bp) were clearly detected in rat bronchial smooth muscle. Thirty cycles for CPI-17 and 25 cycles for GAPDH generated submaximal but distinct bands. The band intensity for GAPDH was equal in each group. To estimate the expression level of CPI-17 mRNA, the ratios of the band intensity of CPI-17 mRNA to that of GAPDH were calculated. As shown in Fig. 1B, the level of expression of CPI-17 mRNA was significantly increased in bronchus from rats repeatedly challenged with antigen.

**Western Blot Analyses.** To determine the expression of CPI-17 protein in bronchial smooth muscle of the rats, immunoblottings were performed in the homogenates of bronchi. As shown in Fig. 2A, immunoblotting with CPI-17 antibody gave a single band with a molecular mass of 17 kDa in the bronchial smooth muscle of each group. In antigen-challenged rats, the expression of CPI-17 protein was significantly augmented compared with the control group (Fig. 2B).

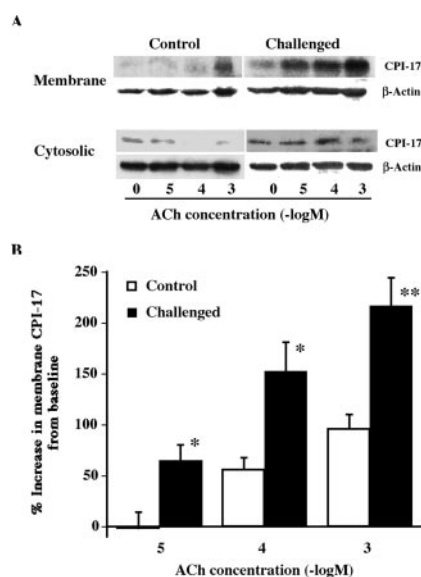
To determine the ACh ( $10^{-5}$ – $10^{-3}$  M)-induced phosphorylation of CPI-17 in bronchial smooth muscle of the rat, im-

munoblottings were performed by using phospho-[Thr<sup>38</sup>]-specific antibody. The ACh-induced phosphorylation of CPI-17 was increased in a concentration-dependent manner in both groups (Fig. 3). It is noteworthy that the ACh-induced CPI-17 phosphorylation at Thr<sup>38</sup> was significantly augmented in bronchus from rats repeatedly challenged with antigen.

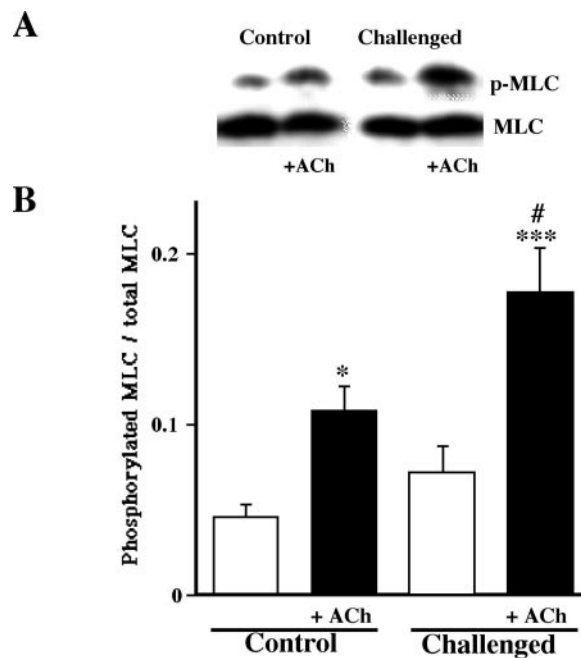
As shown in Fig. 4A, CPI-17 protein was expressed in both the membrane and cytosolic fractions of bronchial smooth muscles at resting state (no ACh stimulation). No significant difference in the ratio of membrane to total CPI-17 at resting state was observed between the control ( $0.216 \pm 0.048$ ) and repeated antigen challenge ( $0.172 \pm 0.029$ ) groups. The CPI-17 contents in the membrane fractions were significantly increased by ACh ( $10^{-5}$ – $10^{-3}$  M) stimulation in a concentration-dependent manner, although the ratio of cytosolic to total CPI-17 was significantly decreased in each group (data not shown) (i.e., ACh-induced translocation of CPI-17 to plasma membrane). As shown in Fig. 4B, the ACh-induced translocation of CPI-17 was significantly augmented in repeated antigen challenge group compared with the control group.

MLC phosphorylation was represented a distinct single band. Treatment of ACh ( $10^{-3}$  M) induced a significant increase in MLC phosphorylation between groups; that is, ACh induced MLC phosphorylation. The ACh-induced MLC phosphorylation was significantly augmented in the repeated antigen challenge group. However, no significant difference in the phosphorylation of MLC of basement was observed between groups (Fig. 5).

In the bronchial preparations of repeatedly challenged



**Fig. 4.** ACh-induced translocation of CPI-17 to plasma membrane in rat bronchial smooth muscle from control rats and rats repeatedly challenged with antigen (Challenged). The bronchi were stimulated with ACh ( $10^{-5}$ – $10^{-3}$  M), and the reaction was stopped by liquid nitrogen 20 min after stimulation. Then, the tissue was homogenized to prepare cytosolic and membrane fractions. A, typical immunoblots for CPI-17 of membrane and cytosolic fractions. Immunoblots were performed by using cytosolic and membrane fractions both on CPI-17 and  $\beta$ -actin. B, CPI-17 translocation to plasma membrane was expressed as membrane CPI-17/total CPI-17 ratio. Values are the means with S.E. from five experiments. ACh-induced translocation of CPI-17 to membrane was augmented in the repeated antigen challenge group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus control.



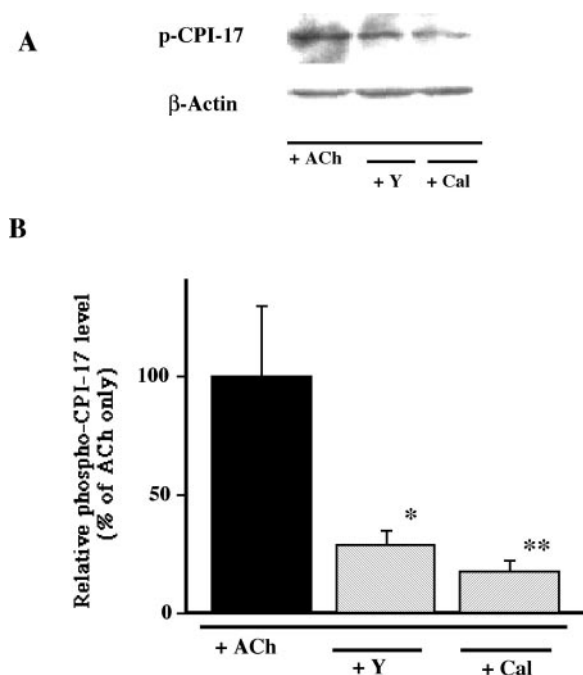
**Fig. 5.** A, typical immunoblots of ACh-induced phosphorylation of myosin light chain (p-MLC; top) and MLC in the bronchial smooth muscle from the control and repeated antigen challenge groups. B, relative densities of p-MLC to MLC in the repeated antigen challenge and control groups. Values are means with S.E. from four different animals. MLC was significantly phosphorylated by ACh ( $10^{-3}$  M) treatment (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  versus each nonstimulated). In the repeated antigen challenge group, ACh-induced phosphorylation was significantly augmented compared with the control + ACh (#,  $p < 0.05$ ).

rats, the ACh-induced phosphorylation and translocation of CPI-17 was significantly inhibited by pretreatment of Y-27632 (ratio of inhibition,  $71.0 \pm 5.9\%$  and  $55.6 \pm 7.9\%$ ) or calphostin C (ratio of inhibition,  $82.6 \pm 4.7\%$  and  $60.6 \pm 10.2\%$ ) (Figs. 6 and 7). Moreover, MLC phosphorylation induced by ACh was also inhibited by pretreatment of Y-27632 (ratio of inhibition,  $59.5 \pm 15.9\%$ ) or calphostin C (ratio of inhibition,  $78.3 \pm 3.5\%$ ) (Fig. 8).

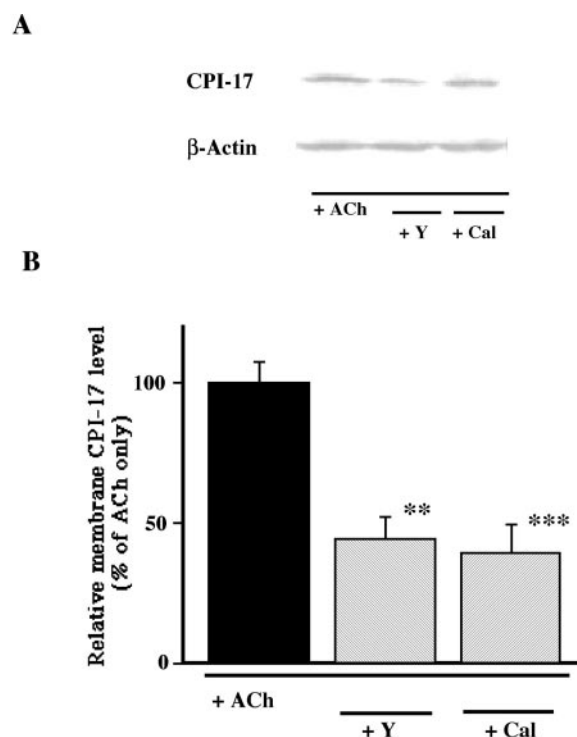
## Discussion

Our previous study demonstrated the *in vivo* AHR to inhaled ACh in rats that were sensitized and repeatedly challenged with antigen by the method described in the present study and elsewhere (Misawa and Chiba, 1993). Furthermore, isolated smooth muscle of the bronchus from the AHR rat had a hyper-responsiveness (Misawa and Chiba, 1993; Chiba and Misawa, 1995). The augmented contractile responsiveness of bronchi to ACh obtained from AHR rats in the present study was concordant with our previous results. It has been demonstrated, with the use of permeabilized bronchial smooth muscles, that ACh-induced  $\text{Ca}^{2+}$  sensitization was inhibited by C3 exoenzyme in normal bronchial smooth muscle (Chiba et al., 1999b). Moreover, the contraction of permeabilized muscle, but not the increase in  $[\text{Ca}^{2+}]_i$ , induced by ACh was much enhanced in bronchial smooth muscle of the airway hyper-responsive rats (Chiba et al., 1999b). Thus, the increased smooth muscle contractility is proposed to be related to augmented agonist-induced  $\text{Ca}^{2+}$  sensitization of myofilaments in AHR rats. At least two pathways might be responsible for the increased  $\text{Ca}^{2+}$  sensitization: the RhoA/ROCK and PKC (Hori and Karaki, 1998). Our

previous studies demonstrated that enhancement of the ACh-induced  $\text{Ca}^{2+}$  sensitization in hyper-responsive muscle was effectively inhibited by C3 exoenzyme, a RhoA inhibitor, in  $\beta$ -escin-permeabilized rat bronchial smooth muscle. The augmented contraction of the intact (nonpermeabilized) bronchial smooth muscle in response 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 augmented, agonist-induced, RhoA-mediated  $\text{Ca}^{2+}$  sensitization of myofilaments. It is possible that the increased expression of RhoA in the bronchial smooth muscle causes an enhancement of RhoA-mediated  $\text{Ca}^{2+}$  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. In the present study, we observed that the levels of CPI-17 mRNA and protein were significantly increased in bronchial smooth muscle from AHR rats. In addition, the ACh-induced phosphorylation of CPI-17 was significantly increased in bronchial smooth muscle from AHR rats. It is interesting that the ACh-induced phosphorylation and translocation of CPI-17 was inhibited by the pretreatment of Y-27632 or calphostin C in bronchial tissue of rats repeatedly challenged with antigen. Moreover, pretreatment with Y-27632 or calphostin C also inhibited the ACh-induced phosphorylation of MLC. The inhibitory effects of Y-27632 and PKC inhibitor on agonist-induced phosphor-



**Fig. 6.** The inhibitory effects of Y-27632 (Y;  $10^{-6}$  M, for 30 min) and calphostin C (Cal;  $10^{-6}$  M, for 30 min) on ACh-induced phosphorylation of CPI-17 in bronchial preparations from repeatedly challenged rats. A, typical immunoblots for phosphorylated CPI-17 (p[Thr<sup>38</sup> CPI-17]) and  $\beta$ -actin. B, relative phospho-CPI level was expressed as percentage of the phosphorylated CPI-17 induced by ACh in the absence of Y-27632 and calphostin C. Values are mean with S.E. from four experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus ACh only.

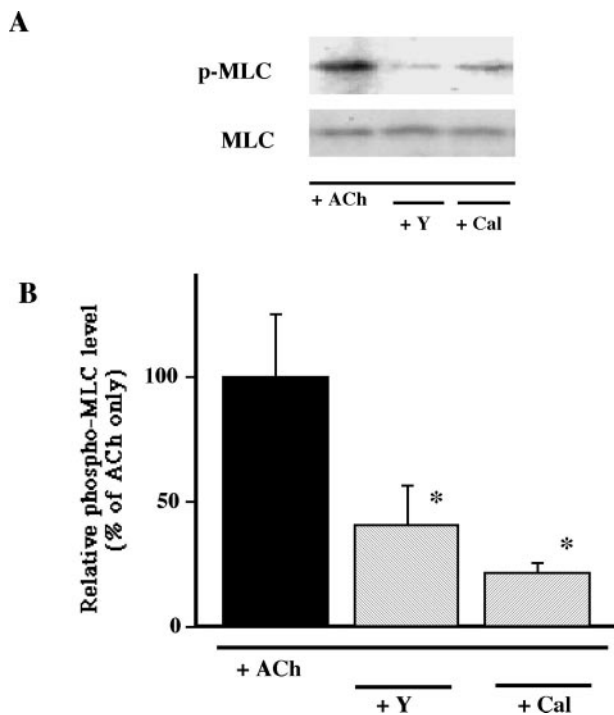


**Fig. 7.** The inhibitory effects of Y-27632 (Y;  $10^{-6}$  M, for 30 min) and calphostin C (Cal;  $10^{-6}$  M, for 30 min) on ACh-induced increase in membrane-associated CPI-17 in bronchial membrane preparations from repeatedly challenged rats. A, typical immunoblots for CPI-17 and  $\beta$ -actin of membrane fractions. B, membrane CPI-17 level was expressed as percentage of the membrane CPI-17 of ACh-stimulated tissues in the absence of Y-27632 and calphostin C. Values are mean with S.E. from four experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  versus ACh only.

ylation of CPI-17 has also been demonstrated in rabbit femoral arterial smooth muscle (Kitazawa et al., 2000). We therefore propose here that  $\text{Ca}^{2+}$  sensitization mediated not only by RhoA/ROCK and PKC/CPI-17 but also by cross-talk of RhoA/ROCK and CPI-17 pathways may play important roles in the ACh-induced bronchial smooth muscle contraction and phosphorylation of MLC. In the state of antigen-induced AHR, these  $\text{Ca}^{2+}$  sensitization pathways may be activated much more intensely than in the non-AHR state.

We here investigated the ACh-induced membrane associated CPI-17. The translocation of CPI-17 was observed by ACh-stimulation in rat bronchial smooth muscle. In the antigen-induced AHR rats, the translocation of CPI-17 was much more augmented. Taggart et al. (1999) showed that receptor agonist stimulation of uterine smooth muscle cell causes a redistribution of RhoA, ROCK, and PKC- $\alpha$  from the cytosol to the cell periphery. Furthermore, myosin phosphatase (MYPT1) has also been shown to translocate to membrane in vascular smooth muscle cells treated with prostaglandin  $\text{F}_{2\alpha}$  (Shin et al., 2002). It is thus possible that the phosphorylation and translocation to plasma membrane of CPI-17 have important roles in agonist-induced smooth muscle contraction and  $\text{Ca}^{2+}$  sensitization with relevance of RhoA, ROCK, and PKC.

In conclusion, we have for the first time suggested that enhancement of the  $\text{Ca}^{2+}$ -sensitizing effect mediated by markedly up-regulated expression and increased activity of CPI-17 might contribute to the augmented contractility of airway smooth muscle at the antigen-induced AHR.



**Fig. 8.** The inhibitory effects of Y-27632 (Y;  $10^{-6}$  M, for 30 min) and calphostin C (Cal;  $10^{-6}$  M, for 30 min) on ACh-induced phosphorylation of myosin light chain (MLC) in bronchial preparations of repeatedly challenged rats. A, typical immunoblots of ACh ( $10^{-3}$  M)-induced phosphorylation of myosin light chain (p-MLC) and total MLC in the bronchial smooth muscle from repeated antigen challenge group. B, relative phospho-MLC level was expressed as percentage of the phosphorylated MLC induced by ACh in the absence of Y-27632 and calphostin C. Values are mean with S.E. from four experiments. \*,  $p < 0.05$  versus ACh only.

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**Address correspondence to:** Hiroyasu Sakai, Department of Pharmacology, School of Pharmacy, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. E-mail: sakai@hoshi.ac.jp