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Possible Involvement of CPI-17 in Augmented Bronchial Smooth Muscle Contraction
in Antigen-induced Airway Hyperresponsive Rats

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Short running title: CPI-17 in airway hyperresponsiveness

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Non-standard abbreviations: AHR, airway hyperresponsiveness; CPI-17,
PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of
17 kDa; ROCK, Rho associated coiled-coil forming protein kinase; MLC, myosin light
chain; MLCK, MLC kinase

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Abstract

Airway hyperresponsiveness (AHR) associated with heightened airway resistance and inflammation is a characteristic feature of asthma. It has been demonstrated that contractile responsiveness and Ca^{2+} sensitization to ACh in repeated antigen challenge-induced airway hyperresponsive bronchial preparation were significantly increased. The CPI-17 (PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa) is activated by PKC 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 hyperresponsiveness 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 repeatedly antigen challenged rats. ACh-induced CPI-17 phosphorylation and translocation to membrane fraction were also significantly increased in bronchus from antigen-challenged rats. In conclusion, it is suggested that augmented expression and activation of CPI-17 observed in the hyperresponsive bronchial smooth muscle might be responsible for the enhanced ACh-induced Ca^{2+} sensitization of bronchial smooth muscle contraction associated with AHR.

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Introduction

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 Ca^{2+} /calmodulin (CAM)-dependent MLC kinase (MLCK)-mediated pathway but also by a Ca^{2+} independent mechanism (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) (Jensen et al., 1996; Walsh et al., 1994) pathways, have been reported linked to the 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 myosin light chain phosphatase 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 Thr³⁸ in CPI-17 converts it to a potent MLCP inhibitor with an IC₅₀ of ~5 nM (Eto et al., 1995; Eto et al., 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

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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 Ca^{2+} sensitization (Woodsome et al., 2001). Assays with purified kinases showed that Thr³⁸ of CPI-17 can be phosphorylated by multiple kinases such as PKC, ROCK, PKN and Zip-like kinase (Eto et al., 1995; Koyama et al., 2000; Hamaguchi et al., 2000; MacDonald et al., 2001).

The airway hyperresponsiveness (AHR) associated with heightened airway resistance and inflammation is the asthmatic characteristic feature (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 for asthmatic medical treatment.

Our previous studies found the occurrence of both *in vivo* and *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^{2+}

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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 post-receptor signaling including augmented Ca^{2+} sensitization. Indeed, Ca^{2+} sensitization in bronchial preparation of repeatedly antigen challenged rats was significantly enhanced as compared with that of control rats (Chiba et al., 1999b). However, the mechanism of augmented 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 hyperresponsiveness of bronchial smooth muscle in antigen-induced AHR rats.

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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 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. 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 (DNP-Asc) by the method described previously (Chiba and Miwawa, 1999a, 1999b). 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 et al., 1995). Therefore, in the present study, age-matched non-sensitized 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 CryopressTM (CP-100W; Niti-on, Co. Ltd., Japan: 15 sec x 3). Total RNA was isolated from each frozen tissue powder by the method of acid guanidium thiocyanate/phenol/chloroform

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extraction (Mullis *et al.*, 1989) and stored at -85°C until use. cDNAs were prepared from the total RNA (0.5 μg) by using a Takara RNA PCR Kit (Ver. 2.1; Takara, Tokyo, Japan) in a total volume of 20 μL reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM MgCl_2 , 1 mM dNTP mixture, 1 U/mL RNase inhibitor, 2.5 μM random 9 mers, and 0.25 U/mL avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated for 10 min at 30°C followed by 60 min at 42°C to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at 99°C for 5 min. Then the RT reaction mixture (10 μL) was added by 0.5 μL of 0.1 mM forward primer, 0.5 μL of 0.1 mM reverse primer, 4 μL of 10 X amplification buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl), 3 μL of 25 mM MgCl_2 , 31.8 μL of H_2O , and 0.25 μL 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'-TACTCCTTGGAGGCCATGTAGG-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 sec at 95°C , 2) annealing primers for 30 sec at 60°C , 3) extending the primers for 60 sec at 72°C . The PCR amplifications were performed 25, 30 and 35 cycles for CPI-17 and 20, 25 and 30 cycles for GAPDH. A portion (10 μL) of the PCR mixture was subjected to electrophoresis on 2 % agarose gel and visualized by densitometer (Atto Densitograph; Atto Co., Tokyo, Japan). The ratios

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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 the method described previously (Chiba et al., 1999) 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 °C) for 60 min with 10 min washout intervals. After the equilibration period, the tissue segments were stimulated by an indicated concentration of 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 Ca^{2+} -induced contraction itself bronchial smooth muscle (Chiba et al., 2001; Sakai et al., in press). Thus, 10^{-6} M concentration 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 (mM): Tris-HCl (pH 7.5) 10, $MgCl_2$ 5, EDTA 2, sucrose 250, dithiothreitol 1,4-(2-aminoethyl) benzenesulphonyl fluoride 1, 20 μ g/mL leupeptin and 20 μ g/mL

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aprotinin. The homogenate was used to quantify the expression of CPI-17. The tissue homogenate was centrifuged (105,000 x g, 4 °C for 30 min) and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 3 mL homogenization buffer and recentrifuged (105,000 x g, 4 °C for 30 min). The resultant pellet was resuspended in 2 mL ice-cold homogenization buffer and used as the membrane fraction. These preparations were stored at –80 °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). Briefly, the samples (10 µg protein per lane) were subjected to 15% SDS-PAGE. Proteins were then electrophoretically transferred for 4 h onto PVDF membranes (Hybond-ECL, Amersham, Little Chalfont, U.K.) in cold transfer buffer (20% methanol containing Tris 25 mM and glycine 192 mM). After repeated washing with Tris buffer (Tris 20 mM, NaCl 500 mM, pH 7.5) containing 0.1% (v/v) Tween 20 (TTBS), the PVDF membranes were incubated with blocking buffer (3% gelatine 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-[Thr³⁸-] phospho-CPI-17 (1: 5000 dilution, Santa Cruz Biotechnology) in antibody buffer (1% gelatine 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 (HRP)-conjugated anti-goat IgG (Amersham) for 1.5 h at room temperature, and then washed five times with TTBS. The blots were

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detected with an enhanced chemiluminescent method (ECL System; Amersham) and quantified by densitometry (Atto Densitograph Software ver. 4.0; Atto Co., Japan). To normalize the CPI-17 contents to an internal control protein, β -actin, immunoblotting was also performed on the same gel by using monoclonal mouse anti- β -actin N-terminal (Sigma, St. Louis, Missouri, U.S.A.) and goat anti-mouse IgG (Amersham). The ratios of corresponding phosphorylated CPI-17/ β -actin and CPI-17/ β -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 $(\text{membrane CPI-17}/\beta\text{-actin})/[(\text{membrane CPI-17}/\beta\text{-actin})+(\text{cytosolic CPI-17}/\beta\text{-actin})]$. In the ACh-induced of MLC phosphorylation study, The bronchial preparation were stimulate by ACh 10^{-3} M for 10 min. Then the samples were homogenized with T-PERTM Tissue Protein Extraction Reagent (Pierce). After the samples (20 μ g) were subjected to 15 % SDS-polyacrylamide gel electrophoresis, western blot was performed. The membranes were incubated with the primary antibodies. As the primary antibodies were used goat anti-p-MLC (Thr 18/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 immunoglobulin (Ig) G (1:5000 dilution; Santa Cruz Biotechnology, Inc) and goat anti-rabbit immunoglobulin (Ig) G (1:5000 dilution; Amersham), detected by an ECL system. The ratio of corresponding p-MLC/MLC was calculated as an index of p-MLC.

Statistical analyses. All the data were expressed as the mean with S.E. Statistical

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significance of difference was determined by Bonferroni/Dunn's test.

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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 25-35 cycles (CPI-17) and for 20-30 cycles (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 level. In order 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 repeatedly antigen-challenged rats.

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 17 kDa molecular weight in the bronchial smooth muscle of each group. In antigen-challenged rats, the expression of CPI-17 protein was significantly augmented when 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, immunoblottings were performed by using phospho

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[Thr³⁸]-specific antibody. The ACh-induced phosphorylation of CPI-17 was increased in a concentration-dependent manner in both groups (Fig. 3). Interestingly, the ACh-induced CPI-17 phosphorylation at Thr³⁸ was significantly augmented in bronchus from repeatedly antigen-challenged rats.

As shown in Fig. 4A, CPI-17 protein was expressed both in 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 control (0.216 ± 0.048) and repeatedly antigen-challenged (0.172 ± 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, while 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 repeatedly antigen-challenged group when compared with the control group.

Myosin light chain (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 repeatedly antigen-challenged group. However, No significant difference in the phosphorylation of MLC of basement was observed between groups (Fig. 5).

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In the bronchial preparations of repeatedly challenged rats, the ACh-induced phosphorylation and translocation of CPI-17 was significantly inhibited by pretreatment of Y-27632 (ratio of inhibition: 71.0 ± 5.9 % and 55.6 ± 7.9 %) or calphostin C (ratio of inhibition: 82.6 ± 4.7 % and 60.6 ± 10.2 %)(Fig. 6 and 7). Moreover, MLC phosphorylation induced by ACh was also inhibited by pretreatment of Y-27532 (ratio of inhibition: 59.5 ± 15.9 %) or calphostin C (ratio of inhibition: 78.3 ± 3.5 %) (Fig. 8).

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Discussion

Our previous study demonstrated the *in vivo* AHR to inhaled ACh in rats that were sensitized and repeatedly challenged with antigen by the same method described in the present study (Misawa and Chiba, 1993). Furthermore, isolated smooth muscle itself of the bronchus from the AHR rat had a hyperresponsiveness (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. By using permeabilized bronchial smooth muscles, it has been demonstrated that the ACh-induced Ca^{2+} sensitization was observed and 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 hyperresponsive rats (Chiba et al., 1999b). Thus, the increased smooth muscle contractility is proposed to be related to augmented agonist-induced Ca^{2+} sensitization of myofilaments in AHR rats. At least two pathways might be responsible for the increased Ca^{2+} sensitization: the RhoA/ROCK and PKC (Hori and Karakai, 1998). 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 β -escin-permeabilized rat bronchial smooth muscle. The augmented contraction of the intact (non-permeabilized) bronchial smooth muscle 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 Ca^{2+} sensitization of

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myofilaments. It is possible that the increased expression of RhoA in the bronchial smooth muscle 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. 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. Interestingly, the ACh-induced phosphorylation and translocation CPI-17 was inhibited by the pretreatment of Y-27632 or calphostin C in bronchial tissue of repeatedly antigen challenged rats. Moreover pretreatment of 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 phosphorylation of CPI-17 has also been demonstrated in rabbit femoral arterial smooth muscle (Kitazawa et al., 2000). We therefore propose here that Ca^{2+} sensitization mediated not only by RhoA/ROCK and PKC/CPI-17 but also crosstalk 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 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

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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- α 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 PGF_{2 α} (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 Ca²⁺ sensitization with relevance of RhoA, ROCK and PKC.

In conclusion, we for the first time suggested that the enhancement of Ca²⁺ sensitizing effect mediated by markedly upregulated expression and increased activity of CPI-17, might contribute to the augmented contractility of airway smooth muscle at the antigen-induced AHR.

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Footnotes

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Figure legends

Figure 1. The levels of expression of CPI-17 mRNA levels in the bronchial smooth muscles from the control (Control) and repeatedly antigen-challenged (Challenged) rats. (A) Typical photographs of RT-PCR product bands for CPI-17 and GAPDH. The PCR amplifications were performed for 25-35 cycles (CPI-17) and 20-30 (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, and the data were summarized in B. Values are the means with S.E. from 4 to 5 experiments. The levels of CPI-17 mRNA were augmented in the repeatedly antigen-challenged group. ** $p < 0.05$ vs. Control.

Figure 2. The levels of CPI-17 protein in the bronchial smooth muscles from the control (1 or Normal) and repeatedly antigen-challenged (2 or Challenged) rats. (A) Typical photographs of bands for CPI-17 and β -actin. The expression levels of CPI-17 were calculated as ratios of the intensities of CPI-17 to β -actin proteins and summarized in B. Values are the means with S.E. from 5 to 6 experiments. The levels of CPI-17 protein were augmented in the repeatedly antigen-challenged group. ** $p < 0.01$ vs. Control.

Figure 3. Acetylcholine (ACh)-induced phosphorylation of CPI-17 in rat bronchial smooth muscles from the control (Control) and repeatedly antigen-challenged (Challenged) rats. (A) Typical immunoblots for phosphorylated CPI-17 (p[Thr³⁸

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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[Thr³⁸ CPI-17]) to total CPI-17 protein. Values are the means with S.E. from 5 experiments. ACh-induced phosphorylation was augmented in the repeatedly antigen-challenged group. *p<0.05 vs. Control.

Figure 4. Acetylcholine (ACh)-induced translocation of CPI-17 to plasma membrane in rat bronchial smooth muscle from the control (Control) and repeatedly antigen-challenged (Challenged) rats. 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 β -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 5 experiments. ACh-induced translocation of CPI-17 to membrane was augmented in the repeatedly antigen-challenged group. *p<0.05 and p<0.01 vs. Control.

Figure 5. (A) Typical immunoblots of acetylcholine (ACh)-induced phosphorylation of myosin light chain (p-MLC; upper) and myosin light chain (MLC) in the bronchial smooth muscle from control and repeatedly antigen-challenged group. (B) Relative densities of p-MLC to MLC in the repeatedly antigen-challenged and control groups.

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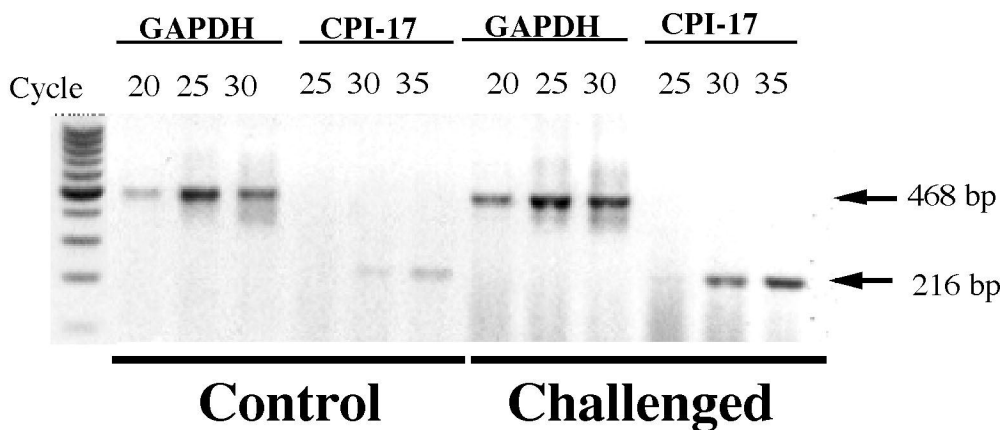
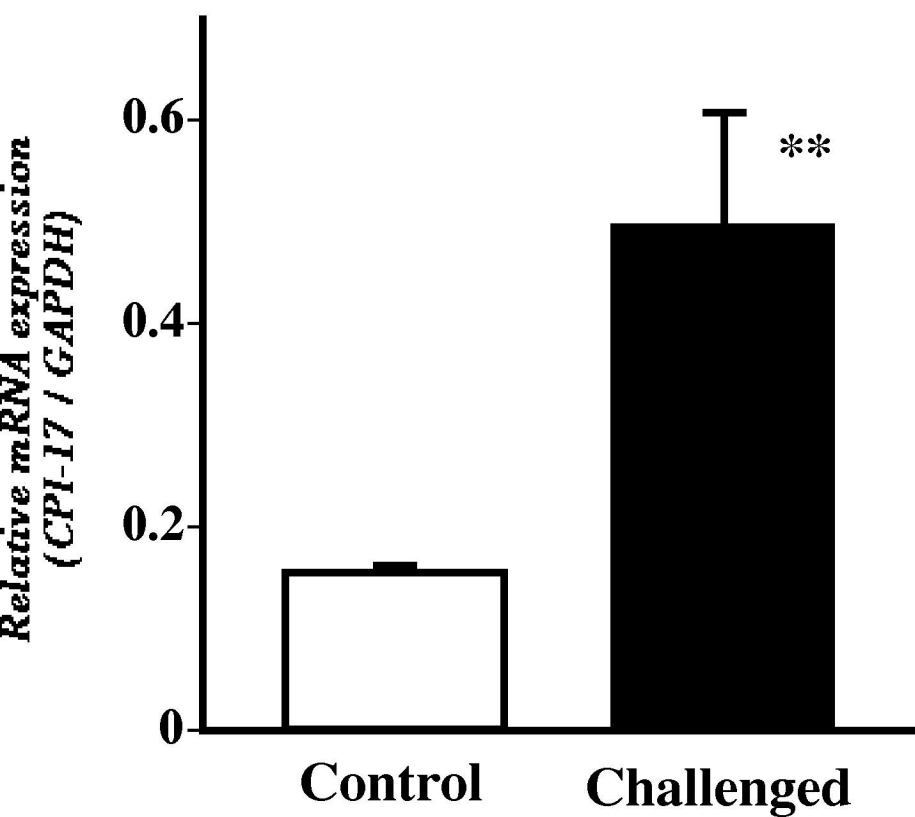
Values are means with S.E. from 4 different animals, respectively. MLC was significantly phosphorylated by ACh (10^{-3} M) treatment (* $p < 0.05$ and *** $p < 0.001$ vs. each non-stimulated). In the repeatedly antigen-challenged group, ACh-induced phosphorylation was significantly augmented as compared to the control + ACh (# $p < 0.05$ vs. Control + ACh).

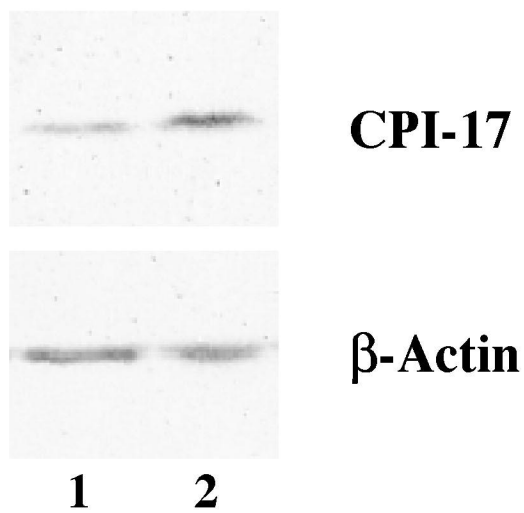
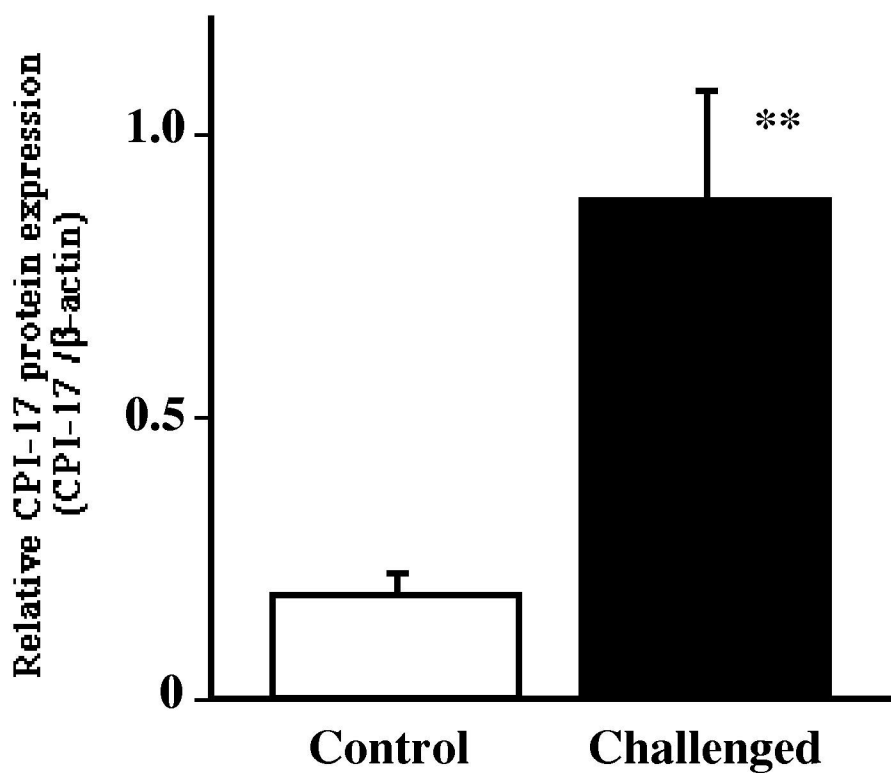
Figure 6. The inhibitory effects of Y-27632 (Y; 10^{-6} M, for 30 min) and calphostin C (Cal; 10^{-6} M, for 30 min) on acetylcholine (ACh)-induced phosphorylation of CPI-17 in bronchial preparations from repeatedly challenged rats. (A) Typical immunoblots for phosphorylated CPI-17 (p[Thr³⁸ CPI-17]) and β -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 4 experiments. * $P < 0.05$ and ** $P < 0.01$ vs. ACh only.

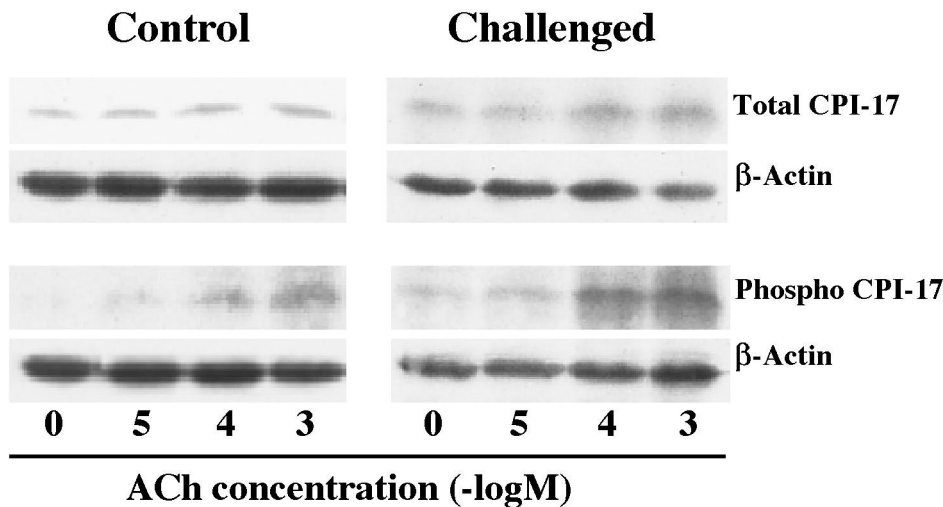
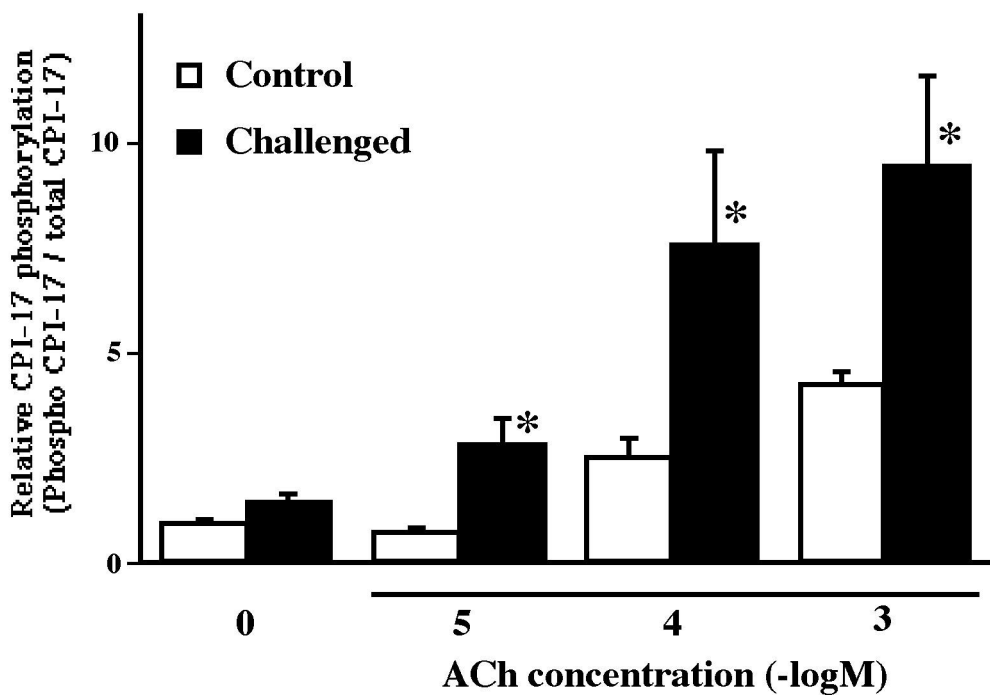
Figure 7. The inhibitory effects of Y-27632 (Y; 10^{-6} M, for 30 min) and calphostin C (Cal; 10^{-6} M, for 30 min) on acetylcholine (ACh)-induced increase in membrane-associated CPI-17 in bronchial membrane preparations from repeatedly challenged rats. (A) Typical immunoblots for CPI-17 and β -actin 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 4 experiments. ** $P < 0.01$ and *** $P < 0.001$ vs. ACh only.

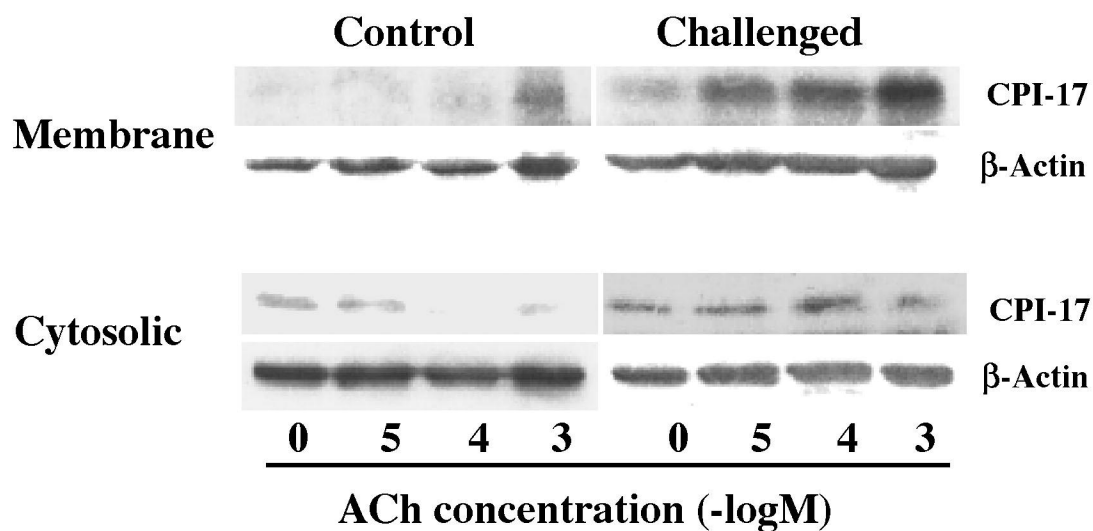
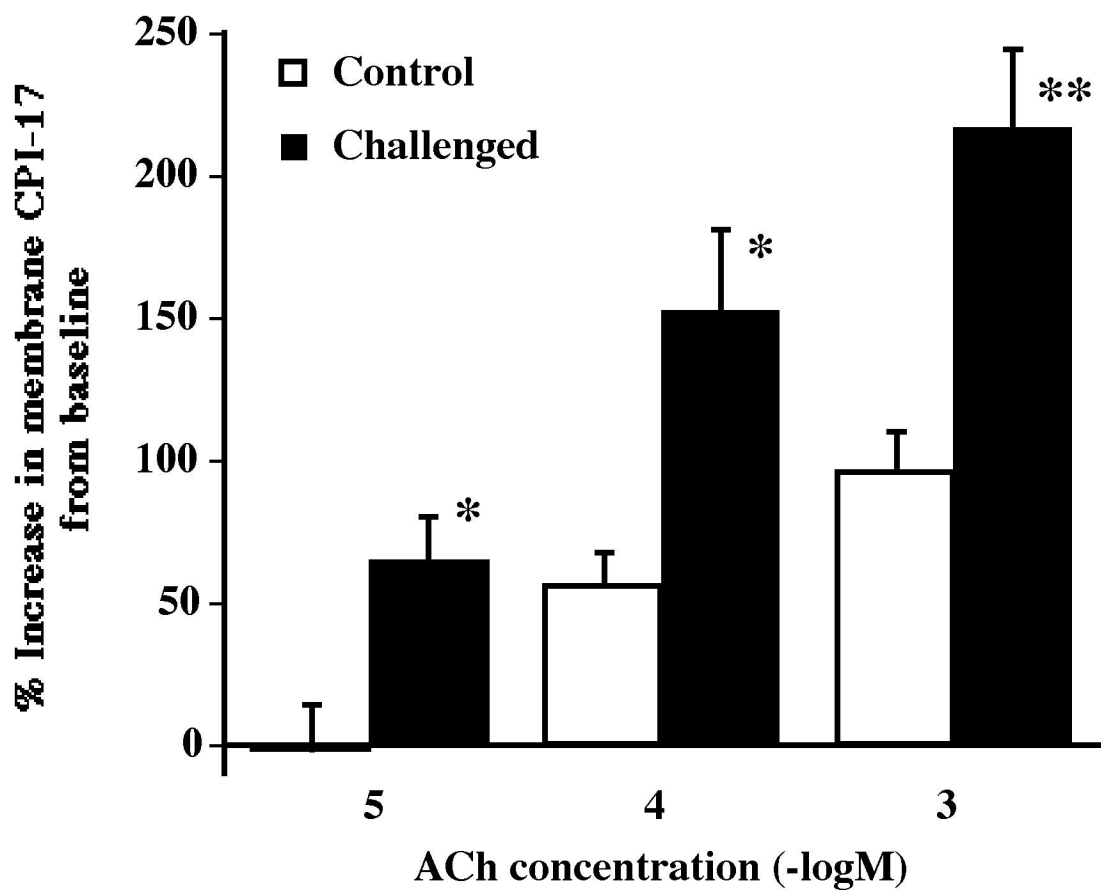
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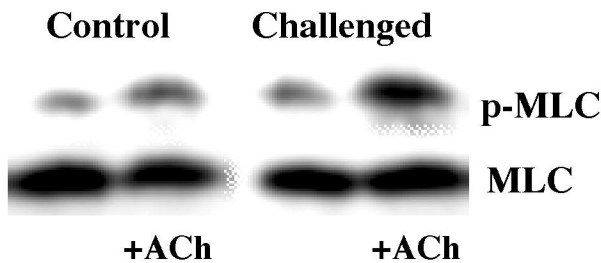
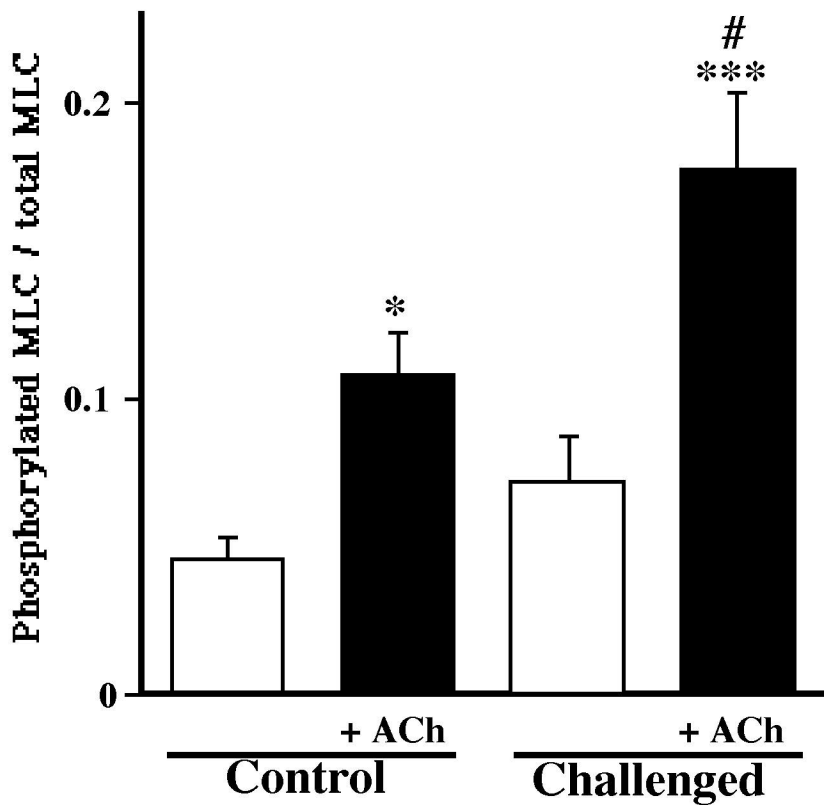
Figure 8. The inhibitory effects of Y-27632 (Y; 10^{-6} M, for 30 min) and calphostin C (Cal; 10^{-6} M, for 30 min) on acetylcholine (ACh)-induced phosphorylation of myosin light chain (MLC) in bronchial preparations of repeatedly challenged rats. (A) Typical immunoblots of ACh (10^{-3} M)-induced phosphorylation of myosin light chain (p-MLC) and total myosin light chain (MLC) in the bronchial smooth muscle from repeatedly antigen-challenged 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 4 experiments. * $P < 0.05$ vs. ACh only.

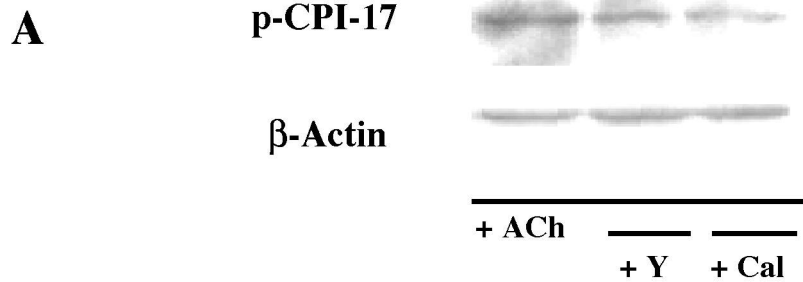
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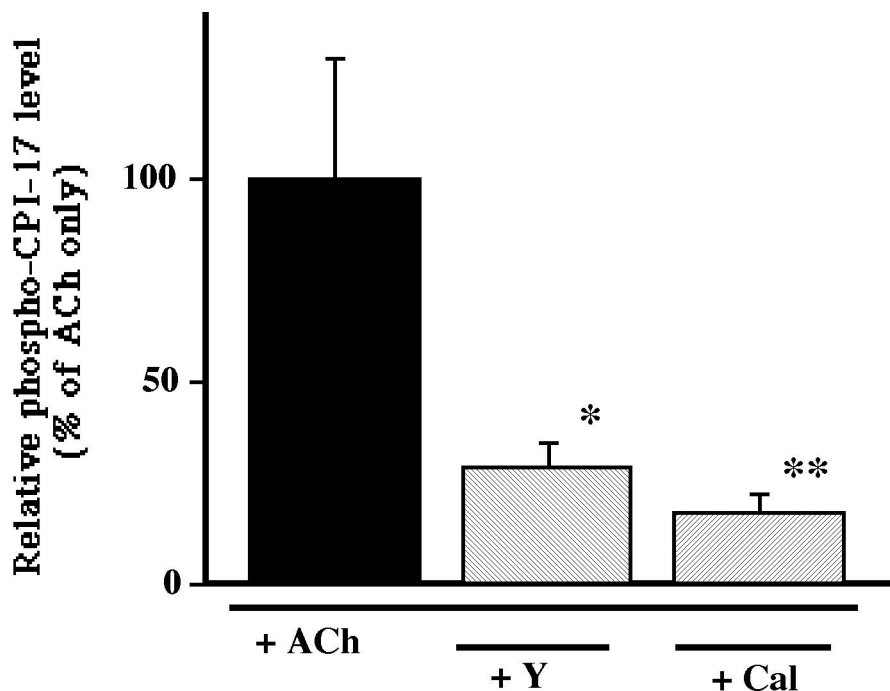
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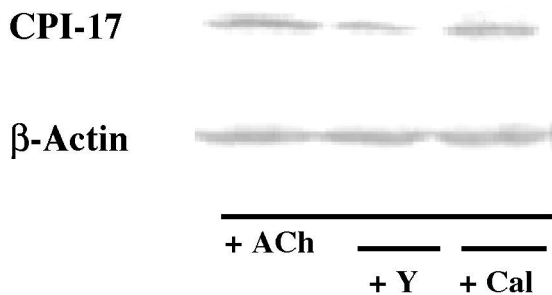
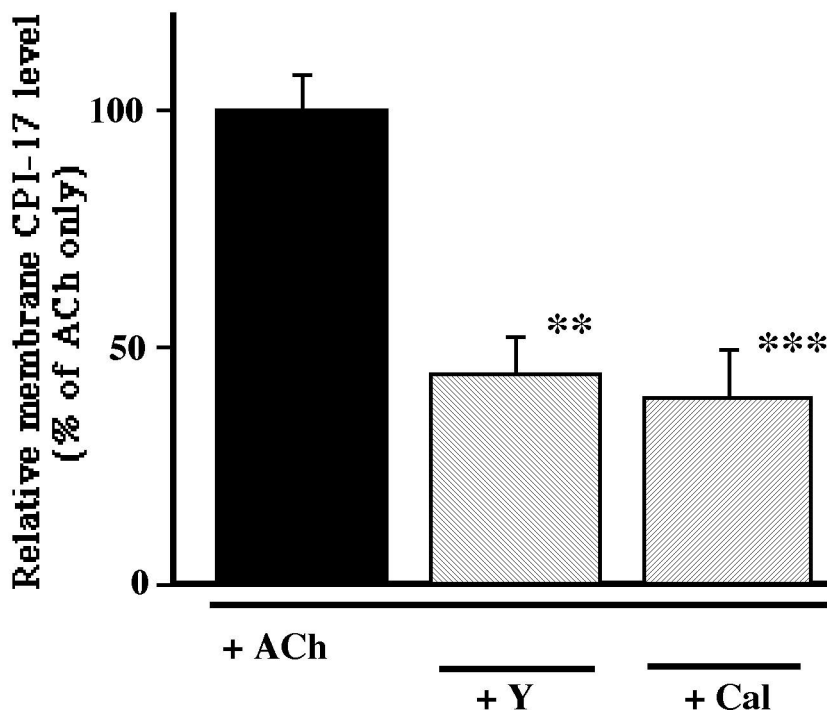
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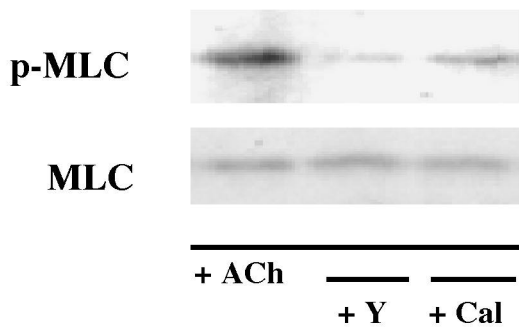
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