

Accepted Manuscript

Inflammatory Mediators Mediate Airway Smooth Muscle Contraction through a GPCR-TMEM16A-VDCC Axis and Contribute to Bronchial Hyperresponsiveness in Asthma

Pei Wang, PhD, Wei Zhao, Bachelor of Medicine, Jie Sun, BS, Tao Tao, BS, Xin Chen, PhD, Yan-Yan Zheng, MS, Cheng-Hai Zhang, PhD, Zhong Chen, MS, Yun-Qian Gao, PhD, Fan She, BS, Ye-Qiong Li, BE, Li-Sha Wei, BS, Ping Lu, PhD, Cai-Ping Chen, PhD, Ji Zhou, Master of Medicine, Da-Quan Wang, Master of Medicine, Liang Chen, MD, Xiao-Hao Shi, PhD, Linhong Deng, PhD, Ronghua ZhuGe, PhD, Hua-Qun Chen, PhD, Min-Sheng Zhu, PhD

PII: S0091-6749(17)31167-3

DOI: [10.1016/j.jaci.2017.05.053](https://doi.org/10.1016/j.jaci.2017.05.053)

Reference: YMAI 12929

To appear in: *Journal of Allergy and Clinical Immunology*

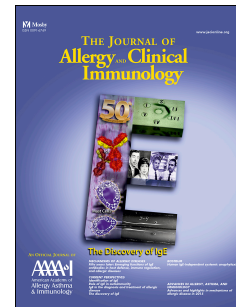
Received Date: 9 February 2017

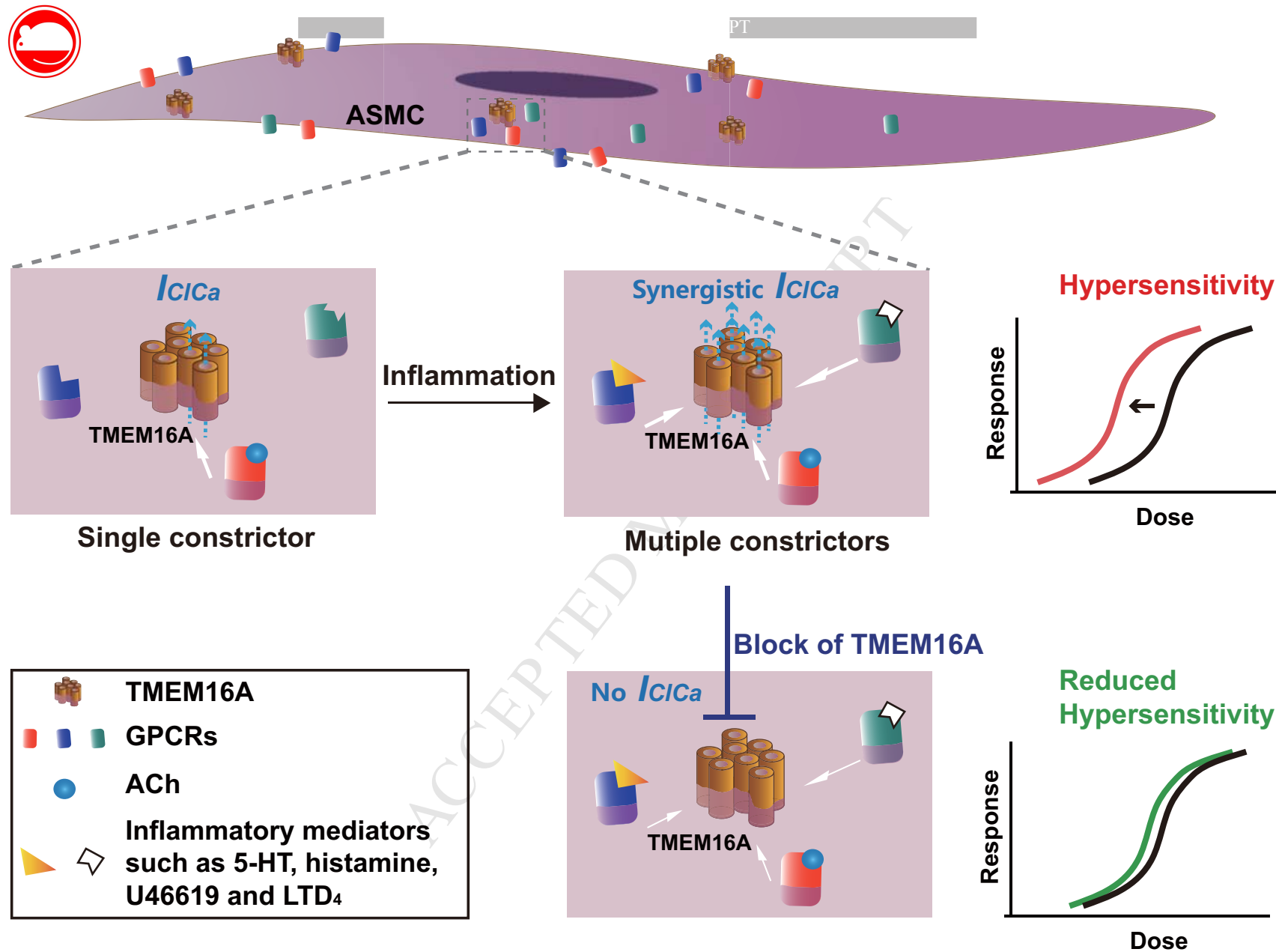
Revised Date: 27 April 2017

Accepted Date: 19 May 2017

Please cite this article as: Wang P, Zhao W, Sun J, Tao T, Chen X, Zheng Y-Y, Zhang C-H, Chen Z, Gao Y-Q, She F, Li Y-Q, Wei L-S, Lu P, Chen C-P, Zhou J, Wang D-Q, Chen L, Shi X-H, Deng L, ZhuGe R, Chen H-Q, Zhu M-S, Inflammatory Mediators Mediate Airway Smooth Muscle Contraction through a GPCR-TMEM16A-VDCC Axis and Contribute to Bronchial Hyperresponsiveness in Asthma, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.05.053.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.





5-HT: 5-hydroxytryptamine; ACh: Acetylcholine; ASMC: Airway smooth muscle cell; GPCRs: G-protein-coupled receptors; I_{ClCa} : Ca^{2+} -activated Cl^- current; LTD₄: Leukotriene D₄

Inflammatory Mediators Mediate Airway Smooth Muscle Contraction through a GPCR-TMEM16A-VDCC Axis and Contribute to Bronchial Hyperresponsiveness in Asthma

Pei Wang PhD^{a,*}, Wei Zhao Bachelor of Medicine^{a,*}, Jie Sun BS^a, Tao Tao BS^a, Xin Chen PhD^a, Yan-Yan Zheng MS^{a,b}, Cheng-Hai Zhang PhD^a, Zhong Chen MS^a, Yun-Qian Gao PhD^a, Fan She BS^a, Ye-Qiong Li BE^a, Li-Sha Wei BS^a, Ping Lu PhD^c, Cai-Ping Chen PhD^{a,d}, Ji Zhou Master of Medicine^e, Da-Quan Wang Master of Medicine^f, Liang Chen MD^f, Xiao-Hao Shi PhD^g, Linhong Deng PhD^g, Ronghua ZhuGe PhD^c, Hua-Qun Chen PhD^{b,#}, Min-Sheng Zhu PhD^{a, h,#}

^a Model Animal Research Center and MOE Key Laboratory of Model Animal for Disease Study and the Medical School of Nanjing University, Nanjing 210061, China.

^b College of Life Science, Nanjing Normal University, Nanjing 210023, China.

^c Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA.

^d Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases, Center of Drug Discovery, China Pharmaceutical University, Nanjing 210009, China.

^e Department of Respiratory, Jiangsu Province Hospital, Nanjing 210029, China;

^f Department of Thoracic and Cardiovascular Surgery, Jiangsu Province Hospital, Nanjing 210029, China;

^g Institute of Biomedical Engineering and Health Sciences, Changzhou University, Changzhou 213164, China.

^h Innovation Center for Cardiovascular Disorders, Beijing 100029, China.

* These authors contributed equally to this work.

[#] **Address for correspondence:** Min-Sheng Zhu, Ph.D., Model Animal Research Center, Nanjing University, Nanjing 210061, China. E-mail: zhums@nju.edu.cn; or to Hua-Qun Chen, Ph.D., College of Life Science, Nanjing Normal University, Nanjing 210023, China. E-mail: chenhuaqun@njnu.edu.cn.

Funding: This work was supported by the National Natural Science Foundation of China (31330034 to M.-S. Z., 31371356/30971540 to H.-Q. C., 31501182 to C.-P.C. and 11532003 to L. D.) and Jiangsu Major Nature Science Foundation of High Education (13KJA180004) and National Key Scientific Research Program of China (973 program) Grants 2014CB964701.

Author contribution: P. W., W. Z., R. Z. G., H.-Q. C and M.-S. Z. designed research; P. W., W. Z., J. S., T. T., X. C., Z. C. and Y.-Y. Z. performed research; Y.-Q. G., C. Z., C.-P.C., F. S., Y.-Q. L. and L.-S. W. contributed new reagents/analytic tools; J. Z., D.-Q. W. and L. C. collected human bronchial smooth muscle samples; X.-H. S. and L. D. provided technical assistance of respiratory function measurements; P. W., W. Z. and M.-S. Z. analyzed data; P. W., R. Z. G., H.-Q. C. and M.-S. Z. wrote the paper.

Acknowledgments: The authors would like to thank Dr. Lawrence M Lifshitz for critical reading of the manuscript and constructive comments; Dr. Min-Min Luo for advices on Ca^{2+} signal measurement and the donation of Ai38D mice.

Competing financial interests: The authors declare no competing financial interests.

Abstract

Background: Allergic inflammation has long been implicated in asthmatic hyperresponsiveness of airway smooth muscle (ASM), but its mechanism underlying remains incompletely understood. Serving as GPCR agonists, several inflammatory mediators can induce membrane depolarization, contract ASM and augment cholinergic contractile response. We hypothesized that the signal cascade integrating on membrane depolarization by the mediators might involve asthmatic hyperresponsiveness.

Objective: We sought to investigate the signalling transduction of inflammatory mediators in ASM contraction and assess its contribution in the genesis of hyperresponsiveness.

Methods: We assessed the capacity of inflammatory mediators to induce depolarization currents by electrophysiological analysis. We analysed the phenotypes of TMEM16A knockout mice, applied pharmacological reagents and measured the Ca^{2+} signal during ASM contraction. To study the role of the depolarization signalling in asthmatic hyperresponsiveness, we measured the synergistic contraction by acetylcholine and inflammatory mediators both *ex vivo* and in OVA-induced mouse model

Results: Inflammatory mediators, such as 5-HT, histamine, U46619 and LTD_4 , are capable of inducing Cl_{Ca} currents in ASM cells, and these currents are mediated by TMEM16A. A combination of multiple analysis revealed that a

GPCR-TMEM16A-VDCC signalling axis was required for ASM contraction induced by inflammatory mediators. Block of TMEM16A activity may significantly inhibit the synergistic contraction of acetylcholine and the mediators, and hence reduces hypersensitivity.

Conclusions: A GPCR-TMEM16A-VDCC axis contributes to inflammatory mediator-induced ASM contraction, and synergistically activating TMEM16A by allergic inflammatory mediators with cholinergic stimuli.

Key Messages

•TMEM16A activation depolarizes the membrane of ASM, and causes calcium influx and contraction, which confers the airway to sensitively respond to several inflammatory mediators and cholinergic stimuli.

•Synergistic activation of TMEM16A by allergenic inflammatory mediators and cholinergic transmitter contributes to asthmatic hyperresponsiveness.

Capsule Summary

The GPCR-TMEM16A-VDCC signalling axis plays important role in inflammatory mediator-induced ASM contraction and hypersensitivity. TMEM16A may be a prospective drug target of asthma.

Key words: asthma, airway smooth muscle, inflammatory mediator, TMEM16A, hypersensitivity.

Abbreviation: ASM, airway smooth muscle; ASMC, airway smooth muscle cell; Cl_{Ca} , Ca^{2+} -activated Cl^- ; GPCR, G protein-coupled receptor; IT- Cl , IP_3 /TMEM16A-mediated calcium influx; MLCK, myosin light chain kinase; PLC, phospholipase C; $ROCl$, receptor-operated Ca^{2+} influx; Rrs, Resistance of the respiratory system; $SOCl$, store-operated Ca^{2+} influx; SR, sarcoplasmic reticulum; TMEM16A, transmembrane protein 16A; T_XA_2 , thromboxane A_2 ; VDCC, voltage dependent Ca^{2+} channel; $VOCl$, voltage-operated Ca^{2+} influx;

92

93 **Words count:** abstract: 229 words, manuscript text: 3513 words.

ACCEPTED MANUSCRIPT

94 INTRODUCTION

95 Mammalian airways are innervated by cholinergic nerve fibres, which regulate *in vivo* airway
96 resistance¹. In asthmatic airways, the smooth muscle tissue may be infiltrated with
97 inflammatory cells, which produce a large amount of cytokines, chemokines, growth factors,
98 and inflammatory mediators^{2,3}. These inflammatory factors may affect multiple processes
99 in asthmatic pathology. Interestingly, in addition to mediating immunological responses,
100 some mediators, such as histamine⁴, 5-HT⁵, prostanoids^{6,7} and cysteinyl leukotrienes
101 (CysLTs)⁸, also directly evoke the contraction of airway smooth muscle cells (ASMCs)⁹.
102 Furthermore, the existence of inflammatory mediators also make ASM more sensitive to other
103 stimuli through a synergistic or augmentation effect^{10,11}, such a sensitivity is one of the
104 essential factors for the formation of asthmatic hyperresponsiveness. The mechanisms by
105 which these mediators regulate smooth muscle contraction and sensitize ASM are
106 incompletely clear. Previous reports have shown that these mediators may bind to their
107 corresponding G protein-coupled receptors (GPCRs)¹² and thus activate G_q/G_i heterotrimeric
108 G proteins, which cause calcium release from the endoplasmic reticulum (ER) through
109 phospholipase C (PLC)/inositol 1,4,5-triphosphate (IP₃) signaling^{13,14}. In smooth muscle
110 cells, the activation of this signalling cascade may lead to calcium release from the
111 sarcoplasmic reticulum (SR), which is required for myosin light chain kinase (MLCK)
112 activation and the initiation of contraction. There are observations implying that, in addition
113 to IP₃ induced Ca²⁺ release, other Ca²⁺ sources may also involve in the contraction of
114 inflammatory mediators, e.g. histamine causes lower phosphoinositide hydrolysis than
115 cholinergic agents but show a comparable capacity to induce contraction^{15,16}; SKF96365, a

non-selective antagonist of Ca^{2+} channels markedly inhibit ASM contraction induced by many different inflammatory mediators^{17, 18}. Three patterns of Ca^{2+} influx, including receptor-operated Ca^{2+} influx (ROCI), store-operated Ca^{2+} influx (SOCI) and voltage-operated Ca^{2+} influx (VOCI) have been implicated in airway smooth muscle contraction¹⁹. As ASM abundantly expresses TMEM16A, a key molecule in Ca^{2+} -activated Cl^- (Cl_{Ca}) channels, we speculate an essential role of VOCI in the contraction of mediators, because Cl^- channels can mediate depolarization currents in smooth muscle cells²⁰.

TMEM16A is a member of the Cl_{Ca} channel family and is expressed in many different tissues²¹. In smooth muscle cells, TMEM16A expression varies according to the tissue origin, e.g., it is abundantly expressed in the airway^{22, 23}, modestly expressed in vascular vessels²⁴, and expressed at low levels in the gut²². TMEM16A activation is sensitive to $[\text{Ca}^{2+}]_{\text{i}}$ ²⁵, by mediating VOCI, which may serve as a sensitive Ca^{2+} signal amplifier to participate in contraction in smooth muscle cells. In ASM and internal anal sphincter smooth muscle, even the intrinsic calcium spark may activate TMEM16A to a certain extent, or even induce contraction^{26, 27, 28}. In this report, we demonstrated that some asthmatic inflammatory mediators may efficiently activate TMEM16A and thereby mediate ASM contraction. These contractile properties of ASM may be conferred by the abundant expression of TMEM16A and mediator receptors. Moreover, we suggest that inflammatory mediator-mediated contraction is regulated by a GPCR/ IP_3 / Ca^{2+} /TMEM16A/VDCC signalling cascade, and this cascade may serve as a sensitive mechanism by which ASM responds to physiological and pathological stimuli. Our results emphasize the importance of inflammatory mediators in some types of asthmatic hyperresponsiveness.

METHODS

Animals

The animal experiments performed in this study were conducted in accordance with the guideline of the Animal Care and Use Committee of the Model Animal Research Center of Nanjing University (Nanjing, China). Establishment of the floxed allele of *Tmem16a* in mice has been described previously²⁷. Hartley guinea pigs were used in some tests, because histamine and LTD₄ are not potent bronchoconstrictors in mice^{4,29}, while guinea pig ASM shows the same response as the case with humans³⁰.

Western blot analysis

Muscle strips were homogenized in 100 µl of lysis buffer as described by Alison J. Davis et al.³¹. The primary antibodies used for the Western blots are summarized in Table E1. ECL (Surdogen Co., Ltd., China) was used to visualize the blots.

Isolation of airway smooth muscle cells

Smooth muscle strips were prepared from the trachea and extra-pulmonary bronchus of the TMEM16A CTR and KO mice (8-20 weeks, both genders) or Hartley guinea pigs (250-300 g, male), and treated with papain and collagenase as previously described²⁸.

Patch-clamp recording

All currents were recorded using a MultiClamp 700B amplifier, Digidata 1440A and pClamp

10 (Molecular Devices). The currents were filtered at 2 kHz and sampled at 10 kHz, and the data were analysed using Clampfit 10. A whole-cell patch clamp was used to record the Cl^- currents. A focal pressurized perfusion (ALA Scientific Instruments) of 3 μM 5-HT, 1 μM U46619, 3 μM histamine, 100 nM LTD_4 , 3 μM MCh and 10 mM caffeine was administered through the application pipette to induce currents.

Isometric contraction measurement

Isometric contraction of mouse left extra-pulmonary bronchi were measured by using a wire myograph (610-M; Danish Myo Technology) with 37°C Krebs solution. Isometric contraction of guinea pig tracheal and human bronchial smooth muscle strips were measured by using a force transducer (MLT0201, AD Instruments). Smooth muscle strips of guinea pig were incubated in a 37°C organ bath with modified Krebs solution (mKrebs)³², while human bronchial strips were measured in Krebs. Human bronchial smooth muscle strips were isolated from lung biopsies of pulmonary cancer patients, from department of thoracic and cardiovascular surgery of Jiangsu province hospital.

Measurement of Ca^{2+} signals

The *Tmem16a*^{SMKO} and CTR mice were crossed with a Cre-dependent GCaMP3 reporter mouse (Ai38D)³³. The GCaMP3 fluorescence of left extra pulmonary bronchial smooth muscle was excited using a 488 nm argon laser, and the images were recorded every 2.5 seconds using a confocal microscope (FV-1000, Olympus). The dynamics of the Ca^{2+} signals were determined by the percent fluorescence ratio (% $\Delta\text{F}/\text{F}_0$).

Measurement of respiratory resistance

We used the flexiVentTM system (SCIREQ) to measure respiratory resistance. The ventilator was set to generate a tidal volume of 10 ml/kg at a frequency of 150 breaths/min. Resistance of the respiratory system (Rrs) was measured by a perturbation of SnapShot.

Statistical analysis

The data are presented as the mean $\pm$ s.e.m. Differences among the groups were determined using an unpaired two-tailed Student's *t*-test or a two-way analysis of variance (ANOVA). A paired two-tailed *t*-test was also used to evaluate differences in some experiments (labelled in the text). The significance levels were set as follows: NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

The detailed description of materials and methods please find in online supplement.

RESULTS

Inflammatory constrictors induce Cl_{Ca} currents through TMEM16A in ASMCs

Many inflammatory mediator receptors belong to the GPCR family, and their activation generates Cl_{Ca} currents to depolarize the membrane in ASMCs^{34, 35}. To determine the molecular nature of these currents, we performed whole cell voltage clamp recording (Figure 1A) in ASMCs from TMEM16A knockout mice and their controls. Before the tests, we measured the knockout efficiency of TMEM16A protein and Cl_{Ca} currents in airway smooth

muscle (see Figure E1 in on line data supplement). In CTR ASMCs, 3 μ M 5-HT elicited inward currents in 11 of 26 smooth muscle cells (Figure 1B and Figure E2A). Similar currents were also detected when cells were treated with 1 μ M U46619, a stable derivative of thromboxane A_2 (T_XA_2)^{36, 37} (Figure 1C and Figure E2B). 3 μ M methylcholine also efficiently induced inward currents, with apparent outward currents (Figure 1D and Figure E2C). Strikingly, deletion of TMEM16A essentially eliminated the inward currents induced by all three constrictors (Figures 1B-D and Figures E2A, C). This effect was highly unlikely to be secondary to TMEM16A deletion because the expression of the constrictor receptors remained unchanged in the mutant ASM (Figure E3). These data indicate that both cholinergic and inflammatory constrictors share a common mechanism by activating TMEM16A to elicit Cl_{Ca} currents.

We wondered whether TMEM16A also underlies Cl_{Ca} currents evoked by other inflammatory mediators. We selected guinea pig to test the effects of histamine and LTD₄, because these two mediators are not potent in mice^{4, 29}, and guinea pig ASM shows the same response as the case with humans³⁰. Figures 1E and F show that histamine and Cys-LTs generated inward currents similar to the Cl_{Ca} current induced by 5-HT and U46619 in mice ASMCs, although the amplitudes varied. These Cl_{Ca} currents results from the opening of TMEM16A channels, because 10 μ M T16A_{inh}-A01, a specific inhibitor of TMEM16A³⁸, essentially blocked these currents (Figures 1E, F and Figures E2D, E). In sum, TMEM16A underlies inflammatory mediator-induced Cl_{Ca} channel activity in ASM cells from different species.

TMEM16A is required for contractile responses to inflammatory constrictors

We sought to determine the role of TMEM16A in constrictor-mediated contraction using TMEM16A KO and T16A_{inh}-A01-treated ASM *ex vivo*. We first examined the morphology of airways from KO and CTR mice. As shown in Figures 2A and B, no apparent morphological difference is noticed, and no apparent change in muscle thickness can be detected in KO bronchus ($P > 0.05$, vs CTR, unpaired two-tail Student's t test). We then compared the contractile responses to different stimuli in ASM from the KO (*flox/flox; Cre+*) and control mice (*flox/+; Cre+* or *flox/flox; Cre-*). Upon stimulation with 60 mM KCl or 10 μ M MCh, the KO ASM developed a force that was comparable to the control muscles (Figures 2C, D and Figure E4A, B), suggesting that these muscles had a normal contractile apparatus. Interestingly, when the KO muscle was treated with MCh at a concentration as low as 300 nM, the evoked force was significantly reduced (KO: 2.58 ± 0.68 mN vs CTR: 5.66 ± 0.95 mN, $P < 0.05$, unpaired two-tail Student's t test, Figure E5). This result indicates that TMEM16A may be necessary for the contractile response to mild cholinergic stimulation. U46619 induced ASM contraction in a dose-dependent manner (Figure 2E and Figure E4C), and the maximal force induced was comparable to MCh (~ 12 mN for MCh, ~ 10 mN for U46619). After TMEM16A deletion, 34%-76% of the force was significantly inhibited ($P < 0.0001$ by two way ANOVA, Figure 2E), and the extent of inhibition varied with the dosage, as TMEM16A contribute more contraction at low U46619 concentration. 5-HT also evoked CTR ASM contraction in a dose-dependent manner, the maximal force developed was ~ 5 to 6 mN, which was only half of the force induced by U46619 or MCh (Figure 2F). To our surprise, TMEM16A-deficient ASM displayed an almost abolished contractile response to

various doses of 5-HT ($P < 0.0001$, two way ANOVA; Figure 2F and Figure E4D), indicating that the force induced by 5-HT was primarily mediated by TMEM16A. This effect could be further confirmed *in vivo*, as the elevated Rrs of the mice challenged with 5-HT was significantly inhibited by TMEM16A deletion ($P < 0.001$, two way ANOVA, Figure 2G).

We also used ASM from guinea pig to measure the contractile responses to histamine and LTD₄. Both constrictors generated force in a dose-dependent manner, as previously reported³⁰ (Figures 3C and D). Next, we used the inhibitor, T16A_{inh}-A01, to assess the role of TMEM16A in histamine- and LTD₄-induced contraction. The specificity of this inhibitor in ASM was first confirmed using *Tmem16a*^{SMKO} mice (Figure 3A and B). Pre-incubating with T16A_{inh}-A01 largely dropped the basal tone of guinea pig ASM, and decreased the capacity of histamine and LTD₄ to induce contraction (Figures 3C, D and Figure E6A-C). In particular, the addition of T16A_{inh}-A01 almost completely abolished the force induced by low doses of histamine (10-300 nM) and LTD₄ (0.01-0.3 nM) (Figures CH, D and Figure E6A, B). As the doses increased, the inhibition due to T16A_{inh}-A01 was rapidly reduced. When the histamine and LTD₄ doses reached 3 μ M and 10 nM, respectively, T16A_{inh}-A01 showed no apparent inhibition effect. Inhibition of TMEM16A shifts the EC₅₀ for histamine from 0.18 ± 0.07 μ M to 1.54 ± 0.45 μ M ($P < 0.05$, unpaired two-tail Student's t test, n=5), for LTD₄ from 69.09 ± 18.76 pM to 2.58 ± 0.85 nM ($P < 0.05$, unpaired two-tail Student's t test, n=5). These results suggest that TMEM16A is required for the sensitized response to weak stimulation with histamine and LTD₄. We also tested T16A_{inh}-A01 activity on human bronchial smooth muscle. The addition of 10 μ M T16A_{inh}-A01 didn't alter the basal tone, but significantly inhibited the force induced by histamine (Figure 3E). Taken together, the genetic deletion or

pharmacological inhibition of TMEM16A inhibits the force evoked by inflammatory constrictors, suggesting that TMEM16A is required for the contraction.

Inflammatory constrictors activate GPCR/TMEM16A/VDCC signalling axis, leading to ASM contraction

How do inflammatory constrictors activate TMEM16A, leading to ASM contraction? Based on the results in Figures 1 and 2 that inflammatory mediators induce Cl_{Ca} currents, and that the reversal potential for Cl^- in smooth muscle is ~ -30 to -20 mV²⁰, we hypothesized that inflammatory mediators activate GPCR to release Ca^{2+} from SR, which in turn opens TMEM16A and subsequently VDCCs, leading to Ca^{2+} influx and contraction. To test this hypothesis, we examined the changes in Ca^{2+} signal by interfering with this signalling pathway. To measure the intracellular calcium concentration, we crossed *Tmem16a*^{SMKO} mice with Ai38D mice, a line that expresses the GCaMP3 calcium indicator³³. In ASM from *Tmem16a*^{SMKO}, 60 mM KCl induced a robust calcium elevation similar to the CTR muscle (Figure 4A and Movie E1). 3 μ M MCh induced an increase in calcium, with an average peak $\Delta F/F_0$ value of $39.67 \pm 3.44\%$, which did not differ from the control ($P = 0.167$) (Figure 4B and Movie E2). These calcium elevation patterns appeared to be consistent with the corresponding contractile responses (Figure E5). Because 5-HT is a primary and typical inflammatory constrictor in mice, we measured the substantial calcium-releasing effect of 5-HT. 300 nM 5-HT induced a smaller rise in the $\Delta F/F_0$ peak value in KO ASM compared with the CTR (*Tmem16a*^{SMKO}: $6.56 \pm 1.02\%$ vs CTR: $13.20 \pm 2.15\%$; $P < 0.05$, unpaired two-tail Student's t test; Figure 4C and Movie E3). When the muscle was treated with a

relatively high dose of 5-HT (3 μ M), the $\Delta F/F_0$ peak value was $23.00 \pm 3.29\%$, which was also significantly lower than the control ($34.35 \pm 4.31\%$) ($P < 0.05$, unpaired two-tail Student's t test, Figure 4D and Movie E4). The $\Delta F/F_0$ value in the sustained phase was significantly reduced in 5-HT-treated KO muscle ($P < 0.001$, two way ANOVA; Figure. 4C and D). We also measured the calcium release induced by other constrictor in *Tmem16a*^{SMKO} smooth muscles and observed similar trends (data not shown). These results showed that TMEM16A was required for the calcium elevation in response to constrictors.

To determine whether this calcium release was mediated by VDCCs, we examined the effect of nifedipine, a specific inhibitor of L-type Ca^{2+} channels³⁹ on constrictor-induced rise of contraction. 1 μ M nifedipine reduce both contraction induced by MCh and 5-HT, though to a much greater extent in 5-HT. However, in the KO bronchial smooth muscles, 1 μ M nifedipine did not alter the dose-response curves of either MCh or 5-HT (Figures 5A and B). Although previous report suggested that TMEM16A may also regulate Ca^{2+} release from SR⁴⁰, we didn't observe a greater inhibition effect in TMEM16A KO ASM comparing with nifedipine treated CTR ASM (Figure 5). Collectively, the results suggested that both inflammatory constrictors and a low dose of MCh might regulate ASM contraction through the GPCR/TMEM16A/VDCC axis.

Inflammatory constrictors augment the cholinergic response through TMEM16A and are required for asthmatic hypersensitivity

In asthma, the ASM becomes more sensitive to cholinergic stimulation, which reflects airway hyper-responsiveness. We hypothesized that TMEM16A activation by inflammatory

constrictors might be involved in this process. Thus, we measured the ability of different inflammatory constrictors to augment MCh-induced responses. We pretreated mouse ASM with low doses of the constrictors (5-HT: 100 nM; U46619: 30 nM), and found no apparent or weak contraction. The ASM was then treated with low doses of MCh (30nM or 100 nM), which also evoked no apparent or weak contraction (Figure E7). But the combination of inflammatory constrictors and MCh led to a significant increase in the evoked force (Figure 6A and Figure E7). These augmented effects were significantly inhibited by TMEM16A deletion (Figure 4A, $P < 0.05$, unpaired two-tail Student's t test): The percent inhibition of 5-HT and U46619 in the KO was $42.17 \pm 10.51\%$ and $60.34 \pm 4.56\%$, respectively. These results show that the inflammatory constrictors potentiate ASM contraction in response to cholinergic stimulation through TMEM16A

To determine the possible contribution of this augmented effect in asthmatic constriction, we established an acute asthma model induced by chicken ovalbumin (OVA). Both the CTR and KO asthmatic mice displayed similar inflammatory infiltration and epithelial swelling (Figure E8). The CTR asthmatic animals that were challenged with the allergen (5% OVA) showed an elevated Rrs, as expected, whereas the KO asthmatic animals had a significantly lower Rrs ($P < 0.001$, two way ANOVA, Figure 6B). Moreover, after challenge with various doses of MCh, the knockout animals also displayed reduced airway constriction (Figure 6C), but the extent of inhibition varied with the MCh dose. When the MCh dose was less than 30 μg per mouse, the ΔRrs was inhibited by 40% in the KO mice, but no inhibition was measured when the doses were increased to 100-300 μg per mouse (Insert panel of Figure 6C). Collectively, these results suggest that inflammatory constrictors may increase the cholinergic

response through TMEM16A, thereby regulating asthmatic constriction.

DISCUSSION

In asthmatic airways, amount of inflammatory mediators are released from infiltrated immune cells, and some directly cause smooth muscle spasms¹². The molecular mechanism of inflammatory mediator-induced airway contraction remain incompletely understood. In this study, we found that inflammatory constrictors such as 5-HT, TxA₂, LTD₄ and histamine were capable of inducing Cl_{Ca} currents in ASMCs by activating the TMEM16A chloride channel, the resultant activation of TMEM16A and calcium influx were necessary for their contractile responses. This regulatory mechanism was also predominantly adopted by a weak cholinergic stimulation in terms of contraction. Because of the abundant expression of TMEM16A in ASM, TMEM16A-based signalling may serve as a sensitive mechanism by which ASM responds to physiological and pathological stimuli. Moreover, we found that TMEM16A was also required by the synergistic effect of inflammatory mediators and methylcholine both *ex vivo* and in asthmatic animals. This effect may well explain why the airway becomes more sensitive to neuronal stimuli during allergenic inflammation in asthma. As such a sensitivity is an essential factor for the formation of asthmatic responsiveness, our result suggest that ASM contractility mediated by the activation of TMEM16A may contribute significantly to asthmatic hyperresponsiveness.

The receptors for inflammatory mediators mediate immune responses through multiple signalling cascades¹². Among these cascades, calcium release from the SR is mediated by

the sequential activation of G_q , PLC and IP_3/IP_3R ¹³. In this study we shows that in ASM cells that express both TMEM16A and constrictor receptors, the activation of $G_q/PLC/IP_3/IP_3R$ signalling can also activate TMEM16A. Several lines of evidence from other reports also support such coupled activation of TMEM16A^{41, 42} by this signalling pathway. Thus, inflammatory constrictors may induce one more signalling pathway to promote calcium elevation in asthmatic airways, $IP_3/TMEM16A$ -mediated calcium influx (IT-CI). Based on our observation that TMEM16A inhibition did not always completely abolish the force induced by the constrictors, the functional contribution of IT-CI to the contractile responses may vary according to the constrictor, e.g., the contribution to the 5-HT response is more profound than that to TxA_2 . Here, we propose a working model for the regulation of ASM contraction by constrictors (Figure 7). In this model, ASMC expresses abundant TMEM16A clusters, inflammatory constrictor receptors, and muscarinic receptors. Activation of their corresponding receptors produces IP_3 and promotes calcium release from the SR. The elevated $[Ca^{2+}]_i$, in turn, causes TMEM16A activation to generate Cl^- current and VDCC activation, and VOCl, which activates MLCK. This signalling pathway primarily responds to inflammatory constrictors and cholinergic transmitters, particularly at low concentrations. If the signals triggered by these stimuli are strong enough, then the calcium release from SR or through ROCl and SOCl will be sufficient to directly activate MLCK to contract smooth muscle.

A unique property of asthma is that the ASM becomes more sensitive to cholinergic and other stimuli, which underlies one aspect of hyperresponsiveness⁴³. A synergistic effect of allergenic inflammatory mediators and a cholinergic transmitter is believed to be one possible

cause of this effect^{10, 11}. In this report, we indeed observed an apparent synergistic effect of inflammatory constrictors and MCh. Importantly, we found that this effect could be significantly blocked by TMEM16A deletion. Because the synergistic effect of the inflammatory constrictors and MCh is more profound at low concentrations^{10, 11}, we suggest that the GPCR-TMEM16A-VDCCs cascade used by the constrictors may play an essential role in asthmatic hyperresponsiveness. The genesis of hyperresponsiveness may be due to the synergistic activation of TMEM16A cluster by combined stimulation of nerve transmitter and inflammatory constrictors which are overproduced in asthmatic airway (Figure 7). Considering inhibition of TMEM16A activity is capable of not only directly relaxing ASM but also reducing hyperresponsiveness, we speculate that TMEM16A inhibitors might be an ideal strategy for asthma therapy. In addition, compared with antagonists of inflammatory mediators such as leukotriene antagonists, which have been approved and are effective asthma therapies⁴⁴, an important advantage of TMEM16A inhibition is that it may block multiple constrictors simultaneously and to provide improved efficacy.

REFERENCES

1. Canning BJ, Fischer A. Neural regulation of airway smooth muscle tone. *Respiration physiology* **125**, 113-127 (2001).
2. Brightling CE. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* **346**, 827-830 (2002).
3. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* **454**, 445-454 (2008).
4. Barnes PJ. Histamine and serotonin. *Pulmonary pharmacology & therapeutics* **14**, 329-339 (2001).
5. Moore BD, Hyde D, Miller L, Wong E, Frelinger J, Schelegle ES. Allergen and ozone exacerbate

- serotonin-induced increases in airway smooth muscle contraction in a model of childhood asthma. *Respiration* **83**, 529-542 (2012).
6. Armour CL, Johnson PR, Alfredson ML, Black JL. Characterization of contractile prostanoid receptors on human airway smooth muscle. *European journal of pharmacology* **165**, 215-222 (1989).
 7. Coleman RA, Sheldrick RL. Prostanoid-induced contraction of human bronchial smooth muscle is mediated by TP-receptors. *British journal of pharmacology* **96**, 688-692 (1989).
 8. Adelroth E, Morris MM, Hargreave FE, O'Byrne PM. Airway responsiveness to leukotrienes C4 and D4 and to methacholine in patients with asthma and normal controls. *New England Journal of Medicine* **315**, 480-484 (1986).
 9. Fernandes DJ, Mitchell RW, Lakser O, Dowell M, Stewart AG, Solway J. Invited Review: Do inflammatory mediators influence the contribution of airway smooth muscle contraction to airway hyperresponsiveness in asthma? *Journal of Applied Physiology* **95**, 844-853 (2003).
 10. Catalli A, Janssen LJ. Augmentation of bovine airway smooth muscle responsiveness to carbachol, KCl, and histamine by the isoprostane 8-iso-PGE₂. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **287**, L1035-L1041 (2004).
 11. Gerthoffer WT. Agonist synergism in airway smooth muscle contraction. *The Journal of pharmacology and experimental therapeutics* **278**, 800-807 (1996).
 12. Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. *Pharmacological reviews* **50**, 515-596 (1998).
 13. Billington CK, Penn RB. Signaling and regulation of G protein-coupled receptors in airway smooth muscle. *Respiratory research* **4**, 2 (2003).
 14. Penn RB, Benovic JL. Regulation of heterotrimeric G protein signaling in airway smooth muscle. *Proceedings of the American Thoracic Society* **5**, 47-57 (2008).
 15. Van Amsterdam RG, Meurs H, Brouwer F, Postema JB, Timmermans A, Zaagsma J. Role of phosphoinositide metabolism in functional antagonism of airway smooth muscle contraction by beta-adrenoceptor agonists. *Eur J Pharmacol* **172**, 175-183 (1989).
 16. Langlands JM, Rodger IW, Diamond J. The effect of M&B 22948 on methacholine- and histamine-induced contraction and inositol 1,4,5-trisphosphate levels in guinea-pig tracheal tissue. *British journal of pharmacology* **98**, 336-338 (1989).
 17. Shiraki A, et al. Role of Ca²⁺ mobilization and Ca²⁺ sensitization in 8-iso-PGF₂ α-induced contraction in airway smooth muscle. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **39**, 236-245 (2009).

18. Gorenne I, Labat C, Gascard JP, Norel X, Nashashibi N, Brink C. Leukotriene D4 contractions in human airways are blocked by SK&F 96365, an inhibitor of receptor-mediated calcium entry. *The Journal of pharmacology and experimental therapeutics* **284**, 549-552 (1998).
19. Hirota S, Helli P, Janssen LJ. Ionic mechanisms and Ca²⁺ handling in airway smooth muscle. *The European respiratory journal* **30**, 114-133 (2007).
20. Chipperfield A, Harper A. Chloride in smooth muscle. *Progress in biophysics and molecular biology* **74**, 175-221 (2000).
21. Ferrera L, *et al.* Regulation of TMEM16A chloride channel properties by alternative splicing. *The Journal of biological chemistry* **284**, 33360-33368 (2009).
22. Huang F, *et al.* Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proceedings of the National Academy of Sciences* **106**, 21413-21418 (2009).
23. Gallos G, *et al.* Functional expression of the TMEM16 family of calcium-activated chloride channels in airway smooth muscle. *American journal of physiology Lung cellular and molecular physiology* **305**, L625-634 (2013).
24. Heinze C, *et al.* Disruption of vascular Ca²⁺-activated chloride currents lowers blood pressure. *The Journal of clinical investigation* **124**, 675-686 (2014).
25. Yang YD, *et al.* TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* **455**, 1210-1215 (2008).
26. Zhang C-H, *et al.* The transmembrane protein 16A Ca²⁺-activated Cl⁻ channel in airway smooth muscle contributes to airway hyperresponsiveness. *American journal of respiratory and critical care medicine* **187**, 374-381 (2013).
27. Zhang C-H, *et al.* The molecular basis of the genesis of basal tone in internal anal sphincter. *Nat Commun* **7**, (2016).
28. Zhuge R, Bao R, Fogarty KE, Lifshitz LM. Ca²⁺ sparks act as potent regulators of excitation-contraction coupling in airway smooth muscle. *The Journal of biological chemistry* **285**, 2203-2210 (2010).
29. Yang G, *et al.* Transgenic smooth muscle expression of the human CysLT1 receptor induces enhanced responsiveness of murine airways to leukotriene D4. *American journal of physiology Lung cellular and molecular physiology* **286**, L992-1001 (2004).
30. Drazen JM, *et al.* Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 4354-4358 (1980).

31. Davis AJ, *et al.* Expression profile and protein translation of TMEM16A in murine smooth muscle. *American Journal of Physiology-Cell Physiology* **299**, C948-C959 (2010).
32. Weichman BM, Muccitelli RM, Tucker SS, Wasserman MA. Effect of calcium antagonists on leukotriene D4-induced contractions of the guinea-pig trachea and lung parenchyma. *Journal of Pharmacology and Experimental Therapeutics* **225**, 310-315 (1983).
33. Zariwala HA, *et al.* A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. *The Journal of Neuroscience* **32**, 3131-3141 (2012).
34. Janssen L, Sims S. Acetylcholine activates non - selective cation and chloride conductances in canine and guinea - pig tracheal myocytes. *The Journal of Physiology* **453**, 197-218 (1992).
35. Janssen L, Sims S. Histamine activates Cl⁻ and K⁺ currents in guinea - pig tracheal myocytes: convergence with muscarinic signalling pathway. *The Journal of Physiology* **465**, 661-677 (1993).
36. Coleman RA, Humphrey PP, Kennedy I, Levy GP, Lumley P. Comparison of the actions of U-46619, a prostaglandin H₂-analogue, with those of prostaglandin H₂ and thromboxane A₂ on some isolated smooth muscle preparations. *British journal of pharmacology* **73**, 773-778 (1981).
37. Takami M, Tsukada W. Correlative alteration of thromboxane A₂ with antigen-induced bronchoconstriction and the role of platelets as a source of TXA₂ synthesis in guinea pigs: effect of DP-1904, an inhibitor of thromboxane synthetase. *Pharmacological research* **38**, 133-139 (1998).
38. Namkung W, Phuan PW, Verkman AS. TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells. *The Journal of biological chemistry* **286**, 2365-2374 (2011).
39. Stork AP, Cocks TM. Pharmacological reactivity of human epicardial coronary arteries: phasic and tonic responses to vasoconstrictor agents differentiated by nifedipine. *British journal of pharmacology* **113**, 1093-1098 (1994).
40. Danielsson J, *et al.* Antagonists of the TMEM16A calcium-activated chloride channel modulate airway smooth muscle tone and intracellular calcium. *Anesthesiology* **123**, 569-581 (2015).
41. Jin X, *et al.* Activation of the Cl⁻ channel ANO1 by localized calcium signals in nociceptive sensory neurons requires coupling with the IP3 receptor. *Science signaling* **6**, ra73 (2013).
42. Borchers MT, *et al.* Methacholine-induced airway hyperresponsiveness is dependent on Gαq signaling. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **285**, L114-L120 (2003).
43. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. *Journal of Allergy & Clinical Immunology* **118**, 551-559 (2006).

526

527 44. Price D, et al. Leukotriene Antagonists as First-Line or Add-on Asthma-Controller Therapy. *New*
528 *England Journal of Medicine* **364**, 1695-1707 (2011).

529

ACCEPTED MANUSCRIPT

FIGURES

Figure 1. TMEM16A mediates agonist-induced inward currents in ASMCs. (A)

Recording configuration. (B-D) Left: Representative traces of 5-HT- (B), U46619- (C) and MCh- (D) induced currents in ASMCs from CTR and TMEM16A KO mice. Right: Quantification of the results (5-HT: n=26 cells from 4 mice of each genotype; U46619: n=20 cells from 4 mice for CTR, n=22 cells from 4 mice for KO; MCh: n=12 cells from 3 mice per genotype). (E-F) Left: Representative traces of histamine- (E) and LTD₄- (F) induced currents in DMSO and T16A_{inh}-A01 pre-incubated guinea pig ASMCs. Right: Quantification of the results (histamine: n=14 cells from 3 guinea pigs per group; LTD₄: n=21 cells for the DMSO group and n=23 cells for the T16A_{inh}-A01 group from 4 guinea pigs each). The holding potential in all recordings was -60 mV. The blue blocks indicate the peak values of the outward currents, and the pink circles indicate the peak values of the inward currents. The bars represent the mean $\pm$ s.e.m., **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, two-tailed Student's *t*-test, blue “*” for outward currents and red “*” for inward currents.

Figure 2. TMEM16A is required for contraction in response to constrictors in mouse

ASM. (A) Representative H & E staining of extra-pulmonary bronchi from CTR and TMEM16A KO mice. Scale bars: 200 μ m. (B) TMEM16A deletion did not alter the thickness of the extra-pulmonary bronchus. The bars represent the mean $\pm$ s.e.m., NS, *P* > 0.05, two-tailed Student's *t*-test. (C-F) Dose-response curves of TMEM16A KO (flox/flox

Cre+), CTR (flox/+ Cre+) and (flox/flox Cre-) bronchial smooth muscle contraction in response to high K^+ and different agonists. The bars indicate the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panels, **** $P < 0.0001$ comparing KO (flox/flox Cre+) with CTR (flox/+ Cre+) by ANOVA. (G) *In vivo* dose-response curve of the Rrs (respiratory resistance) of TMEM16A KO and CTR mice in response to 5-HT. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, *** $P < 0.001$, ANOVA.

Figure 3. T16A_{inh}-A01 suppressed contractile response to constrictors in ASMs from guinea pig and human.

(A) Pre-incubation with 10 μ M T16A_{inh}-01 suppressed 3 μ M 5-HT induced left extra-pulmonary bronchi contraction in CTR but not TMEM16A KO (CTR: n=6, KO: n=7). (B) Pre-incubation with 10 μ M T16A_{inh}-01 did not suppress 3 μ M MCh induced contraction in both CTR and KO (CTR: n=5, KO: n=5). Bars represent mean $\pm$ s.e.m., NS $P > 0.05$, ** $P < 0.01$ by two-tailed student's *t*-test. (C-D) Dose-response curves of DMSO- and T16A_{inh}-A01-pre-incubated guinea pig tracheal smooth muscle contraction in response to histamine (C) and LTD₄ (D). All force values are expressed as percentages of the reference contraction induced by 60 mM KCl. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, **** $P < 0.0001$, ANOVA. (E) Dose-response curve of DMSO- and T16A_{inh}-A01-pre-incubated human bronchial smooth muscle contraction in response to histamine (Please find recording traces of each sample in Fig. E6). All force values are expressed as percentages of the reference contraction induced by 60 mM KCl.

572 The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, * $P <$
573 0.05, ANOVA.

Figure 4. Knockout of TMEM16A suppressed Ca^{2+} signal. (A-D) The Ca^{2+} elevation dynamics (left) and peak values(right) of 60 mM KCl (A), 3 μM MCh (B), 300 nM 5-HT (C) and 3 μM 5-HT (D)-induced Ca^{2+} signalling in CTR and KO bronchial smooth muscle. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, NS $P > 0.05$, $*P < 0.05$ by two-tailed Student's *t*-test, $****P < 0.0001$ by ANOVA.

Figure 5. TMEM16A mediates contraction through VDCCs.

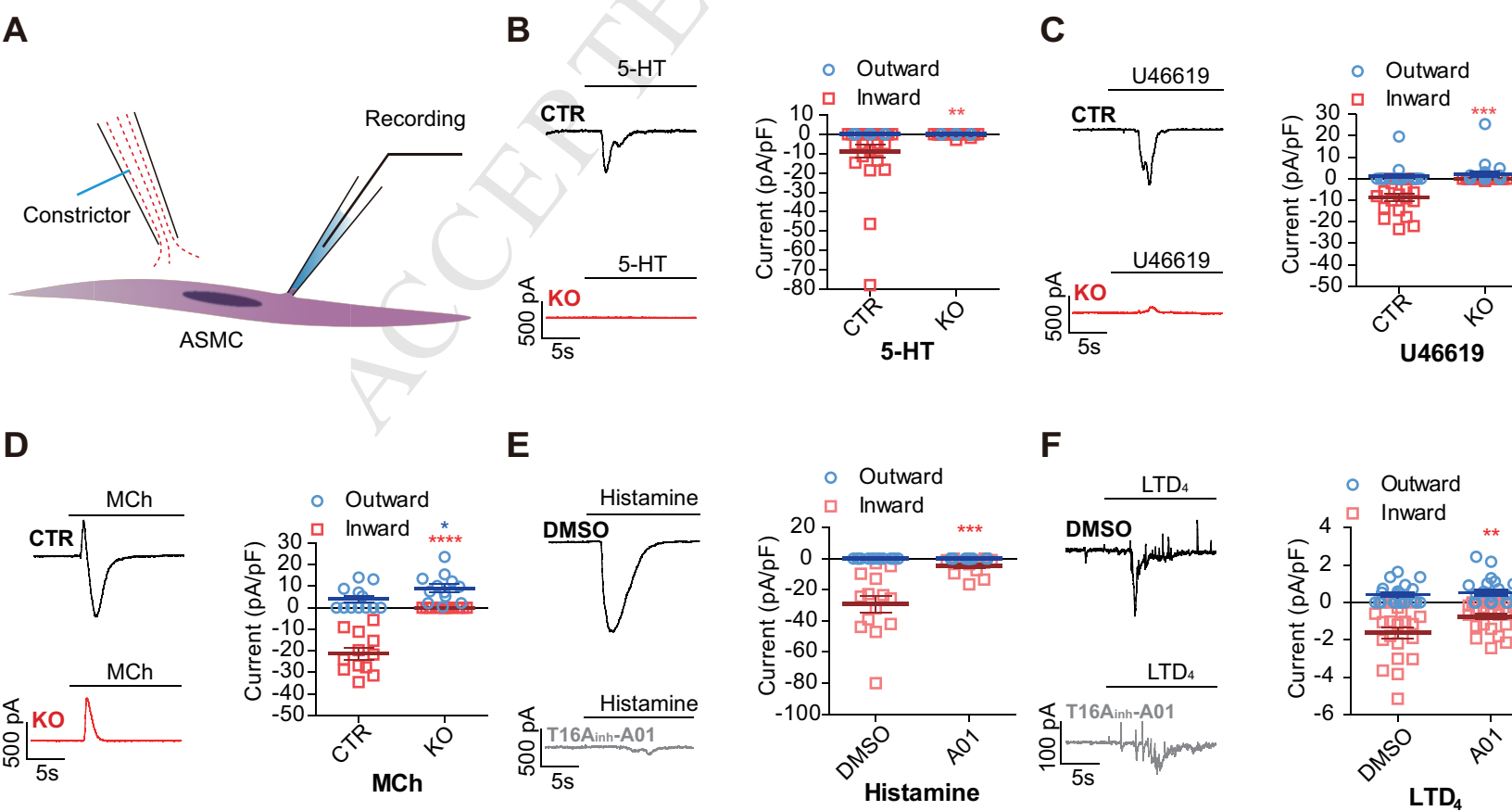
Effect of pre-incubation with nifedipine on the dose-response curves of CTR and KO bronchial smooth muscle contraction in response to MCh (A) and 5-HT (B). Left: representative traces, right: quantitative results. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, $****P < 0.0001$ CTR + nifedipine compared with CTR, ANOVA.

Figure 6. TMEM16A is required for airway hyper-responsiveness. (A) TMEM16A deletion attenuated ability of 5-HT and U46619 to augment MCh stimulation. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, $##P < 0.01$, $####P < 0.0001$ comparing the groups indicated by the lines using a paired two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$ compared with CTR using an unpaired two-tailed Student's *t*-test. (B) Allergen (OVA) inhalation induced Rrs elevation in the asthmatic (OVA groups) and non-asthmatic (PBS groups) CTR and KO mice. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, $****P < 0.0001$ CTR OVA vs CTR PBS by

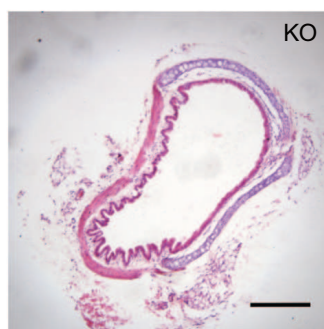
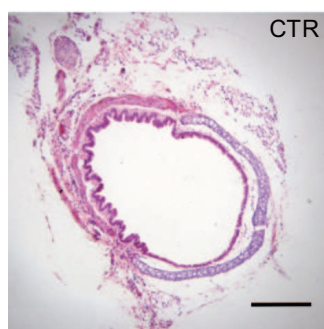
ANOVA, $####P < 0.0001$ KO OVA vs CTR OVA by ANOVA. (C) MCh gradient inhalation induced Rrs elevation in the asthmatic (OVA groups) and non-asthmatic (PBS groups) CTR and KO mice. Inset panel: Percent inhibition of ΔRrs by TMEM16A knockout; the values were normalized by comparing them with the mean ΔRrs value of the corresponding MCh doses. The bars represent the mean $\pm$ s.e.m.; the sample sizes (n) are indicated in the panel, $*P < 0.05$, $**P < 0.01$ compared with CTR PBS by the two-tailed Student's *t*-test, $^{\#}P < 0.05$ compared with CTR OVA by the two-tailed Student's *t*-test.

Figure 7. Schematic showing the hypothesized TMEM16A-based signalling model.

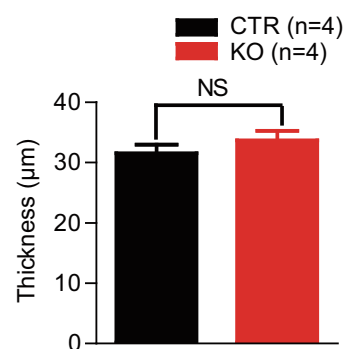
Inflammatory constrictors bind to their corresponding GPCRs and sequentially activate the G_q -PLC- IP_3 axis. IP_3 activates IP_3Rs and induces Ca^{2+} release from SR. Ca^{2+} released from the SR can directly (IP-CR) or indirectly activate MLCK through the TMEM16A-VDCCs axis, mediate Ca^{2+} influx (IT-CI), amplify the cytosolic Ca^{2+} signal and activate more MLCK through this indirect pathway. Under simultaneous stimulation of inflammatory constrictors and ACh, ASMCs always show augmented contraction. This augmentation effect may be due to the interaction of the signalling network formed by different receptors. If the spatial distance between different GPCRs is small enough, the activation of these receptors may enhance local activation of IT-CI, hence augment contractile response. IP-CR: IP_3 -mediated calcium release from the SR; IT-CI: IP_3 /TMEM16A-mediated calcium influx.



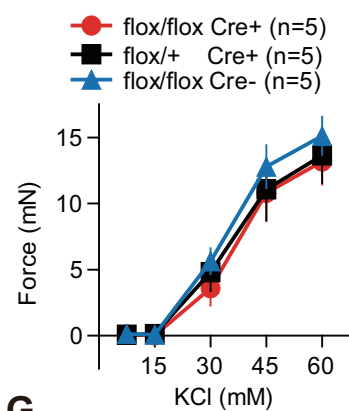
A



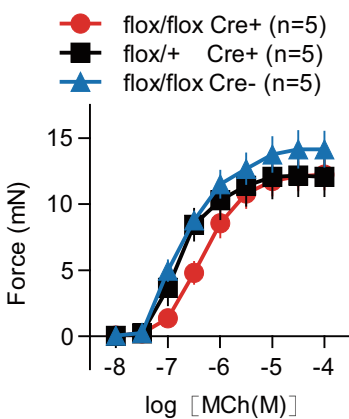
B



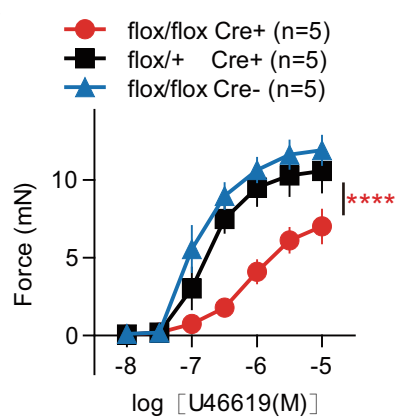
C



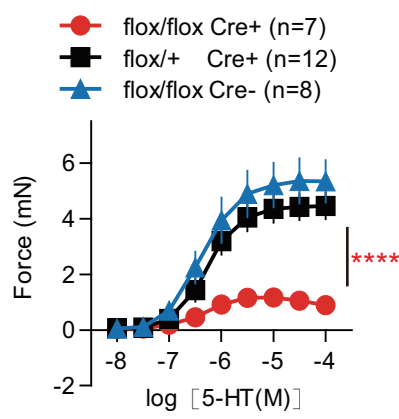
D



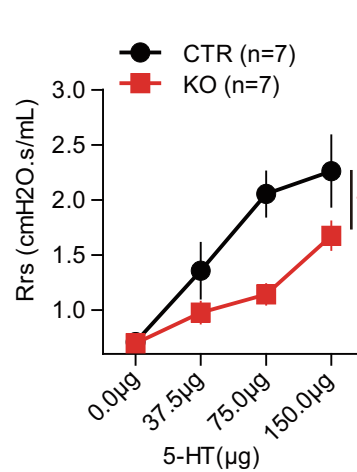
E

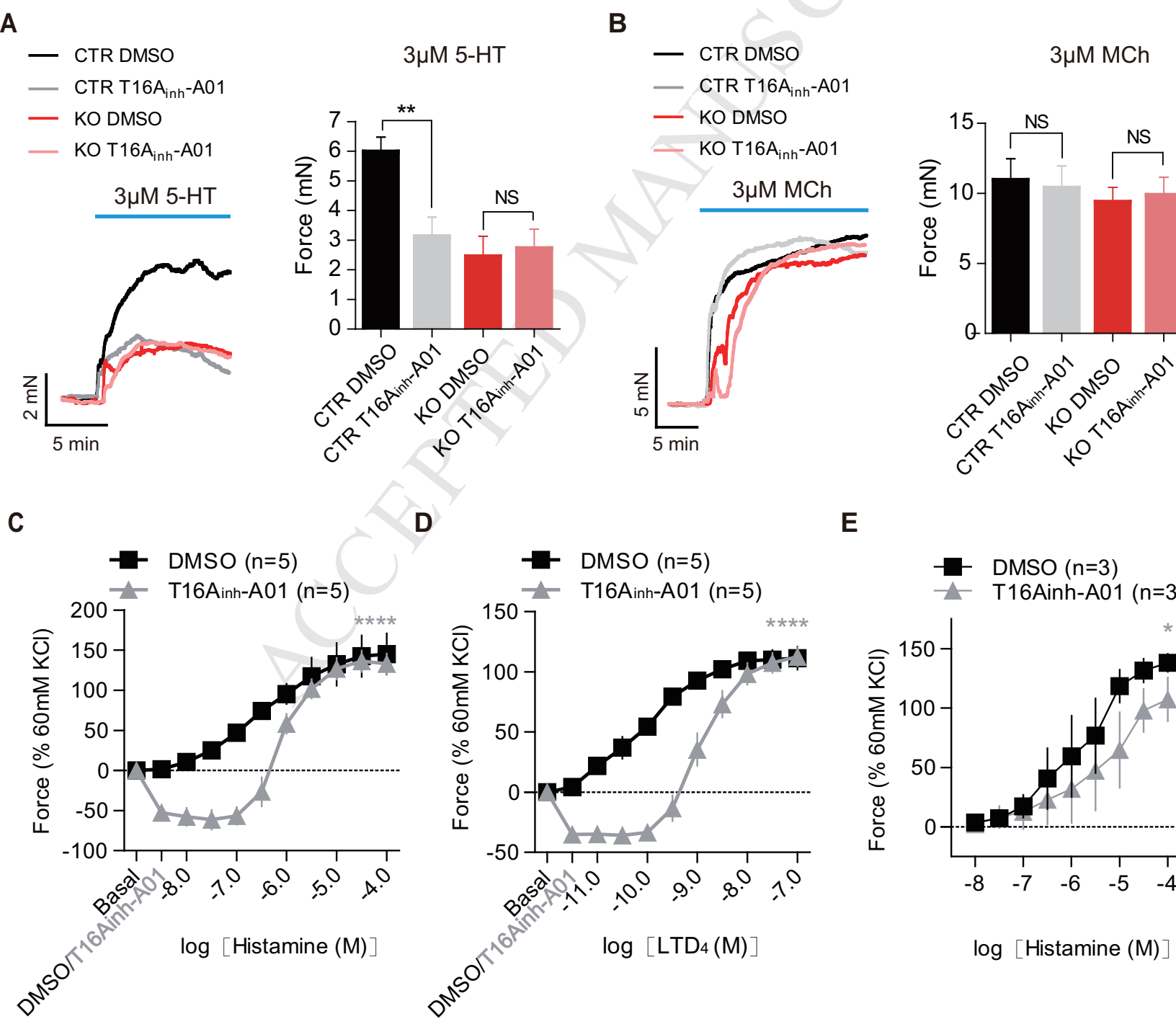


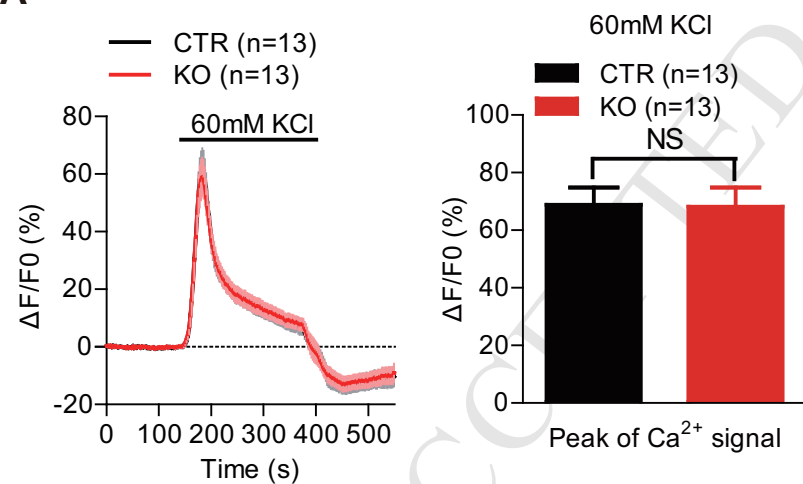
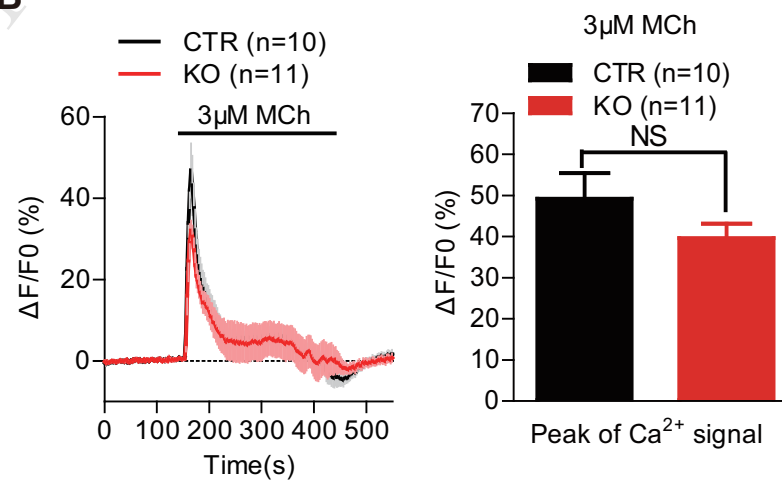
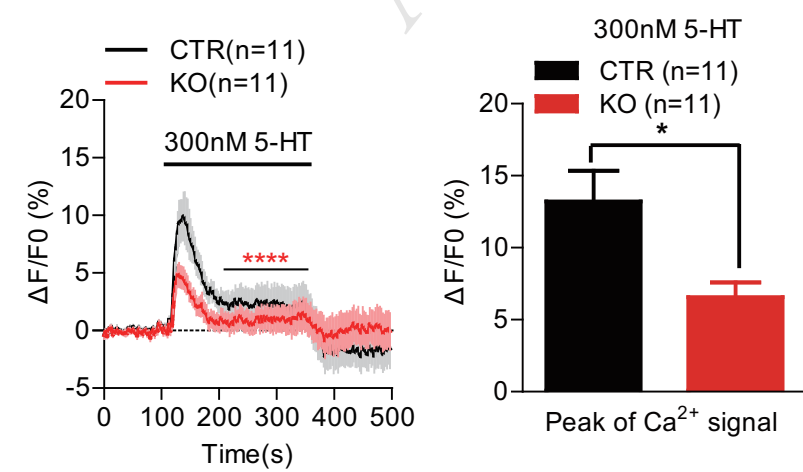
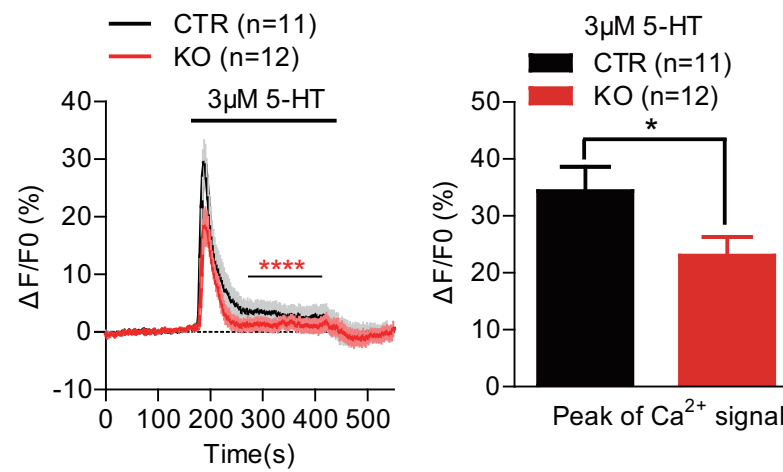
F



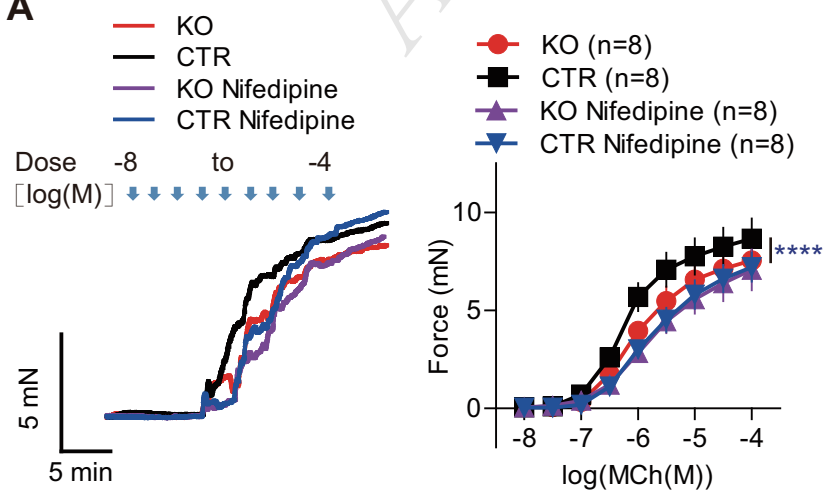
G



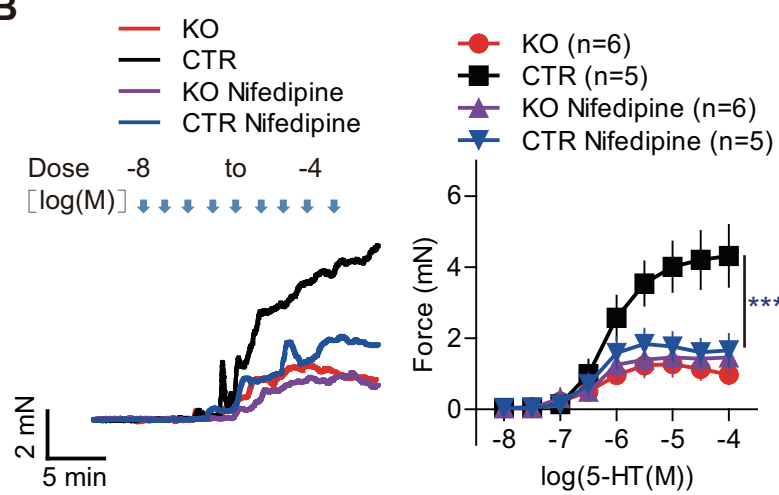


A**B****C****D**

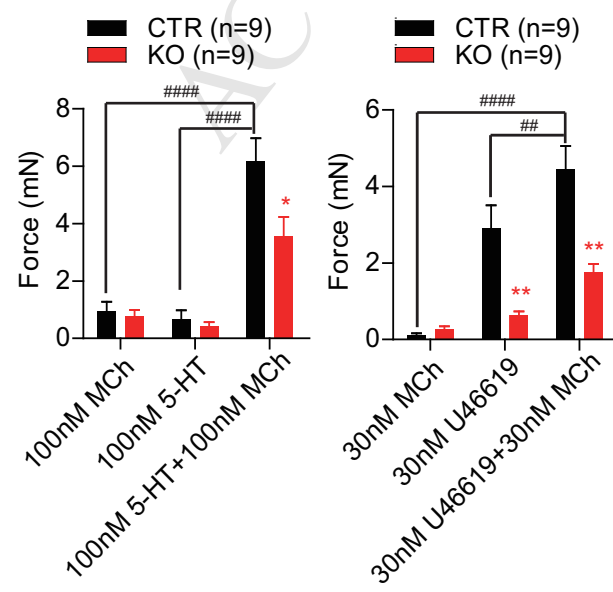
A



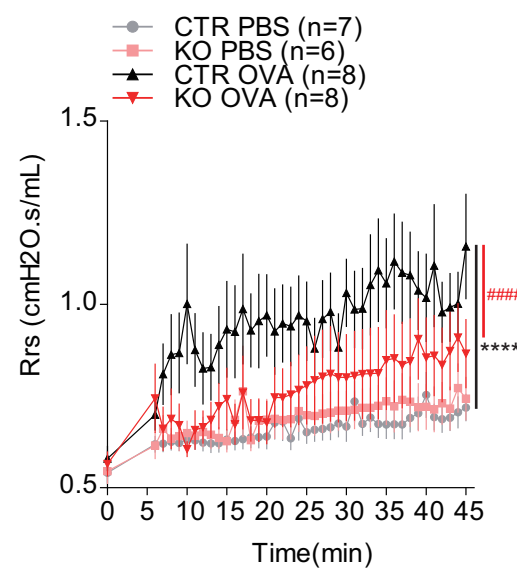
B



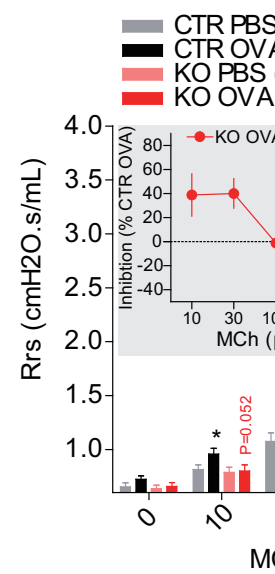
A



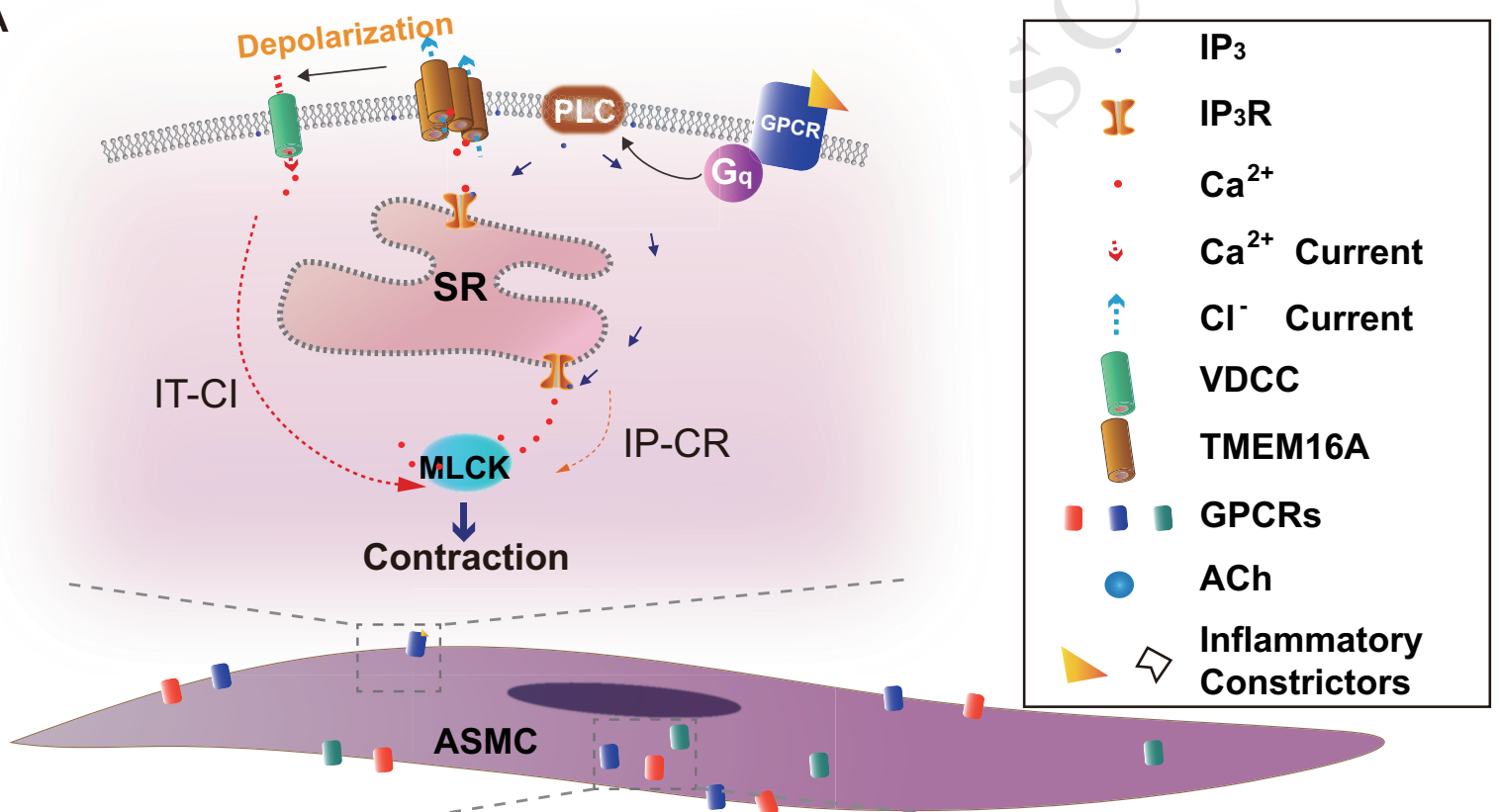
B



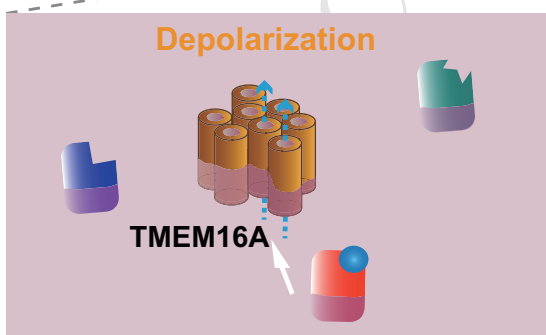
C



A



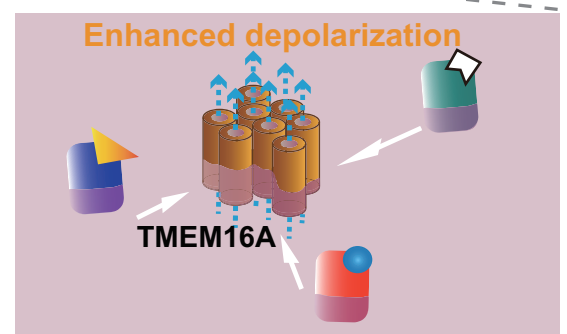
B



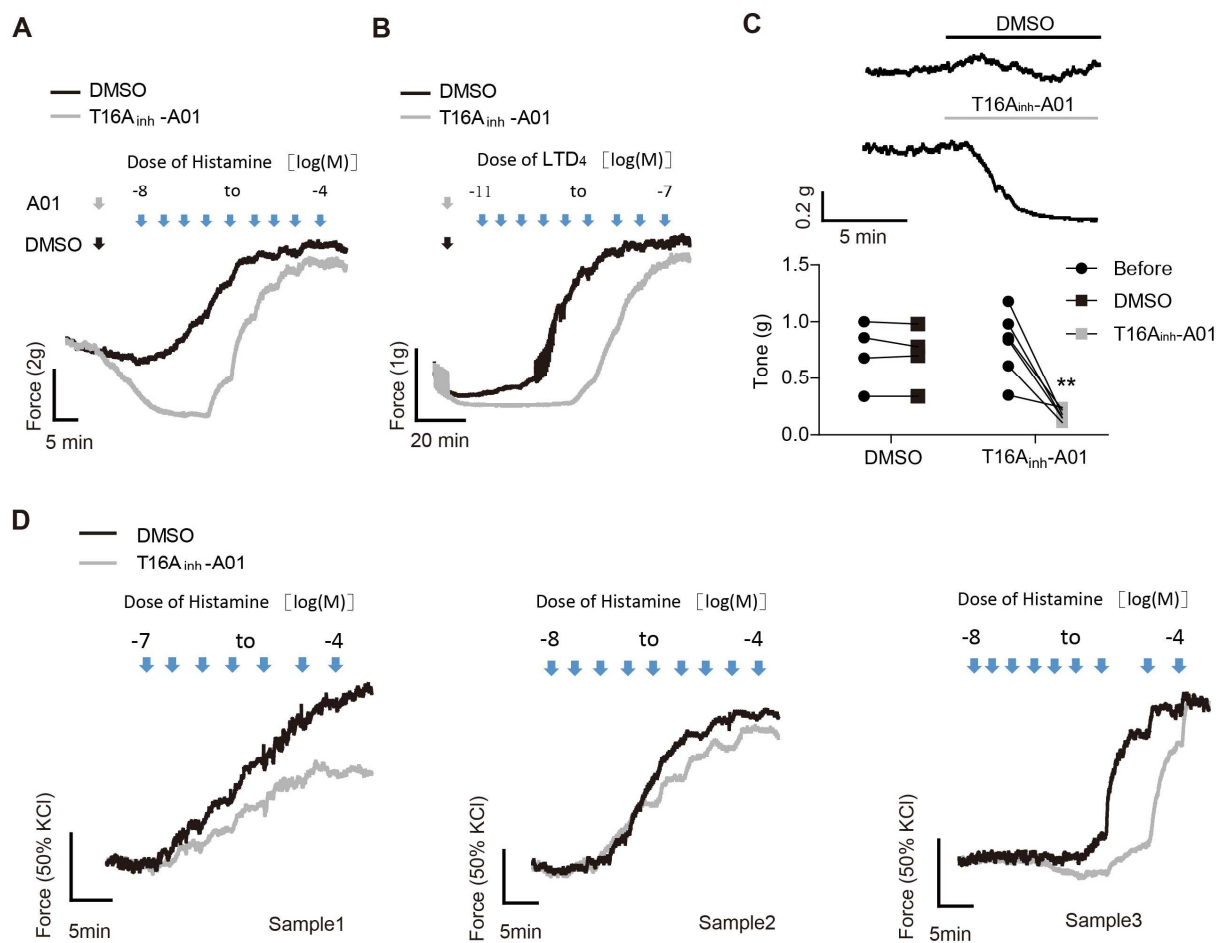
Single constrictor

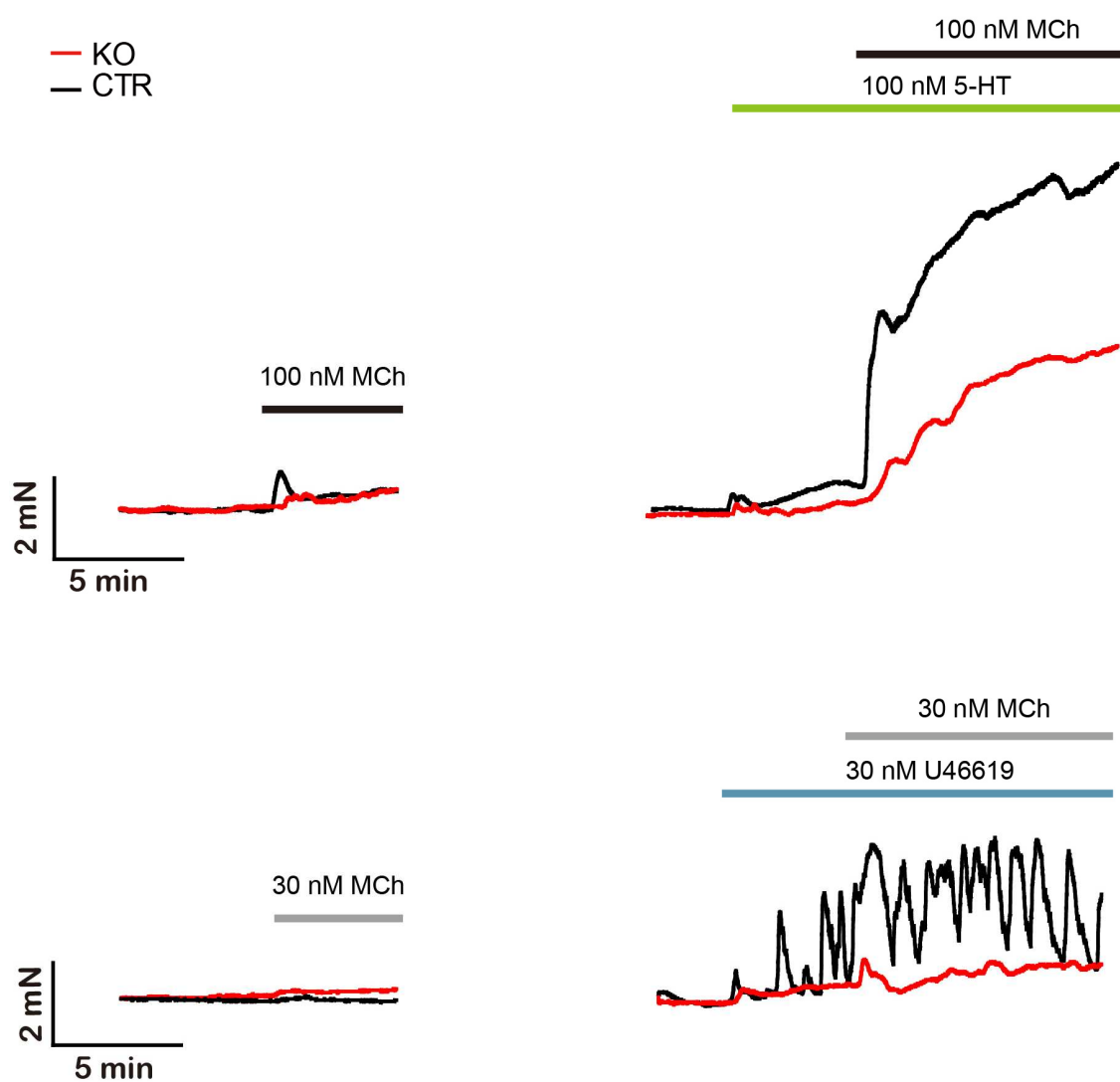
Inflammation

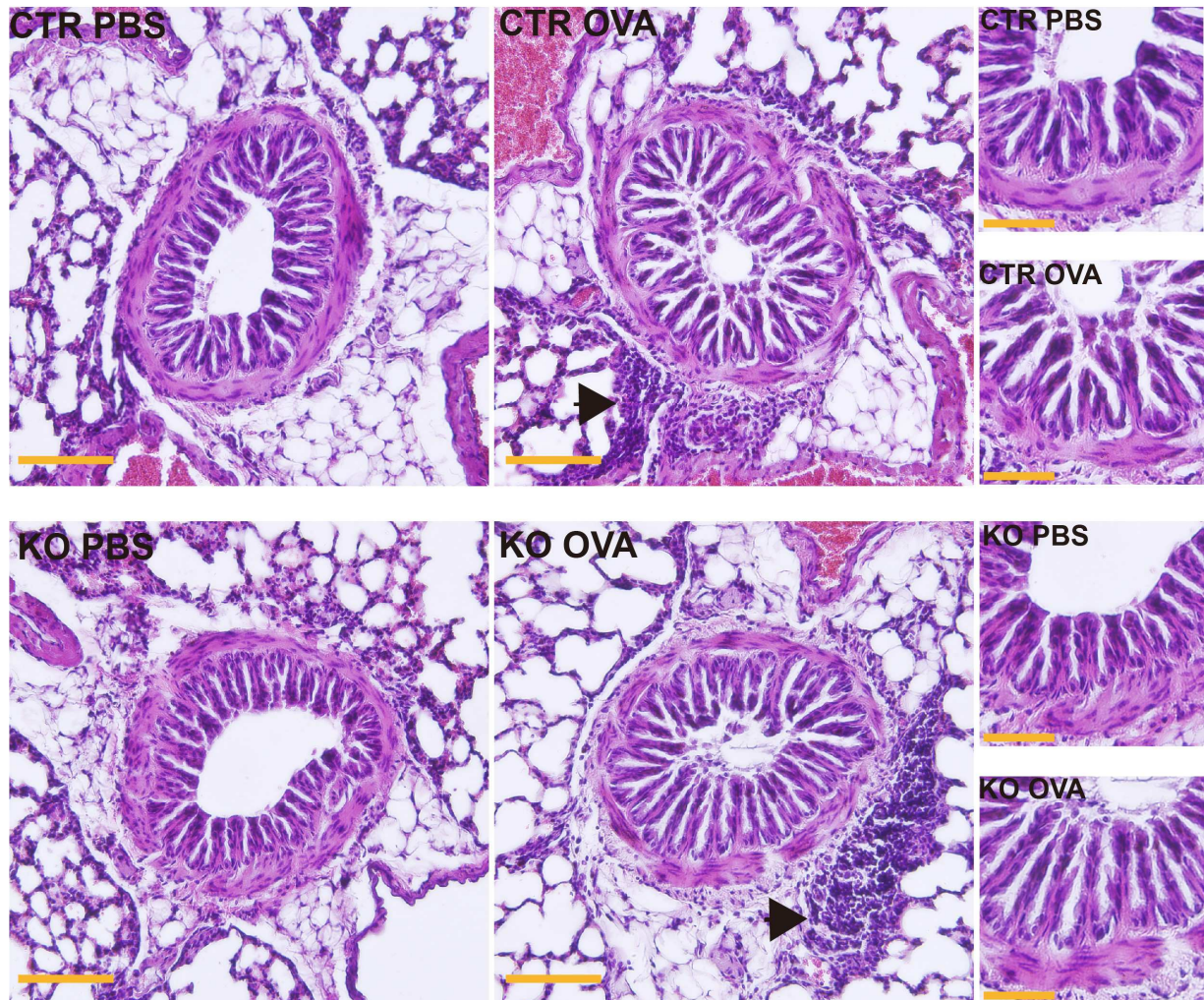
C

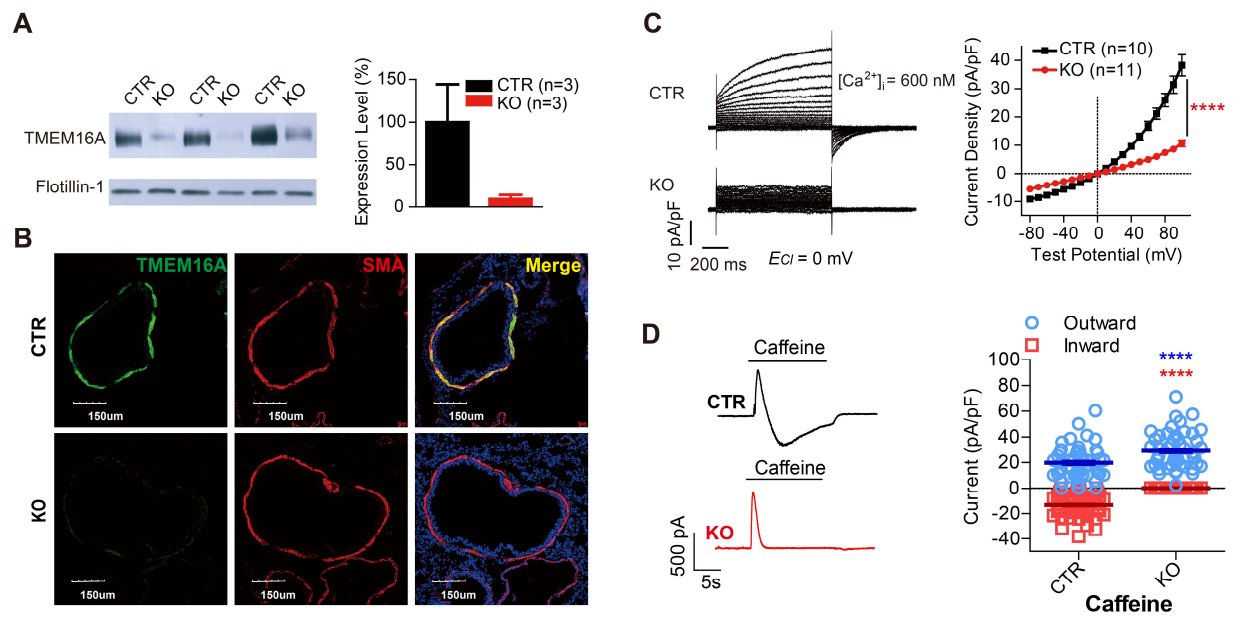


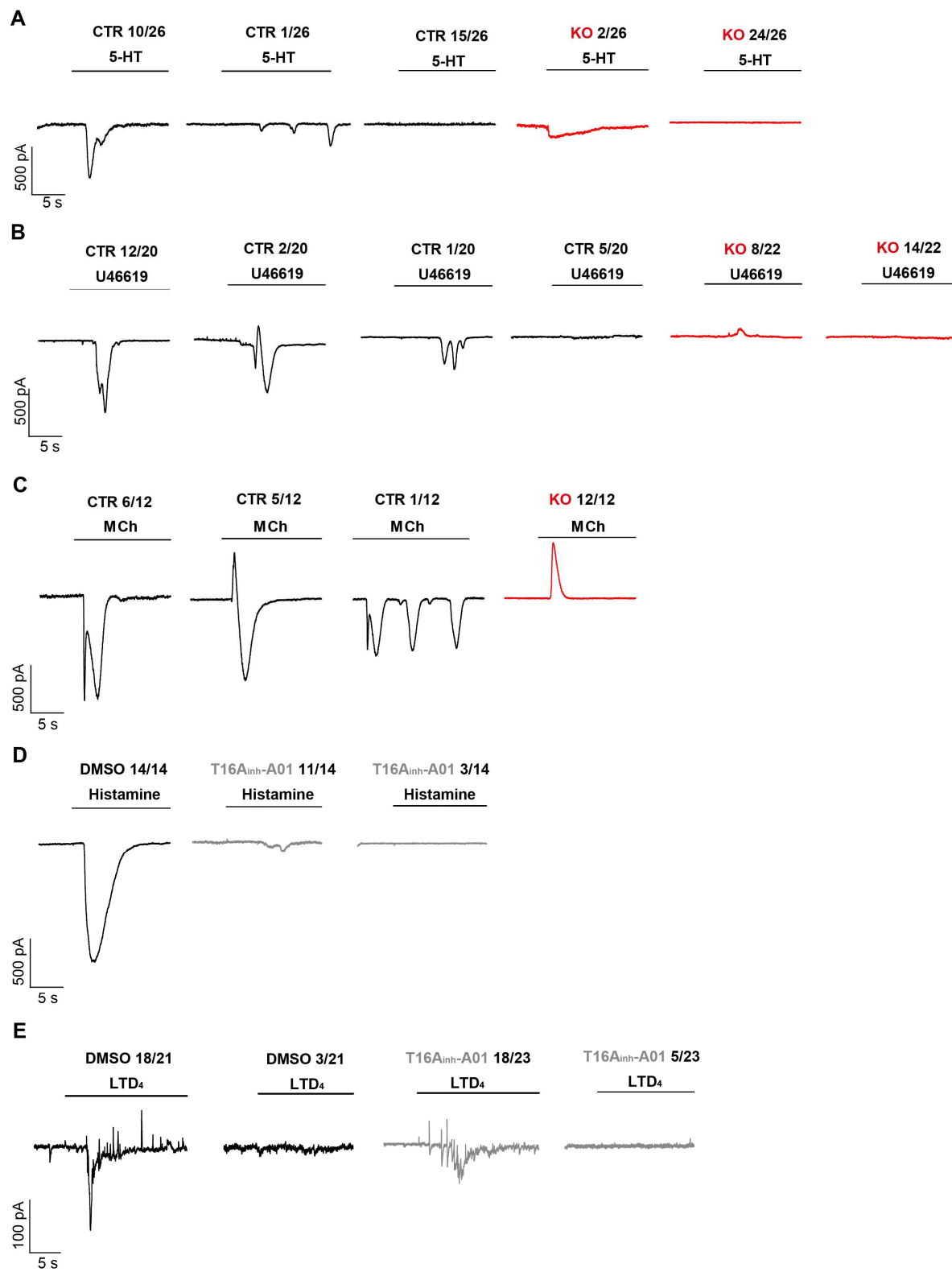
Mutiple constrictors

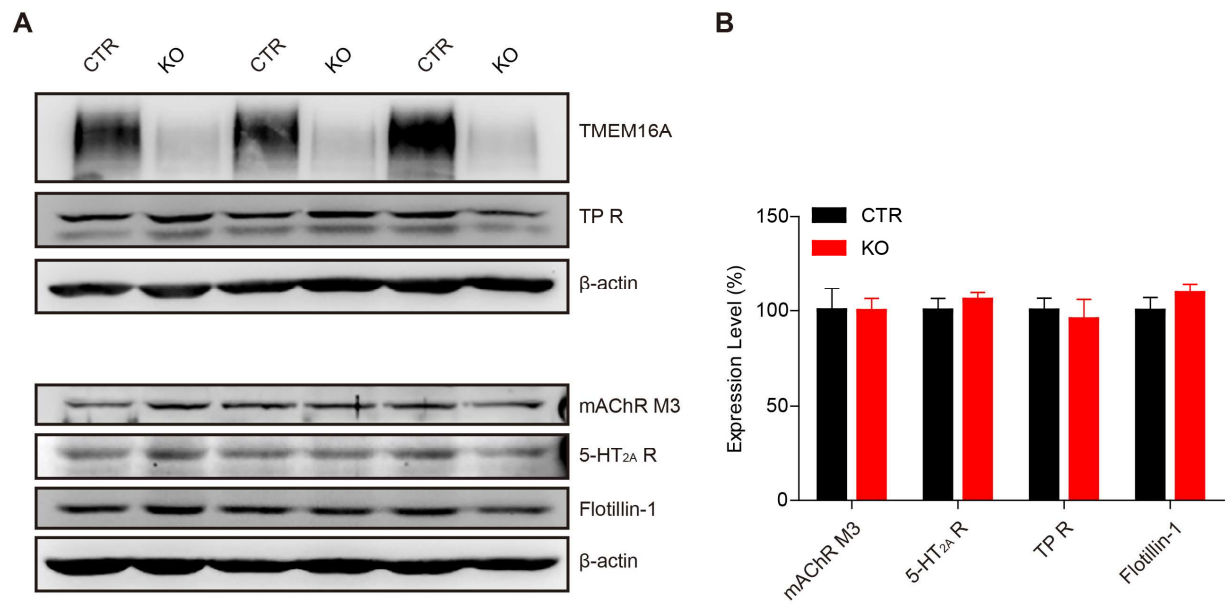


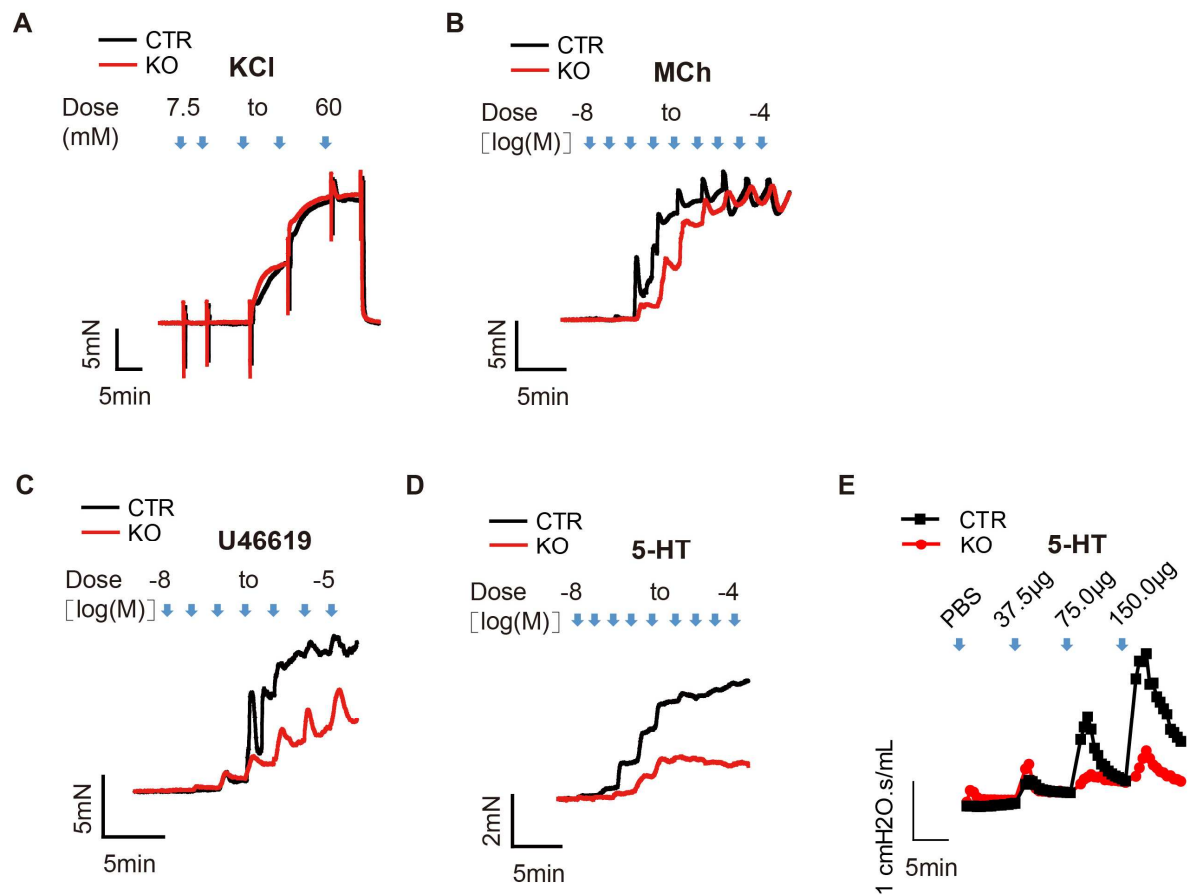


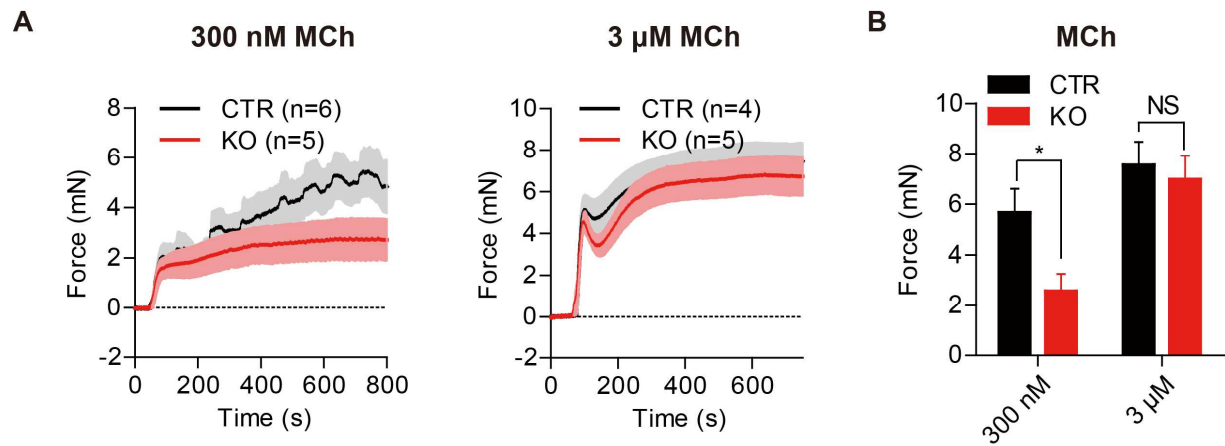












SUPPLEMENTAL ONLINE MATERIAL

MATERIALS AND METHODS

Genetic mouse models

TMEM16A CTR (*Tmem16a*^{fllox/+}; *SMA-Cre*⁺) and KO/*Tmem16a*^{SMKO} (*Tmem16a*^{fllox/fllox}; *SMA-Cre*⁺) mice of both genders were subjected to a series of analyses at 8-20 weeks, including Western blot, immunofluorescence, patch-clamp, isometric tension and respiratory resistance measurements. *Tmem16a*^{fllox/fllox}; *SMA-Cre*⁻ mice of both genders were also used as one more control group in some isometric tension measurements as indicated in Figure 2. TMEM16A CTR and KO mice were crossed with Ai38D mice¹, and both genders were used for the Ca²⁺ measurements at 8-20 weeks. All mice were in a mixed C57BL/6 and Sv/129 background.

Western blot analysis

Muscle strips isolated from the trachea and extra-pulmonary bronchus of CTR and KO mice were homogenized in 100 µl of lysis buffer (20 mM Tris base, 137 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol, protease inhibitor cocktail (Roche, Germany), pH=8)². Next the homogenates were incubated on ice for 15 min and centrifuged at 3,000 g to remove tissue debris. 80 µl of each sample was transferred to a new tube. The protein concentrations were measured with a bicinchoninic acid (BCA) kit (BCA protein assay kit, Pierce, USA). The proteins were mixed with 5 × sample buffer (10% SDS, 20% glycerol, 0.05% bromophenol blue, 10 mM β-mercapto-ethanol, 200 mM Tris-HCl, 8 M urea) and then denatured at 95°C for 5 min. After resolution by SDS-PAGE, the proteins were transferred to a PVDF membrane and blocked with 5% non-fat milk in TBST, followed by sequential incubation

with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. The primary antibodies used for the Western blots are summarized in Supplementary Table 1. ECL (Sudogen Co., Ltd., China) was used to visualize the blots.

Immunofluorescence

Paraffin slices (10- μ m thick) of the left lobe of the mouse lung were used for immunofluorescence staining. Non-specific binding of the primary antibodies was blocked by incubating the slices with PBST buffer containing 1% BSA and 5% non-immune goat serum. A rabbit anti-TMEM16A polyclonal antibody (1:200 dilution, ab53212, Abcam) and mouse anti-SMA monoclonal antibody (1:500 dilution, Clone 1A4, Thermo) were used as primary antibodies. An Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen) diluted 1:250 and an Alexa Fluor 555-conjugated goat anti-mouse antibody (Invitrogen) diluted 1:250 were used as secondary antibodies. The cell nuclei were stained with DAPI. The images were captured using a confocal microscope (FV-1000, Olympus).

Isolation of airway smooth muscle cells

Smooth muscle strips were prepared from the trachea and extra-pulmonary bronchus of the TMEM16A CTR and KO mice (8-20 weeks, both genders) or Hartley guinea pigs (250-300 g, male). The resulting tissues were dissected in dissociation medium (DM: 136 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4 , 4.16 mM NaHCO_3 , 0.34 mM Na_2HPO_4 , 20 mM HEPES, 5.56 mM glucose, 4.9 mM MgCl_2 , pH 7.1). The first digestion was performed with MgCl_2 -free DM containing 30 U/mL papain, 0.2 mM dithiothreitol, and 0.02 mM EDTA, and

the second digestion was performed with a solution containing 3 U/mL collagenase 1A, 1 mg/mL bovine serum albumin, and 0.1 mg/ml deoxyribonuclease I in MgCl_2 -free DM. The tubes from the second digestion were shaken vigorously until most of the clumps disappeared (~20 min). After centrifugation at 300 g for 2 min, the collected cells were washed with MgCl_2 -free DM once by centrifugation at 300 g for 2 min. The isolated cells were re-suspended in DM, transferred to dishes, and stored at 4°C. DM was replaced with the bath solution for patch-clamp recording prior to use.

Patch-clamp recording

All currents were recorded using a MultiClamp 700B amplifier, Digidata 1440A and pClamp 10 (Molecular Devices). The currents were filtered at 2 kHz and sampled at 10 kHz, and the data were analysed using Clampfit 10.

To record the Cl^- currents following stimulation with 600 nM $[\text{Ca}^{2+}]_i$, the bath solution contained 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM D-glucose, 10 mM mannitol and 10 mM HEPES; the pH was adjusted to 7.4 with NaOH. The pipette solution contained 130 mM CsCl, 10 mM EGTA, 1 mM MgCl_2 , 8 mM CaCl_2 , 10 mM HEPES, and 1 mg ATP; the pH was adjusted to 7.3 with NaOH³. $E_{\text{Cl}}=0$ mV under these conditions. Whole-cell currents were recorded in freshly isolated smooth muscle cells in response to 1 s voltage pulses from -80 to +100 mV in 10 mV increments, followed by 700 ms pulses to -60 mV.

To record the currents induced by the constrictors and caffeine, the bath solution contained 130 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 20 mM HEPES, and 10 mM D-glucose; the pH was adjusted to 7.4 with NaOH. The pipette solution contained 140

mM KCl, 1 mM MgCl₂, 20 mM HEPES, and 0.025 mM EGTA; the pH was adjusted to 7.2 with KOH. $E_{Cl}=0$ mV under these conditions. Cells were held at -60mV. A focal pressurized perfusion (ALA Scientific Instruments) of 3 μ M 5-HT, 1 μ M U46619, 3 μ M histamine, 100 nM LTD₄, 3 μ M MCh and 10 mM caffeine was administered through the application pipette to induce currents. The currents were recorded no longer than the 5th minute after breaking the patch of membrane under the pipette.

Histopathology

The extra-pulmonary bronchi and left lobes of the lung were immersed in 4% PFA at 4°C overnight and dehydrated in a graded series of ethanol solutions. The tissue was embedded in paraffin, and 10- μ m sections were prepared for standard H&E staining. Standard H&E staining was performed on paraffin slices (10 μ m thick) of the extra-pulmonary bronchi and left lobes of the lung as previously described⁴. The thickness of the bronchial smooth muscle layer was measured from the innermost edge to the outermost edge using ImageJ software. The thickness of the smooth muscle layer of the extra-pulmonary bronchus was assessed at three predetermined bronchiole sites (two sites near each the end close to the cartilage and one site in the middle of the smooth muscle layer). Three slices per mouse were measured.

Isometric contraction measurement

For the force measurements, the left extra-pulmonary bronchi from TMEM16A CTR and KO mice were dissected from the surrounding tissues in ice-cold Krebs solution (118.07 mM NaCl, 4.69 mM KCl, 2.52 mM CaCl₂, 1.16 mM MgSO₄, 1.01 mM NaH₂PO₄, 25 mM

NaHCO₃, 11.1 mM glucose), rings of approximately 1.5 mm in length were cut and mounted on a wire myograph (610-M; Danish Myo Technology), and the isometric tension was recorded using a Powerlab recording device (AD Instruments). The bronchus rings were kept in Krebs buffer and oxygenated with an O₂-CO₂ mixture (95% O₂-5% CO₂) at 37°C. A proper preload was administered to obtain an approximately 5 mN resting tension after one 60 mM K⁺ stimulation and a 40-min equilibrium. After one more stimulation with 60 mM K⁺, the contractile responses to other stimuli were measured.

To measure the contraction of guinea pig trachea, the tracheas of male Hartley guinea pigs (250-300 g) were quickly removed and placed in ice-cold modified Krebs solution (mKrebs, 118 mM NaCl, 4.6 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 1 mM KH₂PO₄, 24.9 mM NaHCO₃, 11.1 mM glucose)⁵, and dissected from the surrounding tissues. Rings comprising two tracheal cartilages were cut from the tracheas. To obtain strips, the cartilage opposite the muscle layer was cut. One cartilaginous end of the trachea strip was anchored to the bottom of the muscle bath chamber, and the other end was connected to a force transducer (MLT0201, AD Instruments). Isometric tension was measured using a PowerLab (AD Instruments) recording device. The trachea strips were maintained in mKrebs oxygenated with an O₂-CO₂ mixture (95% O₂-5% CO₂) at 37°C. The resting tension was adjusted to 1 g. The strips were stimulated at least twice with 60 mM KCl before subsequent experiments. The samples were pre-incubated with 10 µM T16A_{inh}-A01 or DMSO vehicle for 10 min before addition of the reagents.

To measure human bronchial smooth muscle contraction, surgically resected samples

from lung cancer patients were quickly incubated in ice-cold, oxygen-saturated HEPES-Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, pH 7.4). After transport to the laboratory, the surrounding tissues were cleaned, and the bronchial smooth muscle tissues were cut into 3 mm-wide strips. One end of the strip was anchored to the bottom of the muscle bath chamber, which was filled with Krebs solution oxygenated with an O₂-CO₂ mixture (95% O₂-5% CO₂) at 37°C. The other end was connected to a force transducer (MLT0201, AD Instruments). Because the diameter and thickness of the smooth muscle layer were different in different samples, the suitable resting tension was tested following stimulation with 60 mM KCl. The samples were pre-incubated with 10 μM T16A_{inh}-A01 or DMSO vehicle for 10 min before addition of the reagents.

Measurement of Ca²⁺ signals in Ai38D reporter mice

The *Tmem16a*^{SMKO} and CTR mice were crossed with a Cre-dependent GCaMP3 reporter mouse (Ai38D). The left extra-pulmonary bronchi of both the CTR and KO mice were mounted on a confocal wire myograph (120 cw; Danish Myo Technology) in HEPES-Tyrode (H-T) buffer (137.0 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, 5.6 mM Glucose, pH 7.4). The agonists were added using a self-made gravity perfusion system with a flow velocity of ~10 ml/min. The GCaMP3 fluorescence was excited using a 488 nm argon laser, and the images were recorded every 2.5 seconds using a confocal microscope (FV-1000, Olympus). The average fluorescence intensity of the ROI (bronchial smooth muscle range) was calculated with SV10-ASW software (Olympus), the average

fluorescence intensity during the 1-2 min before stimulation was defined as the initial fluorescence (F0), and the dynamics of the Ca^{2+} signals were determined by the percent fluorescence ratio ($\% \Delta F/F0$).

Sensitization with OVA

Mice of both genders were used at 8-10 weeks of age. On days 0 and 14, the mice were sensitized with 100 μg of ovalbumin (OVA, Sigma-Aldrich) dissolved in 4 mg of aluminium hydroxide (Imject Alum, Pierce) by intraperitoneal injection⁴. The mice were then challenged with aerosolized 1% OVA in PBS for 60 min on days 24, 25, and 26. The mice that had received the last challenge within 24 hours were used as acute asthma models. Mice that received 4 mg of aluminium hydroxide diluted in PBS via intraperitoneal injection and were challenged with aerosolized PBS were used as a non-asthmatic control model.

Measurement of respiratory resistance

We used the flexiVentTM system (SCIREQ) to measure respiratory resistance. The OVA-sensitized mice were anesthetized with 250-300 mg/kg avertin 24 hours after receiving the last challenge of OVA or PBS. The anesthetized mice were cannulated with a blunt mouth 18-gauge metal needle, and the other end of the needle was connected to the rodent ventilator of the flexivent system. The ventilator was set to generate a tidal volume of 10 ml/kg at a frequency of 150 breaths/min. The resistance of the respiratory system (Rrs) was measured by a perturbation of SnapShot. The Newtonian resistance (Rn) was measured by a perturbation of Quick prime-3. To measure the changes in respiratory resistance after

induction with 5% OVA, Rrs values were recorded every minute for 45 minutes after the mice inhaled nebulized OVA for 5 min. To measure the changes in respiratory resistance in response to 5-HT challenge, sequential doses (37.5 µg, 75 µg, and 150 µg) of 5-HT were nebulized to challenge the airway, and the maximal Rrs was calculated for every dose within 3 min after challenge to generate dose-response curves. To measure the changes in respiratory resistance in response to MCh, sequential doses (10 µg, 30 µg, 100 µg, and 300 µg) of MCh were nebulized to challenge the airway, and the maximal Rrs were calculated for every dose within 3 min of challenge.

REFERENCES

1. Zariwala, H.A., *et al.* A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. *The Journal of Neuroscience* **32**, 3131-3141 (2012).
2. Davis, A.J., *et al.* Expression profile and protein translation of TMEM16A in murine smooth muscle. *American Journal of Physiology-Cell Physiology* **299**, C948-C959 (2010).
3. Manoury, B., Tamuleviciute, A. & Tammaro, P. TMEM16A/anoctamin 1 protein mediates calcium-activated chloride currents in pulmonary arterial smooth muscle cells. *J Physiol* **588**, 2305-2314 (2010).
4. Zhang, W.-C., *et al.* Myosin light chain kinase is necessary for tonic airway smooth muscle contraction. *Journal of Biological Chemistry* **285**, 5522-5531 (2010).
5. Weichman, B.M., Muccitelli, R.M., Tucker, S.S. & Wasserman, M.A. Effect of calcium antagonists on leukotriene D4-induced contractions of the guinea-pig trachea and lung parenchyma. *Journal of Pharmacology and Experimental Therapeutics* **225**, 310-315 (1983).

SUPPLEMENTAL FIGURES

Figure E1. Specific deletion of TMEM16A Cl_{Ca} channels in ASM. (A) Western blots (Left) showed more than 90% KO efficiency (quantitative data in right panel) in extra-pulmonary bronchi. (B) Representative immunofluorescence staining showed a high KO efficiency in smooth muscle layer of intra-pulmonary bronchus. (C) Cl_{Ca} currents were significantly inhibited in KO ASMCs. Left: Representative recording traces, right: I-V curves of CTR and KO ASMCs. 600 nM $[\text{Ca}^{2+}]_{\text{i}}$ was set by pipette solution, $E_{\text{Cl}} = 0$ mV. Bars represent mean $\pm$ s.e.m., CTR: n=10 cells from 3 mice, KO: n=11 cells from 3 mice, **** $P < 0.0001$ by ANOVA. (D) 10 mM caffeine-induced Cl_{Ca} currents were eliminated in the KO ASMCs. Left: Representative recording traces; right: quantitative results. Bars represent mean $\pm$ s.e.m., CTR: n=49 cells from 10 mice; KO: n=52 cells from 10 mice, **** $P < 0.0001$ by two-tailed student's *t*-test, blue “*” for outward currents and red “*” for inward currents.

Figure E2. Representative recording traces of agonist-induced currents in ASMCs. (A) Currents induced by 5-HT (3 μM in application pipette) in mouse ASMCs. In CTR cells, upon 5-HT stimulation, 11/26 of cells exhibited typical inward currents, and 1/26 of cells showed oscillated inward currents; In KO cells, only 2/26 of cells exhibited weak inward currents. (B) Currents induced by U46619 (1 μM in application pipette) in mouse ASMCs. For CTR cells, 15/20 of cells exhibited inward currents, and 2/20 cells simultaneously showed outward currents and 1/20 cell showed oscillated inward currents; for KO cells, none of total 22 cells exhibited inward currents, 8 cells exhibited outward currents. (C) Currents induced

by MCh (3 μ M in application pipette) in mouse ASMCs. For CTR, all the 12 cells showed inward currents, among them, 5 cells exhibited outward currents and 1 cells showed oscillated inward currents; for KO, all the 12 cells exhibited only outward currents. (D) Currents induced by histamine (3 μ M in application pipette) in guinea pig ASMCs. For DMSO pre-incubated cells, all the 14 cells showed inward currents; for 10 μ M T16A_{inh}-A01 pre-incubated cells, 11/14 cells showed reduced inward currents, and 3/14 cells didn't show apparent currents. (E) Currents induced by LTD₄ (100 nM in application pipette) in guinea pig ASMCs. For DMSO group, 18/21 cells showed inward currents; for T16A_{inh}-A01 group, 18/23 cells showed inward currents, but the amplitudes were suppressed.

Figure E3. Expression of agonist receptors in TMEM16A KO ASM. (A) Western blots assay for the expression of TMEM16A, mAChR M3, 5-HT_{2A} R, TP R and Flotillin-1 in TMEM16A KO and CTR extra-pulmonary bronchial smooth muscle, in which β -actin was used as internal control. (B) Quantitation of mouse ACh receptor M3 (mAChR M3) (n=6), 5-HT_{2A} R (n=3), TP R (n=5) and Flotillin-1 (n=6) in extra-pulmonary bronchial smooth muscle. Bars represent mean $\pm$ s.e.m..

Figure E4. Recording traces of ASM contraction and airway constriction in vivo. (A-D) Typical contraction recording traces of TMEM16A KO and CTR bronchial smooth muscle contraction evoked by cumulative doses of KCl (A), MCh (B), U46619 (C) and 5-HT (D). (E) Representative recording traces of 5-HT inhalation induced airway constriction in CTR and KO mice.

Figure E5. TMEM16A deletion inhibited ASM contraction induced by a low dose of MCh. (A) The merged contraction tracking of bronchial smooth muscle. The forces induced by a low dose (300 nM) and a relative high dose (3 μ M) of MCh were inhibited in TMEM16A KO muscle. The force of each time point was represented as mean $\pm$ s.e.m. Sample sizes (n) were labeled in both panels. (B) Quantitation for the maximal forces. Bars represent mean $\pm$ s.e.m., sample sizes (n) were the same as panel (a), $*P < 0.05$ by two-tailed student's *t*-test.

Figure E6. Inhibitory effects of T16A_{inh}-A01 on the contractile response of guinea pig and human ASM to inflammatory mediators. (A) Representative recording traces of DMSO or T16A_{inh}-A01 pre-incubated guinea pig trachea smooth muscle (TSM) contraction induced by cumulative doses of histamine. (B) Representative recording traces of DMSO or T16A_{inh}-A01 pre-incubated guinea pig TSM contraction induced by LTD₄ gradients. (C) 10 μ M T16A_{inh}-A01 induced relaxation of guinea pig TSM basal tone. Upper panel: representative recording traces; lower panel: quantitative data. DMSO: n=4, T16A_{inh}-A01: n=6, $**P < 0.01$ by paired two-tailed student's *t*-test. (D) Recording traces of three human bronchial smooth muscle samples showing inhibitory effects of T16A_{inh}-A01 on histamine-induced contraction.

Figure E7. Representative recording traces of the augmentation effects. Extra-pulmonary bronchial smooth muscles were treated with combined addition of 100 nM 5-HT and 100 nM MCh, or 30nM U46619 and 30nM MCh. The addition of agonists were represented by the color bars (100 nM MCh: black, 100 nM 5-HT: green, 30nM MCh: grey,

30 nM U46619: blue).

Figure E8. Histology of CTR and TMEM16A^{SMKO} lungs with and without asthma.

Arrows point positions with inflammatory cells infiltration, Bar = 100 μ m (long) or 50 μ m (short).

Table E1: Western blot primary antibodies

Catalog No.	Target	Host Species	Vendor	Final Concentration
ab53212	TMEM16A	rabbit	abcam	1:500
ab134959	TP R	rabbit	abcam	1:500
sc-9108	mAChR M3	rabbit	santa cruz	1:200
ab66049	5-HT _{2A} R	rabbit	abcam	1:100
sc-25506	Flotillin-1	rabbit	santa cruz	1:1000
a5441	β -actin	Mouse	Sigma aldrich	1:10000

SUPPLEMENTAL MOVIE LEGENDS

Video E1: 60 mM KCl induced GCaMP3 fluorescence signal of CTR and *Tmem16a*^{SMKO}

ASMs

This video shows 60 mM KCl induced Ca²⁺ dynamics in CTR and *Tmem16a*^{SMKO} ASMs by recording GCaMP3 fluorescence. The color bar indicates fluorescent intensity (arbitrary units).

Video E2: 3 μ M MCh induced GCaMP3 fluorescence signal of CTR and *Tmem16a*^{SMKO}

ASMs

This video shows 3 μ M MCh induced Ca^{2+} dynamics in CTR and *Tmem16a*^{SMKO} ASMs by recording GCaMP3 fluorescence. The color bar indicates fluorescent intensity (arbitrary units).

Video E3: 300 nM 5-HT induced GCaMP3 fluorescence signal of CTR and *Tmem16a*^{SMKO} ASMs

This video shows 300 nM 5-HT induced Ca^{2+} dynamics in CTR and *Tmem16a*^{SMKO} ASMs by recording GCaMP3 fluorescence. The color bar indicates fluorescent intensity (arbitrary units).

Video E4: 3 μ M 5-HT induced GCaMP3 fluorescence signal of CTR and *Tmem16a*^{SMKO} ASMs

This video shows 3 μ M 5-HT induced Ca^{2+} dynamics in CTR and *Tmem16a*^{SMKO} ASMs by recording GCaMP3 fluorescence. The color bar indicates fluorescent intensity (arbitrary units).