Acetylcholine-induced phosphorylation and membrane translocation of CPI-17 in bronchial smooth muscle of rats

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Sakai, Hiroyasu, Tomona Hirano, Yoshihiko Chiba, and Miwa Misawa. Acetylcholine-induced phosphorylation and membrane translocation of CPI-17 in bronchial smooth muscle of rats. Am J Physiol Lung Cell Mol Physiol 289: L925–L930, 2005. First published July 22, 2005; doi:10.1152/ajplung.00054.2005—A translocation of protein kinase C (PKC) from cytosol to plasma membrane has been reported as an association with agonist-induced Ca²⁺ sensitization in smooth muscle contraction. Therefore, it is possible that a downstream target of PKC, CPI-17 [PKC-potentiated inhibitory protein for heterotrimeric myosin light chain (MLC) phosphatase of 17 kDa], might also be translocated to membrane when activated. To confirm this hypothesis, cytosolic and membrane CPI-17 was measured in acetylcholine (ACh)- and high-K⁺ depolarization-stimulated bronchial smooth muscle of rats. An active form of CPI-17, i.e., Thr38-phosphorylated CPI-17, was also measured in cytosolic and membrane fractions. Immunoblot analyses demonstrated a translocation of CPI-17 from cytosolic to membrane fraction by ACh, but not high-K⁺ depolarization, stimulation in time- and concentration-dependent manners. Interestingly, phosphorylated CPI-17 was detected only in membrane fractions in the ACh-stimulated tissues. However, in the high-K⁺ depolarization-stimulated tissues, phosphorylated CPI-17 was not detected both in membrane and cytosolic fraction. To estimate downstream of activated CPI-17, immunoblotting for phosphorylated MLC was performed in ACh- or high-K⁺ depolarization-stimulated tissues. ACh- and high-K⁺ depolarization-induced phosphorylation of MLC was observed in its contraction-dependent manner. In conclusion, we, for the first time, suggested that CPI-17 is a key to the PKC-mediated Ca²⁺ sensitization (3). Assays with purified kinases show that Thr38 of CPI-17 can be phosphorylated by multiple kinases such as PKC, ROCK, protein kinase N (PKN), and Zip-like kinase (2, 8, 9, 10). However, function and expression of CPI-17 in bronchial smooth muscle are unclear. Ca²⁺ sensitization involves the coordination of complex signal transduction events at the plasma membrane and contractile apparatus (11). Muscarinic receptor stimulation has been shown to cause the translocation of PKC, RhoA, and ROCK to cell periphery in uterine smooth muscle (12, 13). Therefore, it is possible that CPI-17 might also be translocated to membrane when activated, although the mechanism of activation of CPI-17 is unclear. To confirm this hypothesis, cytosolic and membrane CPI-17 protein were measured in acetylcholine (ACh)- or high-K⁺ depolarization-stimulated bronchial smooth muscle of rats. An active form of CPI-17, i.e., Thr38-phosphorylated CPI-17 (5), was also measured in cytosolic and membrane fractions. Moreover, to estimate downstream of activated CPI-17, the phosphorylation of MLC by ACh or high-K⁺ depolarization-stimulated was detected.

MATERIALS AND METHODS

Animals. Male Wistar rats (6 wk of age, specific pathogen-free, 170–190 g, Charles River Japan) were 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-h light-dark cycle and free access to food and water. 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).

Functional study of intact smooth muscle. The airway tissues under the larynx to lungs were removed under chloral hydrate (400 mg/kg ip) anesthesia. Approximately 4-mm length of the left main bronchus was isolated, and the resultant tissue ring preparations were then suspended in a 5-ml organ bath at a resting tension of 9.8 mN. The isometric contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T; Nihon Kohden, Japan). The organ bath contained modified Krebs-Henseleit solution with the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 1.2 KH₂PO₄, and 10 glucose (pH 7.4). The buffer solution was maintained at 37°C and oxygenated with 95% O₂-5% CO₂. During an equilibration period in the organ bath, the tissues were washed four times at 15-min intervals and equilibrated with high-potassium depolarization-stimulated tissues.

CA²⁺ sensitization of smooth muscle contraction produced by receptor stimulation involves a decrease in myosin light chain (MLC) phosphatase (MLCP) activity mediated through the actions of protein kinase C (PKC) and RhoA-associated coiled-coil-forming protein kinase (ROCK) (1). ROCK inhibits MLCP by phosphorylating MLCP targeting subunit (MYPT1), resulting in an increase in MLC phosphorylation and thus contractile force. Recently, an endogenous inhibitory protein specific for MLCP was isolated from pig aorta smooth muscle extracts and named CPI-17 for PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa (2, 3). Phosphorylation of Thr38 in CPI-17 converts it to a potent MLCP inhibitor with an IC₅₀ of ~5 nM (2, 4). Phospho-CPI-17 enhances myosin phosphorylation and contraction of both permeabilized arterial smooth muscle and intact fibroblast (5, 6). Permeabilization of femoral artery strips using β-escin or Triton-X depletes endogenous CPI-17 with loss of the contractile response to phorbol ester. The PKC-induced contraction of permeabilized artery is reconstituted by addition of recombinant CPI-17 (7). 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 a key to the PKC-mediated Ca²⁺ sensitization (3). Assays with purified kinases show that Thr38 of CPI-17 can be phosphorylated by multiple kinases such as PKC, ROCK, protein kinase N (PKN), and Zip-like kinase (2, 8, 9, 10). However, function and expression of CPI-17 in bronchial smooth muscle are unclear. Ca²⁺ sensitization involves the coordination of complex signal transduction events at the plasma membrane and contractile apparatus (11). Muscarinic receptor stimulation has been shown to cause the translocation of PKC, RhoA, and ROCK to cell periphery in uterine smooth muscle (12, 13). Therefore, it is possible that CPI-17 might also be translocated to membrane when activated, although the mechanism of activation of CPI-17 is unclear. To confirm this hypothesis, cytosolic and membrane CPI-17 protein were measured in acetylcholine (ACh)- or high-K⁺ depolarization-stimulated bronchial smooth muscle of rats. An active form of CPI-17, i.e., Thr38-phosphorylated CPI-17 (5), was also measured in cytosolic and membrane fractions. Moreover, to estimate downstream of activated CPI-17, the phosphorylation of MLC by ACh or high-K⁺ depolarization-stimulated was detected.

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slowly to a baseline tension of 9.8 mN. Fifteen minutes after the last washing, higher concentrations of ACh were successively added after attainment of a plateau response to the previous concentration. In another series of experiments, bronchial smooth muscle was depolarized with isotonic high-K\textsuperscript{+} solution prepared by isosmotic replacement of NaCl by KCl in the presence of 10\textsuperscript{-6} M atropine and 10\textsuperscript{-6} M indomethacin. In time-course experiments, ACh (10\textsuperscript{-3} M) and high-K\textsuperscript{+} (60 mM) solution were treated for 30 min. The treatment of atropine (muscarnic receptor antagonist; 10\textsuperscript{-6} M) or washout was performed 20 min after treatment of ACh (10\textsuperscript{-3} M).

**Sample preparation.** Membrane and cytosolic fractions of bronchial tissue were prepared by the method described previously (14, 15) with minor modifications. In brief, the airway tissues below the main bronchi were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The airways were carefully cleaned of adhering connective tissues, blood vessels, and lung parenchyma under stereomicroscopy. The bronchial tissue was then equilibrated in oxygenated Krebs-Henseleit solution (37°C) for 60 min with 10-min washout intervals. After the equilibration period, the tissue fragments were stimulated by an indicated concentration of ACh (10\textsuperscript{-3} M for 1–20 min or 10\textsuperscript{-5} to 10\textsuperscript{-3} M for 20 min) and high-K\textsuperscript{+} solution (60 mM for 1–20 min). In another series of experiments, the treatment of atropine (10\textsuperscript{-6} M for 5 min) or washout was performed 20 min after treatment of ACh (10\textsuperscript{-3} M). 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): 10 Tris-HCl (pH 7.5), 5 MgCl\textsubscript{2}, 2 EDTA, 250 sucrose, 1 dithiothreitol, and 1 4-(2-aminoethyl)benzenesulphonyl fluoride, and also included 20 μg/ml leupeptin and 20 μg/ml apro tin. The tissue homogenate was centrifuged (105,000 g, 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 (105,000 g, 4°C for 30 min). The resultant pellet was resuspended in 2 ml of ice-cold homogenization buffer containing 1% (vol/vol) Triton X-100 and 1% (wt/vol) sodium cholate and used as the membrane fraction. These preparations were stored at −80°C until use.

**Western blots.** To quantify the translocated CPI-17 to membrane fraction and phosphorylated CPI-17 proteins, immunoblotting was performed as described previously (16). Briefly, the samples (10 μg of protein/lane) were subjected to 15% SDS-PAGE. Proteins were then electrophoretically transferred for 4 h onto polyvinylidene difluoride (PVDF) membranes (Hybond-ECL; Amersham, Little Chalfont, UK) in cold transfer buffer (20% methanol containing 25 mM Tris and 192 mM glycine). After repeated washing with Tris buffer (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% (vol/vol) 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; 1:1,000 dilution, Santa Cruz Biotechnology) or polyclonal goat anti-[Thr38]-phospho-CPI-17 (1:1,000 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 goat anti-rabbit IgG (Amersham) for 1.5 h at room temperature and then washed five times with TTBS. The blots were detected with an enhanced chemiluminescence method (ECL System, Amersham) and quantified by densitometry (Atto Densitograph software, ver. 4.0). To normalize the CPI-17 contents to total CPI-17 in each animal sample was calculated according to the formula (membrane CPI-17/β-actin)/(cytosolic CPI-17/β-actin).

In other experiment series, to quantify the expression of MLC and phosphorylated MLC proteins, Western blot was performed. In brief, transferred PVDF membranes were incubated with the primary antibodies. The primary antibodies used were goat anti-phosphorylated MLC (p-MLC; Thr18/Ser19; 1:1,000 dilution; Santa Cruz Biotechnology) or rabbit anti-MLC (1:1,000; Santa Cruz Biotechnology). The membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (1:5,000 dilution; Santa Cruz Biotechnology) or goat anti-rabbit IgG (1:2,500 dilution; Amersham), detected by an ECL system. The ratio of corresponding p-MLC/MLC was calculated as an index of p-MLC.

**Statistical analyses.** Statistical significance of difference was determined by one-way ANOVA and Bonferroni/Dunn’s posttest. A value of P < 0.05 was considered significant.

**RESULTS**

Effects of ACh- and high-K\textsuperscript{+} stimulation on bronchial smooth muscle contraction. Figure 1 shows ACh- and high-K\textsuperscript{+}--induced bronchial smooth muscle contraction. Both ACh
and high-K⁺ depolarization induced contraction in concentration-dependent manners. The maximal contraction induced by ACh and high-K⁺ depolarization was 0.90 ± 0.10 (−logEC₅₀ value: 5.27 ± 0.17) and 0.26 ± 0.04 g, respectively (Fig. 1A). The smooth muscle contraction induced by ACh (10⁻³ M) was in a time-dependent manner, and the peak was observed 20–30 min after ACh stimulation (Fig. 1C). In high-K⁺ (60 mM)-stimulated smooth muscles, the maximum contraction was observed at 1 min, and the contraction was maintained for at least 30 min.

Effects of ACh- and high-K⁺ stimulation on translocation of CPI-17 in bronchial smooth muscle. Fig. 2 shows time-course changes in CPI-17 translocation from cytosolic to membrane fractions induced by ACh (10⁻³ M) stimulation. The immunoblotting with CPI-17 antibody gave a single band with 17-kDa molecular weight, indicating an existence of CPI-17 protein in the rat bronchial smooth muscle. The CPI-17 contents in membrane fraction were increased contrary to it in cytosolic fraction, i.e., CPI-17 translocation, in a time-dependent manner. A translocation of CPI-17 was significantly increased 2 min after ACh stimulation and sustained at least 20 min (Fig. 2, A and B). The CPI-17/β-actin was also increased in membrane fraction and decreased in cytosolic fraction after treatment of ACh (Fig. 2C). The CPI-17 contents in the membrane fractions were significantly increased by ACh (10⁻⁵ to 10⁻³ M) stimulation in its concentration-dependent manner, whereas the ratio of cytosolic to total CPI-17 was significantly decreased (Fig. 2D).

On the other hand, the translocation of CPI-17 from cytosolic to membrane fractions was not observed in the high-K⁺ depolarized smooth muscles (Fig. 3).

Effects of ACh- and high-K⁺ stimulation on the phosphorylation of CPI-17 in rat bronchial smooth muscle. ACh-induced phosphorylation of CPI-17 was also determined by using Thr38-phosphorylated CPI-17-specific antibody in membrane and cytosolic fractions. As shown in Fig. 4, A–C, ACh (10⁻³ M, for 0–20 min) induced a phosphorylation of CPI-17 in membrane fraction of bronchial smooth muscle. The phosphorylation of CPI-17 in membrane was in a time-dependent manner and was significantly increased 10 and 20 min after treatment of ACh (Fig. 4, A and B). However, phosphorylated CPI-17 was not detected in cytosolic fraction even in the tissues that were stimulated by ACh for 20 min (Fig. 4A). The CPI-17 phosphorylation in the membrane...
fractions was not observed in the high-K$^+$ depolarized smooth muscle. In high-K$^+$ (60 mM)-stimulated smooth muscles, the maximum phosphorylation of MLC was observed at 1 min, and the phosphorylation was maintained for at least 20 min after its stimulation.

Effects of atropin and washout on ACh-induced phosphorylation and translocation of CPI-17 in membrane fraction of rat bronchial smooth muscle. Treatment of atropine (muscarinic receptors antagonist; 10$^{-5}$ M) abolished the ACh-induced bronchial smooth muscle contraction (Fig. 6A). In membrane fraction of rat bronchial smooth muscle, the phosphorylation of CPI-17 and increase in membrane-associated CPI-17 were also abolished by treatment of atropine (Fig. 6, B and C). Similar results were also obtained by the washout of ACh (Fig. 7).

**DISCUSSION**

In the present study, CPI-17 was translocated from cytosol to membrane by ACh stimulation (Fig. 2). In ACh-stimulated tissue, phosphorylated CPI-17 was detected only in membrane but not in cytosolic fraction (Fig. 4). The ACh-induced phosphorylation of CPI-17 was significantly increased in membrane fraction (Fig. 4). On the other hand, no significant change in the ratio of phosphorylated to nonphosphorylated CPI-17 was observed in membrane fraction of ACh-stimulated bronchial smooth muscle (Fig. 4, D and F), suggesting that the translocated CPI-17 might be the phosphorylated CPI-17 in ACh-stimulated bronchial smooth muscle.

Taggart et al. (12) showed that receptor agonist stimulation of smooth muscle causes a translocation of RhoA, ROCK, and PKC-α from the cytosol to the cell periphery. CPI-17 has been known as a target of these protein kinases (2, 8). Furthermore, myosin phosphatase (MYPT1) has also been shown to translocate to membrane in vascular smooth muscle cells treated with PGF$_2\alpha$ (17). It is thus possible that CPI-17 has important roles at the site of membrane in agonist-induced smooth muscle contraction and Ca$^{2+}$ sensitization as well as RhoA, ROCK, and PKC. On the other hand, the ratios of phosphorylated CPI-17 to CPI-17 in membrane fractions of ACh (for 0–20 min and 10$^{-5}$ to 10$^{-3}$ M)-stimulated bronchial smooth muscles were not changed compared with those of resting-state muscles. This finding suggests that membrane-associated CPI-17 is the phosphorylated CPI-17.

We previously demonstrated an ACh-induced Ca$^{2+}$ sensitization of bronchial smooth muscle contraction in rat (14). The Ca$^{2+}$-sensitizing effect was sensitive to C3 exoenzyme (a Rho inactivator) and Y-27632 (a ROCK inhibitor) (14, 18), indicating that a RhoA/ROCK pathway is involved in the Ca$^{2+}$ sensitization. A subcellular fractionation experiment of ACh-stimulated bronchial smooth muscle strips also suggested a translocation of RhoA proteins to plasma membrane (16).

**Fig. 3.** Effect of high-K$^+$ stimulation on the translocation of CPI-17 in rat bronchial smooth muscle. A: typical immunoblots for CPI-17 and β-actin of membrane (top) and cytosolic fractions (bottom). B and C: time course of ACh-induced translocation of CPI-17 after treatment of high-K$^+$ (60 mM) solution in rat bronchial smooth muscle. The levels of CPI-17 in plasma membrane and cytosolic fraction were expressed as ratio to total CPI-17 (B) and as ratio to β-actin (C). Values are means with SE from 4–5 experiments. The translocation of CPI-17 was not induced by high-K$^+$ depolarization.

Effects of ACh- and high-K$^+$ stimulation on the phosphorylation of MLC. Both ACh and high-K$^+$ depolarization induced phosphorylation of MLC in membrane fractions (Fig. 5, A and B). ACh-induced phosphorylation of MLC was increased in a time-dependent manner, and the maximum phosphorylation was observed at 20 min. In high-K$^+$ (60 mM)-stimulated smooth muscles, the maximum phosphorylation of MLC was observed at 1 min, and the phosphorylation was maintained for at least 20 min after its stimulation.

Effects of atropin and washout on ACh-induced phosphorylation and translocation of CPI-17 in membrane fraction of rat bronchial smooth muscle. Treatment of atropine (muscarinic receptors antagonist; 10$^{-5}$ M) abolished the ACh-induced bronchial smooth muscle contraction (Fig. 6A). In membrane fraction of rat bronchial smooth muscle, the phosphorylation of CPI-17 and increase in membrane-associated CPI-17 were also abolished by treatment of atropine (Fig. 6, B and C). Similar results were also obtained by the washout of ACh (Fig. 7).
high-K⁺ depolarization, the translocation and phosphorylation of CPI-17 were not induced by high-K⁺ depolarization. Accordingly, translocation and phosphorylation of CPI-17 induced by ACh might be mediated via an activation of musca-

Fig. 4. Effects of ACh and high-K⁺ stimulation on the phosphorylation (p) of CPI-17 in rat bronchial smooth muscle. The bronchial smooth muscle was incubated in Krebs-Henseleit solution in the absence or presence of ACh, and the reaction was stopped by rapid freezing in liquid nitrogen. The cytosolic and membrane fractions were then prepared as described in MATERIALS AND METHODS, and immunoblots were performed. A: typical immunoblots for phosphorylated CPI-17 (top) and β-actin in membrane (bottom) and cytosolic fraction (bottom) after treatment of ACh (10⁻³ M). C and D: time course of ACh-induced phosphorylation of CPI-17 in membrane fraction of rat bronchial smooth muscle. E and F: concentration-response curve of ACh (10⁻⁵ to 10⁻³ M for 20 min)-induced phosphorylation of CPI-17. The phosphorylation levels of CPI-17 were calculated as the ratio of the intensities of phospho-CPI-17 (mem p-CPI-17) to membrane β-actin (mem β-actin) (C and E) or membrane CPI-17 (mem CPI-17) (D and F). B: typical immunoblots for phosphorylated CPI-17 (top) and β-actin (bottom) of membrane. Time course of high-K⁺ (60 mM) depolarization-induced phosphorylation of CPI-17 in rat bronchial smooth muscle. The phosphorylation of CPI-17 was not induced by high-K⁺ depolarization. Values are means with SE from 4–5 experiments. *P < 0.05 vs. none (0, in the absence of ACh).

Fig. 5. Effects of ACh and high-K⁺ stimulation on the phosphorylation of myosin light chain (MLC) in rat bronchial smooth muscle. The bronchial smooth muscle was incubated in Krebs-Henseleit solution in the absence or presence of ACh (10⁻³ M) or high K⁺ (60 mM), and the reaction was stopped by rapid freezing in liquid nitrogen. The membrane fractions were then prepared as described in MATERIALS AND METHODS, and immunoblots were performed. A: typical immunoblots for phosphorylated MLC (top) and myosin (bottom) in membrane fraction after treatment of ACh (10⁻³ M). B: time course of ACh-induced phosphorylation of MLC in membrane fraction of rat bronchial smooth muscle. The phosphorylation levels of MLC were calculated as the ratio of the intensities of phospho-MLC to MLC (mem p-MLC / mem MLC). Values are means with SE from 4 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. none (0, in the absence of ACh).

Fig. 6. Effects of atropine (Atr; 10⁻⁶ M) on the ACh (10⁻³ M)-induced phosphorylation and translocation of CPI-17 in rat bronchial smooth muscle. A: typical trace of ACh (10⁻³ M)-induced bronchial smooth muscle contraction. The membrane fractions of rat bronchial smooth muscles were prepared at the time points as indicated, and immunoblottings for phosphorylated (B) and nonphosphorylated CPI-17 (C) were performed. The lane numbers in B and C indicate the time points shown in A, respectively.
ACh-induced phosphorylation and membrane translocation of CPI-17 in rat bronchial smooth muscle. Moreover, ACh-induced phosphorylation of CPI-17 may be mediated by Ca\(^{2+}\)-independent PKC such as novel PKC and atypical PKC. Indeed, CPI-17 has reportedly been phosphorylated by PKC-\(\delta\), which belong to a novel PKC (19).

When the time courses of CPI-17 translocation and phosphorylation induced by ACh were compared each other, the translocation is a comparatively earlier event than the phosphorylation (Figs. 2 and 4). Furthermore, phosphorylated CPI-17 was not detected in cytosolic fraction of the ACh-stimulated tissues. Thus phosphorylation of CPI-17 may be induced after its translocation to membrane.

In conclusion, we for the first time suggested that CPI-17 was translocated and phosphorylated by ACh, but not by high-K\(^{+}\) depolarization stimulation, in rat bronchial smooth muscle.

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Fig. 7. Effects of removal of ACh on the ACh (10\(^{-3}\) M)-induced phosphorylation and translocation of CPI-17 in rat bronchial smooth muscle. A: typical trace of ACh-induced bronchial smooth muscle contraction. The membrane fractions of rat bronchial smooth muscles were prepared at the time points as indicated, and immunoblotting for phosphorylated (A) and nonphosphorylated CPI-17 (C) were performed. The lane numbers in B and C indicate the time points shown in A, respectively.