MYOSIN LIGHT CHAIN KINASE IS NECESSARY FOR TONIC AIRWAY SMOOTH MUSCLE CONTRACTION*

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Different interacting signaling modules involving Ca2+/calmodulin-dependent myosin light chain kinase, Ca2+-independent regulatory light chain phosphorylation, myosin phosphatase inhibition and actin filament-based proteins are proposed as specific cellular mechanisms involved in the regulation of smooth muscle contraction. However, the relative importance of specific modules is not well defined. By using tamoxifen-activated and smooth muscle-specific knockout of myosin light chain kinase in mice, we analyzed its role in tonic airway smooth muscle contraction. Knockout of the kinase in both tracheal and bronchial smooth muscle significantly reduced contraction and myosin phosphorylation responses to K+-depolarization and acetylcholine. Kinase-deficient mice lacked bronchial constrictions in normal and asthmatic airways, while the asthmatic inflammation response was not affected. These results indicate that myosin light chain kinase acts as a central participant in the contractile signaling module of tonic smooth muscle. Importantly, contractile airway smooth muscles are necessary for physiological and asthmatic airway resistance.

Smooth muscles line the walls of hollow organs such as the airways of the respiratory tract. They are essential for maintaining homeostasis but also contribute to stresses imposed by disease processes. Based on their different contractile properties, smooth muscle tissues from different organs systems are classified as phasic and tonic types. The typical phasic muscles (such as ileum, taenia coli, uterus, and portal vein) generate action potentials, shorten rapidly, and typically produce spontaneous contraction (1-2). On the other hand, tonic smooth muscles (airway, vascular and sphincter smooth muscles) do not generate action potentials and spontaneous contractions, and they maintain contractile force for prolonged periods of time (2). Phasic and tonic smooth muscles share some common regulatory signaling pathways centered on the molecular motor myosin as well as membrane properties associated with calcium handling and cell adhesion (3-7). As a common mechanism, smooth muscle contraction may be regulated by Ca2+ through two pathways initiated by depolarization and agonist respectively. Depolarization of the cell membrane activates...
voltage-gated Ca\(^{2+}\)-channels resulting in Ca\(^{2+}\) influx whereas agonist stimulation generally activates GPCR\(^1\) leading to inositol 1,4,5-trisphosphate formation and Ca\(^{2+}\) release from the sarcoplasmic reticulum (8-9). The increase in cytosolic Ca\(^{2+}\) leads to smooth muscle contraction through MLCK activation by Ca\(^{2+}\)/calmodulin and myosin RLC phosphorylation (9-10). Additionally, activation of GPCRs leads to inactivation of MLCP by agonist-induced PKC and RhoA/ROCK activation (11-14). These inhibitory mechanisms thus enhance RLC phosphorylation and force development (Ca\(^{2+}\)-sensitization).

Several other proposed regulatory mechanisms include RLC phosphorylation by Ca\(^{2+}\)-independent kinases (5,15) that may act synergistically with Ca\(^{2+}\)-sensitization leading to the proposal that MLCK is required only for the initial contraction while the sustained contraction involves activities of Ca\(^{2+}\)-independent kinases with myosin light chain phosphatase inhibition. Additionally, activation of actin-associated thin-filament proteins may play a role in smooth muscle contraction (16). Thus, it is crucial to elucidate the functional contributions of these different signaling modules to understand the integrated contraction of airway smooth muscle. In phasic smooth muscle deletion of MLCK abolished K\(^{+}\)-induced contraction and significantly reduced GPCR-mediated contraction, thus indicating the central role of RLC phosphorylation by this Ca\(^{2+}\)-dependent kinase (17). In tonic smooth muscle such as airway smooth muscle, other regulatory elements including Ca\(^{2+}\)-sensitization and Ca\(^{2+}\)-independent kinases may play primary roles in sustained force development (5,15,18-19).

Although bronchial hyper-responsiveness is a component of asthma, the mechanisms underlying this excessive narrowing of the airways are unclear. An intrinsic change in airway smooth muscle may contribute and include increases in MLCK content (20-21), enhancement of Ca\(^{2+}\)-sensitization pathway (22-23), Ca\(^{2+}\)-independent kinases (24-25), and/or remodeling of the actin cytoskeleton (26).

We therefore deleted MLCK in adult tonic airway smooth muscle to address its biochemical importance in physiological contractions as well as its role in airway smooth muscle responsiveness in an animal model of asthma.

**EXPERIMENTAL PROCEDURES**

*Generation of floxed Mlck mice and tissue-specific knockout mice (MLCK\(^{SMKO}\))—* To generate MLCK\(^{SMKO}\) mice, MLCK\(^{flox/flox}\) mice were crossed with SM-CreER\(^{T2}\) (ki) mice expressing a tamoxifen-activated Cre recombinase under control of the SM22 promoter as previously described (17,27). To purify MLCK\(^{SMKO}\) from 129/B6 background, we backcrossed the mice to C57BL/6 for six generations. Female MLCK–deficient and littermate control mice (Mlck\(^{flox/+}\); SM-CreER\(^{T2}\)) at 8–12 weeks of age were used for all experiments. Tamoxifen was injected i.p. for five consecutive days at a dose of 1mg per day as described (27). The tamoxifen (100 mg, Sigma, T5648) was dissolved in 0.5 ml ethanol followed by 9.5 ml sunflower oil at a concentration of 10 mg/ml and stored at -20C for up to one month. All experiments were conducted in accordance with Animal Care and Use Committee of Model Animal Research Center of Nanjing University.

*Tracheal contractility—* Analysis of mouse tracheal contractility was performed as reported previously (28). Tracheae were excised and dissected free of surrounding tissues and cut into rings of 2 mm in length. The tracheal rings were opened and suspended on tissue hooks in individual 10-ml jacketed organ baths containing...
modified Krebs-Henseleit solution maintained at
37°C, pH 7.4, gassed with a mixture of 95% O₂ and
5% CO₂ for an equilibration period of 30 min. The
Krebs-Henseleit solution was of the following
composition: NaCl 118.1 mM, KCl 4.7 mM,
CaCl₂ 2.5 mM, MgSO₄ 1.2 mM, KH₂PO₄ 1.2 mM,
NaHCO₃ 25 mM, glucose 11.1 mM. Force was
recorded isometrically by a transducer (MLT0202,
ADInstruments) connected to a PowerLab
(ADInstruments) recording device. At the end of
the equilibration period, KCl (60 mM), or
acetylcholine (10 μM) was added to measure
contractile responses. Acetylcholine was then
applied cumulatively to obtain dose-dependent
responses. Force per cross-sectional area was
calculated from tracheal muscle cross-sectional
area obtained from histological images of each
tissue.

Bronchial ring isometric contraction
bioassay—Mouse bronchial ring contractility was
measured as reported in detail (29). Briefly, the
entire respiratory tree was rapidly removed from
anesthetized mice and immersed in
Krebs-Henseleit solution. Bronchial rings, 200–400 μm in diameter and 2 mm in length,
were isolated from mouse intrapulmonary bronchi
and mounted in a small-vessel wire myograph
chamber (Danish Myo Technology, Aarhus,
Denmark) by threading on two steel wires (40
μm in diameter) secured to two supports. One support
was attached to a micrometer allowing control of
ring circumference while the other support was
attached to a force transducer for measurements of
isometric force development. The preparation was
immersed in 6 ml Krebs-Henseleit solution,
bubbled with 95% O₂ and 5% CO₂, and
maintained at 37°C. Isometric force was initially
set to 0 and the bronchial ring was allowed to
equilibrare for 10 min. The bronchial ring was
then stretched by applying a total of 7.5 mN force
(in 3 discrete steps of 2.5 mN force with 5 min
between intervals). After equilibration, KCl (60
mM) or acetylcholine (10 μM) was added to the
bath respectively and bronchial ring isometric
force was recorded using a data acquisition and
analysis program (Danish Myo Technology,
Aarhus, Denmark).

Histopathology—Lungs were infused with 4%
formalin through the tracheal cannula at a constant
pressure of 25 cm H₂O to inflation fix the lung.
Specimens were immersed in 4% formalin
overnight and dehydrated in a graded series of
ethanol solutions. Tissue was embedded in
paraffin. Sections were cut at 5 μm thickness and
mounted onto positively charged slides (APES).
Standard HE staining was performed. For
quantification of bronchial smooth muscle, the
thickness of the smooth muscle layer (the
transverse diameter) was measured from the
innermost edge to the outermost edge. The smooth
muscle layer thickness was assessed at four
predetermined bronchiole sites (12, 3, 6, and 9
o’clock) in at least ten bronchioles of similar size
(150–200 μm) on each slide (30). At the same
time, smooth muscle nuclei were counted and
normalized by mm² of muscle layer for trachea
and by the length of the muscle layer for
bronchioles.

Immunohistochemistry—Cryosections (10 μm)
of lung and trachea were fixed in ice-cold acetone
for 10 min and non-specific binding of primary
antibodies was blocked by incubation with PBS
containing 0.1% Triton X-100, 0.1% Tween-20,
1% BSA and 5% non-immune goat serum for 1 h.
Incubation was performed overnight with a mouse
monoclonal antibody to MLCK (K36, Sigma)
diluted 1:100, together with a rabbit monoclonal
α-smooth muscle actin antibody (clone 1A4,
1:100; Neomarkers). After washing in PBST,
the sections were incubated with a
FITC-conjugated goat anti-mouse antibody
(Sigma) diluted 1:200 and a Alexa Fluor
555-conjugated goat anti-rabbit antibody (Invitrogen) diluted 1:100 for 1 h, and after a final wash step, the sections were mounted with phosphate buffered glycerol, pH 7.4. Immunoreactivity was evaluated using a TCS-SP2 AOBS confocal laser scanning microscope (Leica, Heidelberg, Germany).

Western blot analysis—Western blot analyses were performed for measurement of MLCK and other protein expressions (31). Briefly, tissue samples were frozen quickly in 10% trichloroacetic acid and 10 mM dithiothreitol in acetone precooled to a slush at –80 °C. After homogenizing thoroughly, the sample pellet was washed three times with ether for 5 minutes each and dried to remove residual ether. The protein was dissolved completely in 8 M urea solution. Protein concentration was measured with bicinechonic acid protein assay reagent (BioRad). Equal amounts of protein were loaded for SDS-PAGE followed by protein transfer to a nitrocellulose membrane. The membrane was then probed with a monoclonal antibody to MLCK (K36, Sigma) and secondary antibody sequentially. The membrane was incubated in Super Signal West Dura substrate (Pierce) before exposure to film. Antibodies for other proteins included ILK (Sigma), total MYPT1 (Upstate), phospho-MYPT1[Thr-696] (Upstate), phospho-MYPT1[Thr-850] (Upstate), ZIP kinase (Sigma), ROCK II (Santa Cruz), sGC (Cayman Chemical) and PKG (Stressgen).

Measurement of myosin regulatory light chain phosphorylation—Urea/glycerol-PAGE electrophoresis was used for measurement of RLC phosphorylation where the nonphosphorylated RLC is separated from the monophosphorylated RLC (31). Trachea were excised, dissected free of surrounding tissues and then equilibrated for 30 min in the Krebs-Henseleit solution. The trachea were then incubated with 10 μM acetylcholine or 60 mM KCl for different times and transferred into 10% trichloroacetic acid and 10 mM dithiothreitol in acetone precooled at –80 °C to stop reactions. Tissues were homogenized, dissolved in sample buffer containing 8 M urea, 234 mM sucrose, 23 mM glycine, 10.4 mM dithiothreitol, 20 mM Tris (pH 8.6) and 0.01% bromphenol blue, and loaded into urea/glycerol-PAGE gels.

Immunization and airway challenge—Six- to eight-week-old female mice were sensitized to ovalbumin by i.p. injection of 80 μg OVA (Grade VI; Sigma-Aldrich) adsorbed to 4 mg of aluminum hydroxide (Inject Alum, Pierce, Rockford, IL) in a total volume of 0.2 ml of sterile saline on days 0 and 14. These mice were then challenged for 60 minutes to aerosolized 1% ovalbumin by ultrasonic nebulization on days 24, 25, and 26. Sham-immunized animals received saline-diluted aluminum hydroxide i.p. and aerosolized saline. From day 9 to day 13, both MLCKSMKO mice and control mice were injected with tamoxifen i.p. at a dose of 1 mg per day.

Airway responsiveness—Analysis of airway responsiveness to methacholine was performed as previously described (32). Briefly, on the day after the final aerosol challenge, airway responsiveness was measured noninvasively using a whole-body plethysmograph (model PLY 3211, Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by use of the enhanced pause variable (Penh) according to the following formula: $Penh = \frac{[(Te/RT) - 1] \times (PEF/PIF)}{Pe}$, where $Penh$ = enhanced pause (dimensionless), $Te$ = expiratory time, $RT$ = relaxation time, $PEF$ = peak expiratory flow (ml/s), and $PIF$ = peak inspiratory flow (ml/s) (33). Penh is a calculated parameter that reflects changes in the waveform of the measured box pressure signal and thus, the shape of the respiratory cycle. The average Penh over 3 min was
determined after 2 min exposure to aerosolized normal saline as a baseline. Aerosolized methacholine in increasing concentrations was nebulized for 3 min and the average Penh over 3 min was then determined. Results were expressed as the percentage increase of Penh following challenge with each concentration of methacholine.

**Whole-lung lavage**—Animals were injected i.p. with a lethal dose of pentobarbital (450 mg/kg). The trachea was cannulated, and the lung was then lavaged with 0.8 ml of PBS three times and the fluid pooled. Cells in the lavage fluid were counted by using a hemocytometer, and BAL cell differentials were determined on slide preparations stained with hematoxylin and eosin. At least 200 cells were differentiated by light microscopy based on conventional morphologic criteria. The levels of cytokine IL-4, IL-5 in lavage fluid and OVA-specific IgE in serum were determined using commercially available ELISA kits (ADL Biotech. and Dev. Co., USA) as per the manufacturer’s instructions.

**Statistical analysis**—Data are presented as the mean±SEM. Differences between groups were determined by Student’s t-test with significance at p<0.05.

**RESULTS**

**Ablation of MLCK expression in airway smooth muscle and phenotypic characterization of MLCKSMKO mice in C57BL/6 background**—A purified genetic background of knockout mice may reduce phenotypic variation in normal animals and animal models of disease. Therefore we back-crossed (129:B6) MLCKSMKO mice to C57BL/6 for six generations. Fig.1A shows a representative time-course for MLCK deletion in tracheal smooth muscle. At 16 days after starting injections of tamoxifen, only a small amount of MLCK protein was detected by Western blotting, while almost no MLCK protein was typically detected by day 20. Immunohistochemistry with co-staining by anti-MLCK and anti-SMA antibody confirmed the absence of MLCK in the trachea of MLCKSMKO mice at 16 days after tamoxifen (Fig.1B). To examine MLCK expression in mutant bronchial smooth muscle, immunohistochemistry was performed on lung sections. The results also showed an absence of MLCK protein in bronchial and lung smooth muscle at 16 days after tamoxifen (Fig.1C and D). Thus, we conclude that MLCKSMKO mice exhibit an efficient deletion of MLCK in airway smooth muscles.

Macrophenotypic analysis for MLCKSMKO mice in the B6 background showed very similar characteristics to mixed background mice as described previously, except a minor difference in the time-course to death after treatment with tamoxifen (17). Mice in the B6 background died at 20±1 days after tamoxifen treatment, whereas the original MLCKSMKO mice died at 17 days after induction. These different times appear consistent with the differences in MLCK protein deletion.

Our previous analysis of gut smooth muscle showed a significant hypertrophy of smooth muscle cells in MLCKSMKO mice (17). To determine whether the airway smooth muscle of MLCKSMKO mice also displays hypertrophy, histological morphometry of tracheal and bronchial smooth muscle was performed. The mutant trachea exhibited a comparable histological morphology relative to control trachea, and no hypertrophy was apparent by measuring the thickness of the muscle layer (157 ± 24 μm vs 160 ± 17 μm from control mice, p>0.05; n = 5 sections from 3 mice) or number of nuclei per mm² of smooth muscle (2885 ± 151 vs 2823 ± 166, p>0.05) (Fig.2A). Similarly, bronchial smooth
muscle thickness was comparable (5.14 ± 0.37 μm for control mice vs 5.03 ± 0.35 μm for MLCK<sup>SMKO</sup>, p>0.05; n = 7 sections from 3 mice) with a comparable number of cell nuclei per mm of muscle layer (41 ± 7 vs 43 ± 6 for control mice, p>0.05) (Fig. 2B). Thus, MLCK deletion in airway smooth muscle did not affect morphological properties.

**Reduced contraction of MLCK-deficient airway smooth muscle**—To determine the contractile properties of MLCK-deficient airway smooth muscle, we measured force development by tracheal and bronchial smooth muscles in response to KCl and muscarinic agonists and calculated stress values (force normalized to tissue cross sectional area). Tracheal rings isolated from MLCK-deficient mice developed an average 16.4% of the stress relative to control tissues in response to 60 mM KCl (Fig.3A, B and C). The responses to KCl with and without the muscarinic antagonist atropine were similar (data not shown), indicating KCl did not stimulate ACh release from parasympathetic nerve endings in tracheal tissues. The tracheal smooth muscle from MLCK-deleted mice developed an average 12.4% stress responses to 10 μM ACh compared to control muscles (Fig.3 A, B and C). Treatment with atropine abolished the ACh-induced contraction in smooth muscles, indicating a muscarinic-specific response (data not shown). These results clearly show that deletion of MLCK results in a significant reduction in responses to K<sup>+</sup>-depolarization and muscarinic receptor activation in tracheal smooth muscle.

Bronchial smooth muscle distributes evenly around the bronchus, and is important for producing airway resistance. We measured the contractile responses of bronchial rings in response to KCl and ACh. Greater force responses were obtained in bronchial rings from control mice versus MLCK-deficient mice in response to KCl (Fig.3 D, E and F). Bronchial smooth muscles from knockout mice displayed only 15% force relative to control bronchioles. Similarly, the bronchial smooth muscle rings from MLCK<sup>SMKO</sup> mice developed only 15% of the stresses developed by control tissue in response to ACh (Fig.3 D, E and F). Thus, MLCK depletion also leads to impairment of bronchial smooth muscle contraction in response to KCl and agonist. Due to the similar contractile responses of trachea and bronchial smooth muscles, we used tracheal smooth muscle for our subsequent biochemical analyses.

**RLC phosphorylation responses were inhibited in MLCK<sup>SMKO</sup> tracheal smooth muscles**—Our previous results showed that MLCK-catalyzed myosin light chain phosphorylation is required for phasic smooth muscle contraction (17). To assess the functional contribution of MLCK-mediated RLC phosphorylation in a tonic contraction of tracheal smooth muscle, we measured RLC phosphorylation in response to KCl and ACh. In control tracheal muscle, RLC phosphorylation increased from a resting value of 1.1% to 25.2% by 20 sec after KCl stimulation and then remained elevated; in MLCK-deficient tracheal muscle, RLC phosphorylation increased from 0.8% to only 5.0% after KCl stimulation (Fig.4A and B). With ACh-induced contraction, RLC phosphorylation increased to 28.0% at 20 sec in control tracheal tissues (Fig.4 C and D). In MLCK<sup>SMKO</sup> trachea, however, RLC was phosphorylated only to 4.3% by 20 sec after ACh stimulation. No diphosphorylated RLC was detected in mutant or control trachea. Importantly, there were no additional increases in RLC phosphorylation with a contraction sustained for up to 300 sec (Fig.4 C and D). These results are consistent with the marked attenuation of force development in tracheal muscles from MLCK<sup>SMKO</sup> mice by KCl and agonist stimulation. Thus, MLCK appears to be the primary kinase that
phosphorylates RLC in response to both K⁺-depolarization and muscarinic receptor activation. These results show that MLCK-mediated RLC phosphorylation is important for both the initial and sustained contractile responses of airway smooth muscle. 

The residual contraction of MLCK-deleted trachea is calcium-dependent and blebbistatin-sensitive, but less sensitive to acetylcholine. There were small contractile and RLC phosphorylation responses to KCl and agonists in MLCK-deficient airway smooth muscle. To understand the production of residual contraction, we determined the properties in terms of sensitivity to ACh and dependency on calcium. As the concentration of ACh cumulatively increased up to 3 uM, MLCK-deficient tracheal smooth muscle started to contract, reaching a maximum response at 100 uM. In contrast, tracheal from control animals started to contract 0.1 uM ACh, developing a maximum contractile response at 10 uM (Fig.5A and C). The EC50 values for ACh were 5.4 ± 2.8 (control; n=7) and 14.5 ± 3.3 uM (MLCK SMKO; n=5), respectively, showing that tracheal smooth muscles from MLCK SMKO mice have significantly lower sensitivity to ACh (p<0.01).

To determine if the small contraction of MLCK-deleted tracheal muscle is Ca²⁺-dependent, we depleted intracellular Ca²⁺ by repeated ACh applications in the presence of EGTA. Results showed that Ca²⁺-depletion inhibited the robust contractile response in control trachea as well as the smaller contraction in MLCK SMKO trachea (Fig.5B and D). Thus, the small contraction induced by ACh in MLCK SMKO tonic muscle is Ca²⁺-dependent.

We also determined the inhibitory effect of the selective myosin II inhibitor blebbistatin, (34-35). Results showed that addition of 30 uM blebbistatin inhibited both the robust (KO: 34 ± 18%, n=3; CTR: 14 ± 8%, n=5) and sustained (KO: 81 ± 10%, n=3; CTR: 37%, n=5) contractile response to KCl in the MLCK SMKO trachea as well as control trachea (Fig.5E and F). The higher percent inhibition in mutant trachea might be due to the smaller amount of force or the atypical contraction waveform. Thus, the small contraction in MLCK SMKO tonic muscle is sensitive to blebbistatin.

Normal cGMP/PKG and RhoA/ROCK signaling in trachea from MLCK SMKO mice—Reduced contractile responses may be contributed by decreased Rho/ROCK signaling or by increased cGMP/PKG signaling (5). To rule out possible compensatory effects during the contraction response of MLCK-deleted trachea, we measured relative amounts of key proteins in these signaling modules (17,36). Compared with control muscle, the knockout tracheal smooth muscle had a comparable expression level of ILK, ROCK II, MYPT1 and ZIP kinase (Fig.6A). Phosphorylation responses for MYPT1 (MYPT1-p696 and MYPT1-p850) to agonist were not altered in MLCK-deficient tracheae compared with controls (Fig.6B). cGMP/PKG signaling may affect smooth muscle contraction so we measured sGC and PKG amounts (31). Results showed a comparable expression of sGC and PKG in the trachea from the knockout vs control mice (Fig.6C). These observations indicate normal amounts of key proteins involved in the cGMP/PKG and RhoA/ROCK pathways in tracheae from MLCK SMKO mice.

Deletion of MLCK abolished physiological and asthmatic bronchial constriction—To assess the effect of MLCK deletion on the respiratory system under physiological conditions, we measured airway constriction and other respiratory parameters in a whole-body plethysmograph with inhalation of increasing amounts of the muscarinic agonist, methacholine. MLCK SMKO mice
appeared to exhibit less change in airway resistance as estimated by the enhanced pause variable (Penh) compared to control mice (p<0.01) (Fig.7A). The time of inspiration of knockout mice was reduced significantly (p<0.05) and the minute volume became larger (Fig.7 B and E). The time of expiration and tidal volume showed no significant difference (p>0.05) (Fig.7C and D).

To determine whether the absence of MLCK activity protected against the development of asthmatic airway constriction associated with allergen-induced airway hyper-reactivity, MLCK$^{SMKO}$ mice were sensitized to ovalbumin and then challenged with aerosol ovalbumin. Airway hyper-reactivity was then assessed by measurements of respiratory parameters as above. Whereas the ovalbumin-immunized control mice demonstrated robust airway hyper-reactivity, exhibited by much stronger airway constriction than non-immunized control mice, ovalbumin-immunized MLCK$^{SMKO}$ mice showed no signs of airway hyper-reactivity (p>0.05), even when challenged with a high concentration of methacholine (Fig.7A). Taken together, our results show that deletion of MLCK abolishes airway constriction under both physiological and asthmatic conditions.

Deletion of MLCK did not affect allergic inflammation in asthmatic airway—To determine whether the abolishment of airway constriction is caused by a failure of allergic sensitization, and whether the reduced constriction affects inflammation intensity, we examined lungs histologically and measured profiles of inflammatory cells and cytokines in lungs from control and MLCK$^{SMKO}$ mice. Following sensitization and challenge with ovalbumin, both control and MLCK$^{SMKO}$ mice showed significant inflammation in lung tissue with dense peribronchiolar and perivascular infiltrates consisting of lymphocytes, eosinophils, and neutrophils (Fig.8 and Fig.9A). Quantification for cellular composition shows about 30% of eosinophils in broncho-alveolar fluid from both asthmatic control and MLCK$^{SMKO}$ mice, suggesting a typical pattern of asthmatic inflammation cells. In contrast to non-sensitized mice, the sensitized mice exhibited significantly higher levels of ovalbumin-specific IgE, IL-4 and IL-5 (Fig. 9B and C), showing a typical immune response of asthmatic inflammation. No differences were observed between knockout and control groups (p>0.05). In conclusion, our results show that the allergic inflammation of the asthmatic airway is similar in control and MLCK-deficient mice.

**DISCUSSION**

Ca$^{2+}$/calmodulin-dependent MLCK was originally proposed as an essential initiator of smooth muscle contraction because its phosphorylation of RLC activated myosin (10,37). A simple cascade of biochemical reactions starting with Ca$^{2+}$ binding to calmodulin, activation of MLCK, RLC phosphorylation and force development for smooth muscle contraction was developed. A decrease in [Ca$^{2+}$], and inactivation of MLCK allows RLC dephosphorylation and relaxation. However, more elaborate signaling modules involving regulation of MLCP activity emerged with additional studies. GPCR activation of RhoA leads to phosphorylation of the regulatory subunit of MLCP, MYPT1, by Ca$^{2+}$-independent kinases leading to inhibition of its phosphatase activity (5-7,22). CPI-17 phosphorylated by PKC may also inhibit MLCP activity. Additionally, Ca$^{2+}$-independent kinases have been proposed to phosphorylate RLC directly. The relative physiological importance of these different signaling proteins is difficult to appreciate. Genetic approaches offer tools to
unravel complexities in defining signaling modules in physiological responses and pathophysiological developments. We have thus used a conditional knockout of MLCK in adult smooth muscle to define its function in airway physiology and pathological airway constrictions associated with asthma.

MLCK appears to play a central role in smooth muscle contractile responses. MLCK is clearly important in phasic smooth muscle contraction because its deletion significantly impairs transient force development and RLC phosphorylation (17). RLC phosphorylation and force are sustained in tonic smooth muscle contractions such as airway smooth muscle (7,38). Many studies suggest that the sustained RLC phosphorylation may be due to contributions from other imposing signaling pathways involving primarily inhibition of MLCP by activation of PKC and RhoA/ROCK pathways (7,12-14) but also Ca²⁺/CaM-independent kinases that act on both RLC and MLCP (5,7,19). Biochemical measurements show lower activities of MLCK and MLCP in tonic smooth muscles, with greater amounts of proteins in the RhoA/ROCK signaling module that could inhibit MLCP activity compared with phasic smooth muscle (39-40). Others predict a potentially important role of Ca²⁺-independent regulation acting directly to phosphorylate RLC (5,7,11,15,41). However, we find that deletion of Ca²⁺/calmodulin-dependent MLCK in tonic airway smooth muscle greatly attenuated RLC phosphorylation as well as force development initially and during the sustained contraction phase with both depolarization and muscarinic receptor-induced contraction. Thus, MLCK and its phosphorylation of RLC play an essential role in airway smooth muscle contraction and most likely other tonic smooth muscles. Furthermore, inhibition of MLCP by phosphorylation of its regulatory subunit MYPT1 by itself appears insufficient for robust RLC phosphorylation and force development without MLCK activity. However, while these studies indicate MLCK plays a central role as the kinase that directly phosphorylates RLC, the results do not diminish the documented important role for inhibition of myosin phosphatase activity in promoting Ca²⁺-sensitization.

Our results do not exclude the importance of MYPT1 phosphorylation in regulating MLCP activity during a contraction, because the function of MLCP will be dependent on RLC phosphorylation catalyzed by MLCK. Ca²⁺-dependency of the residual force developed by airway smooth muscle after MLCK gene ablation may be related to residual MLCK in which only a small fraction is necessary for sufficient RLC phosphorylation (31). Importantly, we could not find evidence that Ca²⁺-independent kinase activity towards direct phosphorylation of RLC is involved in sustained contractions.

We examined the inhibitory effect of blebbistatin on the MLCK-KO muscle, and found that the small contraction could be inhibited. Due to the small amount of force for residual contraction and atypical waveform of contraction, we could not determine if the small contraction was more sensitive to blebbistatin, thereby indicating a primary contribution of non-muscle myosin II. However, we expect that MLCK also catalyzes non-muscle myosin phosphorylation if it contributes to force development in the tonic contraction (42). Blebbistatin is an effective inhibitor of smooth muscle actomyosin and smooth muscle contraction from mammals and chicken arteries whereas it is not as effective for inhibiting gizzard smooth muscle contractions, consistent with the original report that blebbistatin was not effective in inhibiting avian gizzard actomyosin ATPase activity (34).

The importance of MLCK in airway smooth
muscle function is strongly supported by the phenotypes from measurements of airway respiratory resistance in knockout mice where smooth muscle appears critical for airway constriction. Many external signals can invoke airway smooth muscle contraction through different signaling modules in which MLCK may serve as focal point, and hence control airway constriction (43). The loss-of-function evidence resulting from deletion of MLCK that caused almost complete abolishment of both physiological and asthmatic airway constriction clearly defines an essential role of MLCK in airway contraction. Moreover, as the knockout mice displayed an alteration in breathing pattern with shorter inspirations and larger minute volumes compared with measurements from control mice, it appears that MLCK-mediated airway smooth muscle contraction could influence the breathing pattern as effectors of the pulmonary stretch reflex (44). Thus, relaxed airway smooth muscle may lead to reduced respiratory resistance and hence a quicker inspiration; however, the air in the lung is not dispelled efficiently without contraction, resulting in a larger minute volume. Therefore, we propose that airway smooth muscle regulates airflow turnover in a physiological breath by controlling air resistance.

Asthma is a disease characterized by chronic airway inflammation, airway hyper-reactivity and airway remodeling, resulting in high airway constriction and episodic airway obstruction (45). The excessive contraction of airway smooth muscle is a primary contributing determinant for asthmatic airway constriction along with airway inflammation (46). Current treatments for asthma involving cholinergic, histaminergic, and leukotriene antagonists as well as anti-immune modulation and anti-inflammation approaches have provided limited successes and thus, asthma therapeutics continues to be an important area of clinical investigation (47). As asthma is accompanied by abnormal airway smooth muscle contraction in response to a wide variety of excitatory stimuli, specific inhibition of MLCK represents a potential therapeutic approach to treating symptoms.

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FOOTNOTES

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Abbreviations are: GPCR, G-protein coupled receptors’ MLCK, myosin light chain kinase; RLC, regulatory light chain; MLCP, myosin light chain phosphatase; ROCK, Rho kinase; ILK, integrin-linked protein kinase; MYPT1, myosin targeting subunit of myosin light chain phosphatase; SMA, smooth muscle actin; PKG, cGMP-dependent protein kinase; ACh, acetylcholine; OVA, ovalbumin.

FIGURE LEGENDS

**FIGURE 1. Targeted disruption of Mlck gene in airway smooth muscle.** A, Western blots of MLCK in tracheae from tamoxifen-treated mice collected at indicated days (d) after treatment. Total actin stained with Coomassie Brilliant Blue G-250 was used as protein loading control. Immunofluorescence analysis of MLCK expression in tracheal (B) and lung (C and D) smooth muscle from control (CTR) and MLCK<sup>SMKO</sup> mice. At the 16th day after tamoxifen injection, the trachea and lung of MLCK-deficient mice and control were embedded in OCT and sectioned. After acetone fixation, the frozen sections were stained with K36 antibody and α-smooth muscle actin antibody. Scale bars in panels B and C and D are 80 and 100 and 200μm, respectively.

**FIGURE 2. Histological analysis of MLCK-deficient airway smooth muscle.** Fresh tracheal and lung tissues from control (CTR) and MLCK<sup>SMKO</sup> knockout mice were fixed with 4% formalin and dehydrated in a graded series of ethanol solutions followed by standard paraffin section and HE staining. Whole image of tracheal histology (left part of panel A) was made by merging images from different
fields (marker measures 160 μm). Magnification for tracheal smooth muscle layer is shown in right part of panel A with a scale bar of 40 μm. Panel B represents histology of bronchiole in lung tissues (scale bar = 50 μm) and the magnification for the insert frame is shown in right part of panel B (scale bar = 15 μm). The arrows indicate smooth muscle cells.

FIGURE 3. Reduced contractions of tracheal and bronchial smooth muscle from MLCK\textsuperscript{SMKO} mice. Representative recordings of tracheal ring contraction from control (CTR) (A) and MLCK\textsuperscript{SMKO} (B) mice treated with 60 mM KCl or 10 uM ACh. Bars show duration of stimulation. Quantification of contraction responses to KCl and ACh is summarized (C). Columns represent means±SEM, N=4-10, **p<0.01. Representative recordings of bronchial ring contraction from CTR (D) and MLCK\textsuperscript{SMKO} (E) mice treated with 60 mM KCl or 10 uM ACh are shown. Quantification of bronchial contraction responses to KCl and ACh are shown (F). Columns represent means±SEM, N=4-6, **p<0.01.

FIGURE 4. Inhibition of RLC phosphorylation in tracheal smooth muscle from MLCK\textsuperscript{SMKO} mice. RLC phosphorylation (RLCp) was measured in quick-frozen tracheal rings from control and MLCK\textsuperscript{SMKO} mice treated with 60 mM KCl (A and B) or 10 μM ACh (C and D) as shown by representative Western blots of glycerol/urea PAGE gels and quantification. Bars represent means±SEM, N=4-6, **p<0.01.

FIGURE 5. Ca\textsuperscript{2+}-dependence of small contractions of MLCK-deficient trachea. Sensitivity to ACh of MLCK-deficient and control (CTR) trachea was assessed by isometric force measurements in response to cumulative increases in the concentration of ACh (A), and expressed as percent of maximal force with ACh (C). Arrows indicate the point at which the concentration of acetylcholine was increased; means ±SEM from 7 control (CTR) and 5 MLCK\textsuperscript{SMKO} tracheal rings are shown, * P<0.05, ** P< 0.01. Panels B and D show the effects of Ca\textsuperscript{2+}-depletion on tracheal smooth muscle contraction. Depletion of Ca\textsuperscript{2+} in CTR and MLCK\textsuperscript{SMKO} tracheal smooth muscle by adding 1 mM EGTA in the bath solution inhibited the contractile responses to repeated exposure to 10 μM ACh (B). Quantification of contractile responses by CTR and MLCK\textsuperscript{SMKO} muscles with Ca\textsuperscript{2+} depletion and normalized to pre-treatment values (D). Values are means±SEM (N=3-4). Panels E and F represent a typical inhibitory effect of blebbistatin on tracheal smooth muscle contraction. With or without addition of 30μM blebbistatin to the incubation buffer, the isometric force of control (E) and MLCK-deficient (F) trachea developed with 80 mM KCl was measured. Each measurement was repeated in 3 to 5 independent animals.

FIGURE 6. Expression of contractile and related regulatory proteins in tracheae from MLCK\textsuperscript{SMKO} mice. (A) Western blots for ILK, ZIP kinase, ROCK II and MYPT-1 expression in tracheal tissues from control (CTR) and MLCK\textsuperscript{SMKO} mice. The samples were resolved by separate SDS-PAGE and β-actin was stained with anti-actin antibody as a loading control. (B) MYPT1 phosphorylation at residues 696 and 850 in response to 10 μM ACh were measured by anti-MYPT1-P696 and anti-MYPT1-P850 antibodies. (C) Western blots for PKG and sGC expression in CTR and mutant tracheal tissues. Blots shown are representative of at least three measurements.
FIGURE 7. Decreased airway respiratory resistance and altered breathing pattern in MLCK-deficient mice. (A) MLCK<sup>SMKO</sup> and control (CTR) mice were immunized and challenged by OVA. On the day after the final aerosol challenge, airway responsiveness was measured noninvasively using whole-body plethysmography. Data are expressed as percent above baseline. At the same time, some respiratory parameters such as Ti (B), Te (C), Tv (D) and Mv (E) at baseline were analyzed to compare the differences between the MLCK<sup>SMKO</sup> and control mice. Ti = Time of inspiration; Te = Time of expiration; Tv= Tidal volume; Mv = Minute volume. Values are means ± SEM (N=6). * p< 0.05, ** p< 0.01, as compared to same condition without asthma.

FIGURE 8. Histopathology and inflammatory cells of lungs from control (CTR) and MLCK<sup>SMKO</sup> mice sensitized to ovalbumin. Two days after the last challenge with ovalbumin, the mice were anesthetized and killed. The lung and the bronchoalveolar fluid from mice were collected. (A) Histological examination (hematoxylin and eosin (HE) staining) of lung tissue from CTR mice (upper panels) shows dense perivascular and peribronchiolar eosinophils as indicated by arrows. Lower panels show the histology of lung from MLCK<sup>SMKO</sup> mice. The mutant lung shows similar histopathologic changes. Scale bar in left panels is 60 μm; scale bar in right panels is 20 μm. (B) The total number of macrophages, eosinophils, lymphocytes and neutrophils in the bronchoalveolar fluid were determined by counting HE stained cells. Dotted bars, CTR/Saline; white bars, MLCK<sup>SMKO</sup>/Saline; hatched bars, CTR/OVA; black bars, MLCK<sup>SMKO</sup>/OVA. Eos = eosinophils; Lym = lymphocytes; Mac = macrophages; Neu = neutrophils. Values are means ± SEM (N=6).

FIGURE 9. Immunological responses of ovalbumin-sensitized control (CTR) and MLCK<sup>SMKO</sup> mice. After the last challenge with ovalbumin, the bronchoalveolar fluids of mice were collected. OVA-specific IgE in serum (A) and cytokines IL-4 (B), IL-5 (C) in bronchoalveolar fluid from the mice were measured by ELISA assay according to manufacturer’s instructions. Values are means ± SEM (N=6). ** p< 0.01
Figure 1
Figure 2

A
CTR

MLCK<sub>SMKO</sub>

B
CTR

MLCK<sub>SMKO</sub>
Figure 3
Figure 4

A  

RLC  RLCp  CTR  MLCK<sup>SMKO</sup>  

0  10  20  40  60  120  300 (s)  

KCl  

B  

%Phosphorylation  

CTR  MLCK<sup>SMKO</sup>  

Time (s)  

C  

RLC  RLCp  CTR  MLCK<sup>SMKO</sup>  

0  10  20  40  60  120  300 (s)  

ACh  

D  

%Phosphorylation  

CTR  MLCK<sup>SMKO</sup>  

Time (s)
Figure 6

A

|       | ILK | ZIP kinase | ROCK II | MYPT1 |
|-------|-----|------------|---------|--------|
| CTR   |     |            |         |        |
| KO    |     |            |         |        |

actin

B

|       | CTR | KO |
|-------|-----|----|
| 0     |     |    |
| 10    |     |    |

(min) Ach

MYPT1-p696
MYPT1-p850
Total MYPT1

C

|       | sGC | PKG |
|-------|-----|-----|
| CTR   |     |     |
| KO    |     |     |

actin
Figure 7

A

% Change in Penh

CTR  KO  CTR+asthma  KO+asthma

B

Ti (s)

CTR  MLCK^{SMKO}

C

Te (s)

CTR  MLCK^{SMKO}

D

Tv (mL)

CTR  MLCK^{SMKO}

E

Mv (mL)

CTR  MLCK^{SMKO}
Figure 8

A

CTR

MLCK\textsuperscript{SMKO}

B

\begin{align*}
\text{Cell number} \times 10^3 \\
\hline
\text{CTR} & \text{MLCK}\textsuperscript{SMKO} & \text{CTR+asthma} & \text{MLCK}\textsuperscript{SMKO+asthma} \\
\hline
\text{Total} & \text{Mac} & \text{Lym} & \text{Eos} & \text{Neu} \\
\hline
\end{align*}
Figure 9

A

Serum anti-OVA IgE (IU/L)

Sal  OVA  Sal  OVA
CTR   MLCK^{SMKO}

B

BALF IL-4 (pg/mL)

Sal  OVA  Sal  OVA
CTR   MLCK^{SMKO}

C

BALF IL-5 (pg/mL)

Sal  OVA  Sal  OVA
CTR   MLCK^{SMKO}
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