The Transmembrane Protein 16A Ca^{2+} -activated Cl^- Channel in Airway Smooth Muscle Contributes to Airway Hyperresponsiveness

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Rationale: Asthma is a chronic inflammatory disorder with a characteristic of airway hyperresponsiveness (AHR). Ca^{2+} -activated Cl^- [$\text{Cl}_{(\text{Ca})}$] channels are inferred to be involved in AHR, yet their molecular nature and the cell type they act within to mediate this response remain unknown.

Objectives: Transmembrane protein 16A (TMEM16A) and TMEM16B are $\text{Cl}_{(\text{Ca})}$ channels, and activation of $\text{Cl}_{(\text{Ca})}$ channels in airway smooth muscle (ASM) contributes to agonist-induced airway contraction. We hypothesized that *Tmem16a* and/or *Tmem16b* encode $\text{Cl}_{(\text{Ca})}$ channels in ASM and mediate AHR.

Methods: We assessed the expression of the TMEM16 family, and the effects of niflumic acid and benzbromarone on AHR and airway contraction, in an ovalbumin-sensitized mouse model of chronic asthma. We also cloned TMEM16A from ASM and examined the Cl^- currents it produced in HEK293 cells. We further studied the impacts of TMEM16A deletion on Ca^{2+} agonist-induced cell shortening, and on $\text{Cl}_{(\text{Ca})}$ currents activated by Ca^{2+} sparks (localized, short-lived Ca^{2+} transients due to the opening of ryanodine receptors) in mouse ASM cells. **Measurements and Main Results:** TMEM16A, but not TMEM16B, is expressed in ASM cells and its expression in these cells is up-regulated in ovalbumin-sensitized mice. Niflumic acid and benzbromarone prevent AHR and contraction evoked by methacholine in ovalbumin-sensitized mice. TMEM16A produces $\text{Cl}_{(\text{Ca})}$ currents with kinetics similar to native $\text{Cl}_{(\text{Ca})}$ currents. TMEM16A deletion renders Ca^{2+} sparks unable to activate $\text{Cl}_{(\text{Ca})}$ currents, and weakens caffeine- and methacholine-induced cell shortening.

Conclusions: *Tmem16a* encodes $\text{Cl}_{(\text{Ca})}$ channels in ASM and contributes to Ca^{2+} agonist-induced contraction. In addition, up-regulation of TMEM16A and its augmented activation contribute to AHR in an ovalbumin-sensitized mouse model of chronic asthma. TMEM16A may represent a potential therapeutic target for asthma.

Keywords: TMEM16A; airway smooth muscle; airway hyperresponsiveness

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Airway hyperresponsiveness is one of the cardinal features of asthma, a complex disorder with a great deal of mortality and morbidity. Ca^{2+} -activated Cl^- channels have been shown to be involved in airway hyperresponsiveness. However, the molecular basis of these channels and the cell type in which they play their role in airway hyperresponsiveness remain elusive. We hypothesized that the TMEM16 Ca^{2+} -activated Cl^- channel in airway smooth muscle (ASM) cells plays an important role in mediating airway hyperresponsiveness.

What This Study Adds to the Field

We show that TMEM16A, but not TMEM16B, is responsible for Ca^{2+} -activated Cl^- channels in ASM cells and contributes to Ca^{2+} agonist-induced contraction. We also demonstrate that TMEM16A in ASM cells is up-regulated in an ovalbumin-induced mouse model of chronic asthma. Functional blockage of these channels by two pharmacological inhibitors, niflumic acid and benzbromarone, prevents airway hyperresponsiveness *in vivo* and hypercontraction *in vitro*. Thus, TMEM16A in ASM cells represents a key molecule mediating AHR, and it could serve as a therapeutic target in asthma.

Asthma, affecting 300 million people worldwide, is characterized by chronic inflammation, mucus overproduction, reversible airway obstruction, and airway hyperresponsiveness (AHR). AHR is a heightened tendency to airway narrowing in response to non-specific contractile agents, and underlies much of the pathology of asthmatic symptoms. Although intensively studied, the cellular and molecular mechanisms underlying AHR remain only partially understood and controversial. A number of studies have implied that Ca^{2+} -activated Cl^- [$\text{Cl}_{(\text{Ca})}$] channels are involved in AHR. Gene expression profile studies have consistently revealed that type 3 $\text{Cl}_{(\text{Ca})}$ channels (mCLCA3) are highly up-regulated in mouse models of asthma (1–6), and hCLCA1, the human counterpart of mCLCA3, is significantly more abundant in the airway epithelium of patients with asthma (4, 7, 8). Moreover, suppressing the expression of mCLCA3 or hCLCA1 by antisense or its function by niflumic acid, a relatively selective inhibitor of $\text{Cl}_{(\text{Ca})}$ channels, prevents allergen- or helper T-cell type 2 (Th2) cytokine (e.g., IL-13)-induced mucus overproduction and AHR (5, 9–11). Not surprisingly, mCLCA3/hCLCA1

has even been hailed as, potentially, “the asthma channel” (12). However, genetic deletion of mCLCA3 causes no detectable changes in AHR (13, 14), and mCLCA3 has been found to be a secreted protein rather than a membrane protein (15, 16). These studies highlight the necessity to reexamine the molecular nature of $Cl_{(Ca)}$ channels in airways.

Mounting evidence indicates that transmembrane protein 16A (TMEM16A) and TMEM16B are $Cl_{(Ca)}$ channels (17–19). In airway epithelial cells, TMEM16A is expressed, and knocking it out decreases Cl^- secretion in response to Ca^{2+} -dependent agonists (20, 21). Interestingly, TMEM16A is up-regulated in the airway epithelial cells of patients with asthma and in a mouse model of acute asthma (22); Th2 cytokine IL-4 markedly up-regulates TMEM16A expression in human airway epithelial cells (17). Last, deletion of TMEM16A results in congenital cartilaginous defects, a possible reason these mice die neonatally (23). However, it remains unclear whether TMEM16A functions as a $Cl_{(Ca)}$ channel in nonepithelial cells in airways and contributes to AHR.

Airway smooth muscle (ASM) cells are the major resident cells in airways, and their dysfunction has been implicated as contributing to AHR (24–27). It is known that the reversal potential for chloride in smooth muscle cells is about -20 mV, and thus activation of $Cl_{(Ca)}$ channels depolarizes the membrane, leading to contraction (28). We have found that $Cl_{(Ca)}$ channels in ASM cells have a Ca^{2+} sensitivity almost identical to that of TMEM16A (19, 29). Moreover, TMEM16A is highly expressed in ASM (21). We therefore hypothesized that *Tmem16a* encodes $Cl_{(Ca)}$ channels in these cells, and that augmented TMEM16A activity plays an essential role in AHR. To test these possibilities, we studied the currents produced by a TMEM16A clone from ASM, and the effect of TMEM16A deletion on $Cl_{(Ca)}$ currents and contraction in ASM. We also assessed changes in the expression of TMEM16, and the effects of niflumic acid and benzbromarone, a TMEM16A blocker (22), on airway responsiveness and contraction in an ovalbumin (OVA)-sensitized mouse model of chronic asthma. We found that TMEM16A encodes $Cl_{(Ca)}$ channels in ASM cells and is an important determinant in AHR of this OVA-sensitized mouse model of chronic asthma.

METHODS

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and TMEM16A null mutant mice were generated by Rock and colleagues (23). The protocols for animal experiments were approved by the Animal Care Committee of the University of Massachusetts Medical School (Worcester, MA) (A-1473 to R.Z.G.) and Nanjing University (Nanjing, China) (MZ-5 to M.-S.Z.).

Immunization and Airway Challenge

Six- to 8-week-old female C57BL/6 mice were intraperitoneally injected with $80\text{ }\mu\text{g}$ of OVA in 0.2 ml of aluminum hydroxide (2%) on Days 0 and 14, followed by aerosol challenge with 2% OVA for 60 minutes on Days 24, 25, and 26. After Day 26, the mice were persistently challenged with 2% OVA three times per week until the end of the study. As demonstrated previously, mice challenged until some time between Days 35 and 55 exhibited typical features of chronic allergic asthma (30). Therefore these mice were used in the present study. Control animals received 0.2 ml of aluminum hydroxide (2%) via intraperitoneal injection and were challenged with aerosolized phosphate-buffered saline (PBS) on the same days as were animals in the OVA group.

Measurement of Airway Responsiveness

Lung mechanics were measured *in vivo* 24 hours after the last challenge in a custom-made whole-body plethysmography chamber as described

previously (30) or with a computer-controlled ventilator (FlexiVent system; Scireq, Montreal, PQ, Canada) (*see* the online supplement). Results are expressed as the percentage increase in airway resistance above baseline after each dose of Mch.

Reverse Transcription PCR and Real-Time RT-PCR

Total RNA was extracted from mouse tracheal smooth muscle with TRIzol (Invitrogen, CA) and reverse transcribed with Moloney murine leukemia virus transcriptase (TaKaRa Biotechnology Co., Ltd, Dalian, China). PCRs were performed with specific primers as listed in the online supplement. For real-time PCR, TMEM16A mRNA expression was determined with an ABI7300 detection system and SYBR green I (TaKaRa Biotechnology) with β -actin as an internal control.

TMEM16A Cloning, Expression, and Current Recording

TMEM16A from ASM was cloned and expressed in HEK293 cells as described in the online supplement. Cl^- currents were induced by a series of voltage steps in the conventional whole-cell configuration in the presence of 300 nM cytosolic Ca^{2+} .

Western Blotting

Smooth muscle α -actin, α -tubulin, and β -actin expression was assessed in ASM tissues from PBS control mice and OVA-sensitized mice by Western blotting using specific antibody agonists of these three proteins, as described in the online supplement.

Immunohistochemical Staining and Quantification

Immunostaining was the same as described previously (31). Rabbit anti-TMEM16A and mouse monoclonal smooth muscle α -actin antibody were purchased from Abcam (Cambridge, MA) and Neomarkers (Fremont, CA), respectively. Image quantification was performed with custom-written software.

Bronchial Ring Isometric Contraction Bioassay

Airway rings from mouse trachea and mainstem bronchi were attached to mounting support pins that were connected to force transducers, and changes in their force were recorded with a multiwire myograph system (model 610M; Danish Myo Technology, Aarhus, Denmark) as reported previously (32).

Preparation of Single Isolated Mouse ASM Cells

Tracheas from TMEM16A null mice and wild-type mice were treated, first with papain and then collagenase, to obtain isolated single cells as described previously with minor modifications (29) (*see* the online supplement).

Simultaneous Patch-Clamp Recording and Ca^{2+} Spark Imaging

Membrane currents were recorded using a whole-cell patch recording configuration and fluorescence images were obtained with Fluo-3 as the calcium indicator and a custom-built wide-field, high-speed digital imaging system. The conventional fluorescence ratio, $\Delta F/F_0$, was used to quantify Ca^{2+} sparks (localized, short-lived Ca^{2+} transients due to the opening of ryanodine receptors), as described previously (33).

Measurement of Cell Shortening

Cells were imaged with a custom-built wide-field digital imaging system, and their lengths were determined with custom software to manually trace down the center of the cell (34).

Data Analysis and Statistics

Results are presented as means $\pm$ SEM. Data were analyzed by Student *t* test or one-way analysis of variance (ANOVA) with Tukey post-test analysis for significant differences. The significance level was set at $P < 0.05$.

RESULTS

The $\text{Cl}_{(\text{Ca})}$ Channel Inhibitor Niflumic Acid Blocks Airway Hyperresponsiveness in OVA-sensitized Mice

We examined the effect of niflumic acid on airway responsiveness to methacholine (Mch) in an OVA-sensitized mouse model of chronic asthma (30). This model was chosen because asthma is a chronic inflammatory airway disorder. To minimize the potential interference of airway epithelial cells and airway mucus in the delivery of Mch and niflumic acid, we administered them intravenously. Niflumic acid is one of the most specific $\text{Cl}_{(\text{Ca})}$ channel inhibitors (35), but it can also inhibit other Cl^- channels and activate large-conductance Ca^{2+} -activated K^+ (BK) channels in several cell types (36). However, we (and L.J. Janssen and S.M. Sims) have shown that niflumic acid does not activate BK channels in ASM cells (29, 37, 38), and we have confirmed this with evidence from force measurements of mouse airways (*see* Figure E1 in the online supplement). To date, γ -aminobutyric acid A (GABA) and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels are the only additional Cl^- channels to have been found in the plasma membrane of ASM cells (39, 40), yet both of them are insensitive to niflumic acid. Therefore niflumic acid is a specific inhibitor of $\text{Cl}_{(\text{Ca})}$ channels in ASM cells and is a useful reagent for examination of the involvement of $\text{Cl}_{(\text{Ca})}$ channels in airway hyperresponsiveness (AHR).

As shown in Figure 1A, Mch dose-dependently increased airway resistance in OVA-sensitized mice, and it did so to a much greater extent than in PBS control mice. ANOVA shows that there was a significant difference in the responses between OVA-sensitized mice and PBS controls ($P < 0.0001$; $n = 5$ for both groups). At the Mch dose of 2 $\mu\text{g}/\text{kg}$, airway resistance was about twofold greater in OVA-sensitized mice than in the PBS controls ($P < 0.05$); at an Mch dose of 5 $\mu\text{g}/\text{kg}$, it reached about fivefold greater in the sensitized mice than in the controls ($P < 0.001$). Strikingly, niflumic acid (0.64 $\mu\text{g}/\text{kg}$) almost completely prevented airway responses in both PBS controls and OVA-sensitized mice. ANOVA indicates there was no difference in the responses between PBS controls and OVA-sensitized mice in the presence of niflumic acid ($P > 0.05$, $n = 6$ for PBS control, and $n = 7$ for OVA).

We also analyzed airway responsiveness to Mch in terms of the corresponding changes in dynamic compliance (C_{dyn}) (Figure E2). Mch-induced decreases in C_{dyn} were significantly greater in OVA-sensitized mice than in PBS control mice ($P < 0.0005$). Moreover, niflumic acid (0.64 $\mu\text{g}/\text{kg}$) significantly inhibited the Mch-induced decrease in C_{dyn} ($P < 0.0005$, OVA-sensitized mice with and without niflumic acid). Because niflumic acid was administered 5 minutes before Mch application, it is unlikely that the effect of niflumic acid on lung function resulted from its influence on cytokine production, mucin synthesis, or goblet cell degranulation (9–11). To validate this, we examined the effect of niflumic acid on IL-17, a cytokine critical to the pathogenesis of asthma in this mouse model (30). We found that niflumic acid applied 5 minutes before airway resistance measurements did not change the IL-17 level in OVA-sensitized mice (Figure E3). Together, these results indicate that increased activity of $\text{Cl}_{(\text{Ca})}$ channels contributes to the AHR in OVA-sensitized mice.

Niflumic Acid Reverses Augmented Contraction of Isolated Airways from OVA-sensitized Mice

Previous studies and our results in Figure 1A and Figure E2 suggest that $\text{Cl}_{(\text{Ca})}$ channels play an important role in AHR in the mouse model of asthma (5, 9). Airway resistance is the result of the interplay among several factors including mucus production,

Figure 1. Niflumic acid (NFA) prevents airway hyperresponsiveness to methacholine (Mch) by blocking airway smooth muscle (ASM) contraction in an ovalbumin (OVA)-sensitized mouse model of chronic asthma. (A) Response to Mch was significantly increased 24 hours after the last allergen challenge in OVA-sensitized mice ($P < 0.0001$; $n = 5$ for phosphate-buffered saline [PBS] group and OVA group). Moreover, NFA essentially blocked the airway responses to Mch in both groups ($P > 0.05$, $n = 6$ for PBS control, and $n = 7$ for OVA group). Mch doses are shown as micrograms of Mch per kilogram mouse weight, and data are expressed as the percentage above baseline. (B) OVA sensitization and challenge caused stronger contractions in response to Mch compared with PBS-challenged control mice. The isometric force was measured with cumulative addition of Mch in concentrations between 10^{-8} M and 10^{-4} M. Symbols and error bars represent means $\pm$ SEM ($n = 7$, $*P < 0.05$ and $**P < 0.01$, PBS group vs. OVA group). (C) NFA dose-dependently inhibited contractile responses to Mch in both PBS controls and OVA-sensitized mice. NFA was cumulatively added after the contraction was completely elicited by 30 μM Mch. Note that the inhibition in the absolute force was greater in OVA-sensitized mice than in control mice. Results represent means $\pm$ SEM ($n = 4$ –6, $*P < 0.05$).

goblet cell hyperplasia/metaplasia, lung elasticity, and smooth muscle contraction. To ascertain the role of $\text{Cl}_{(\text{Ca})}$ channels in ASM in mediating OVA-induced AHR as shown in Figure 1A, we studied the effect of niflumic acid on the isometric force generation of isolated airways without epithelium. The advantage of this preparation is that the potential contributions of mucus content and lung elasticity to AHR are eliminated and the influence of epithelium on AHR is minimized. As expected,

Mch dose-dependently increased force generation in the airways from PBS control mice (Figure 1B). Yet, in OVA-sensitized mice, the dose-response curve of Mch was shifted to the left and the maximal response to Mch was increased (Figure 1B), an indication that the ASM had become hyperresponsive. More interestingly, when applied after an Mch (30 μ M)-induced maximal contraction, niflumic acid dose-dependently reversed the contraction in both groups but to a much greater extent in the OVA-sensitized group (Figure 1C; $P < 0.05$, OVA group vs. PBS group). At 100 μ M, niflumic acid reduced the force by 4.68 ± 0.64 mN in PBS group ($n = 5$) and by 8.42 ± 1.14 mN in OVA group ($n = 5$, $P < 0.05$), whereas at 33 μ M, it reduced the force by 4.53 ± 0.62 mN ($n = 5$) in the control group and by 8.06 ± 1.04 mN ($n = 5$) in the OVA group ($P < 0.05$; OVA group vs. PBS group).

TMEM16A Is Up-regulated in ASM Cells from OVA-sensitized Mice

Tmem16a and *Tmem16b* are genes encoding $\text{Cl}_{(\text{Ca})}$ channels (17–19). It has been shown that mouse ASM expresses TMEM16A (21), and that $\text{Cl}_{(\text{Ca})}$ channels in these cells have a Ca^{2+} sensitivity almost identical to that of TMEM16A (19, 29). These results raise the possibility that *Tmem16a* and/or *Tmem16b* encode $\text{Cl}_{(\text{Ca})}$ channels contributing to AHR as shown previously. To examine this possibility, we studied the expression of these two genes in airways from PBS-treated control mice and OVA-sensitized mice. Using a pair of specific primers for *Tmem16b*, no mRNA was detected in airways from either the control or OVA-sensitized mice (Figure 2A), indicating this gene is not expressed in airways. In contrast, *Tmem16a* transcripts were detected in airways from both the controls and OVA-sensitized mice (Figure 2A). Using real-time PCR, we detected no significant difference in TMEM16A mRNA between the controls and OVA-sensitized mice (data not shown).

To assess whether the level of TMEM16A protein is different in ASM cells between the controls and OVA-sensitized mice, we performed immunostaining of TMEM16A in airway tissues. To ensure the specific detection of ASM, and to permit quantification of TMEM16A, the airways were costained with smooth muscle α -actin. As shown by Western blotting in Figure E4, smooth muscle α -actin, α -tubulin, and β -actin, three housekeeping genes, did not change their levels between the control mice and OVA-sensitized mice. We therefore normalized the total TMEM16A signal in each sample by dividing it by its smooth muscle α -actin signals. Figure 2B shows the distribution of TMEM16A and smooth muscle α -actin in a PBS control and an OVA-sensitized mouse. By normalizing to the α -actin levels, we found that TMEM16A was up-regulated by 48% ($P < 0.05$; $n = 8$) in ASM cells from OVA-sensitized mice compared with the control mice.

TMEM16A from ASM Cells Generates $\text{Cl}_{(\text{Ca})}$ Currents

To ascertain whether *Tmem16a* from ASM cells encode $\text{Cl}_{(\text{Ca})}$ channels, we cloned this gene from these cells and examined the currents it generated in HEK293 cells. Figure 3A shows that TMEM16A, as marked by green fluorescent protein (GFP), localized predominantly on the plasma membrane. Figure 3B displays a series of currents in a TMEM16A-GFP-positive cell in response to voltage stimuli in the presence of 300 nM cytosolic Ca^{2+} . The currents reversed at the calculated reversal potential for Cl^- (i.e., ~ 0 mV), suggesting that the observed currents were primarily TMEM16A-produced Cl^- currents. Importantly, at this level of Ca^{2+} , these currents exhibited an outward rectification and time-dependent activation at positive potentials, two of the key features of native $\text{Cl}_{(\text{Ca})}$ channels in

Figure 2. Expression of transmembrane protein 16A (TMEM16A) is up-regulated in airway smooth muscle (ASM) cells from ovalbumin (OVA)-sensitized mice. (A) TMEM16A, but not TMEM16B, is expressed in airways from control (Ctr) mice and OVA-sensitized mice as assessed by RT-PCR with primers specific to both genes, respectively. Eye and brain tissues were used as positive controls for TMEM16B expression. (B) Distribution of TMEM16A in ASM as revealed by immunostaining with specific anti-TMEM16A. Smooth muscle cells were marked by smooth muscle α -actin (SMA) antibody. To better reveal the distribution pattern of TMEM16A in ASM cells, TMEM16A stainings in both control mice (i) and OVA-sensitized mice (ii) were scaled up twofold from the original staining intensity and two regions of interest (white boxes) were enlarged. (iii) The ratio of TMEM16A to SMA is 0.57 ± 0.07 ($n = 8$, mean $\pm$ SEM) in the OVA group and 0.39 ± 0.06 in the phosphate-buffered saline (PBS) control group ($n = 6$, $*P < 0.05$). Scale bars: 500 μ m.

a variety of tissues including smooth muscle cells from airways (37) and pulmonary arteries (41).

To directly examine whether TMEM16A generates $\text{Cl}_{(\text{Ca})}$ currents in ASM cells, we compared the spontaneous transient inward currents (STICs) in neonatal ASM cells from TMEM16A knockout mice and their isogenic controls. It is known that in ASM cells, STICs result from the opening of approximately 300 $\text{Cl}_{(\text{Ca})}$ channels activated by Ca^{2+} sparks, that is, short-lived and localized Ca^{2+} transients due to the opening of ryanodine receptors in sarcoplasmic reticulum (29, 42). Moreover, $\text{Cl}_{(\text{Ca})}$ channels in mouse ASM cells appear to localize predominantly in the areas that are reached by Ca^{2+} sparks (29). To record Ca^{2+} sparks and STICs, we simultaneously performed high-speed Ca^{2+} imaging with Fluo-3 as Ca^{2+} indicator, and membrane current recording with patch-clamp in the conventional whole-cell configuration. In neonatal TMEM16A^{+/+} ASM cells, Ca^{2+} sparks were temporally associated with STICs when the membrane potential was held at -80 mV (Figure 4A). Ryanodine (100 μ M) blocked Ca^{2+} sparks and STICs, whereas niflumic acid blocked only STICs (data not shown), confirming that ryanodine receptors mediate Ca^{2+} sparks and $\text{Cl}_{(\text{Ca})}$ channels mediate STICs in neonatal ASM cells, as they do in mature ASM cells from mouse and guinea pig (29, 34, 42). However, in TMEM16A^{-/-} ASM cells, Ca^{2+} sparks did not associate with STICs (Figure 4), but they still activated

Figure 3. Cloned transmembrane protein 16A (TMEM16A) from airway smooth muscle (ASM) generates Ca^{2+} -activated Cl^- currents. (A) TMEM16A localizes predominantly in the periphery of HEK293 cells. Images show a TMEM16A-GFP-positive cell under 488-nm illumination (top) and under visible light illumination (bottom) 1 day after transfection with TMEM16A-GFP cDNA. (B) Representative recordings of a TMEM16A-positive cell (left middle) and a TMEM16A-negative cell (left bottom), and mean values (right, solid circles, $n = 7$ for the positive cells; and open circles, $n = 5$ for the negative cells) as measured at the end of command pulses in the presence of 300 nM cytosolic Ca^{2+} . Note that the slow rise in the currents at the depolarizing voltages (left) and outward rectification in current-voltage relationship (right) are similar to native Ca^{2+} -activated Cl^- channels in smooth muscle (41).

STOCs (i.e., spontaneous transient outward currents as a result of the opening of large-conductance Ca^{2+} -activated K^+ channels) (Figure E5). Compared with the wild-type cells, the amplitude and frequency of Ca^{2+} sparks in the knockout cells were not significantly different (frequency: 1.6 ± 0.4 Hz in WT cells and 1.7 ± 0.5 in TMEM16A $^{-/-}$ cells; amplitude: $45 \pm 11\%$ $\Delta\text{F}/\text{F}_0$ in WT cells ($n = 12$) and $41 \pm 9\%$ $\Delta\text{F}/\text{F}_0$ in TMEM16A $^{-/-}$ cells; $n = 12$ for WT cells and $n = 9$ for KO cells; $P > 0.05$ by unpaired t test) (Figure 4B). These data indicate that (1) lack of associated STICs in TMEM16A $^{-/-}$ cells is not due to a decrease in the amplitude of Ca^{2+} sparks, but rather is due to the absence of TMEM16A, and (2) deletion of TMEM16A does not affect Ca^{2+} sparks.

Impairment in Agonist-induced ASM Cell Shortening from TMEM16A $^{-/-}$ Mice

To assess the role of TMEM16A in bronchoconstrictor-induced contraction, we studied the impact of TMEM16A knockout on ASM cell shortening. Cell length was determined by analysis of images acquired with our digital imaging system as described previously (34). Mch (100 μM) contracted ASM cells from WT mice by $46 \pm 8\%$ ($n = 18$), and those from TMEM16A $^{-/-}$ mice by $30 \pm 8\%$ ($n = 15$) ($P < 0.05$, WT cells vs. TMEM16A $^{-/-}$ cells; Figure 4C). For comparison, caffeine (20 mM), a ryanodine receptor agonist, induced contraction by $38 \pm 7\%$ in WT cells and by $19 \pm 5\%$ in TMEM16A $^{-/-}$ cells ($P < 0.05$, $n = 14$ for WT cells and $n = 12$ for TMEM16A $^{-/-}$ cells; Figure 4C). These results suggest that TMEM16A deletion impairs contractile agonist-mediated contraction in ASM.

TMEM16A Antagonist Benzbromarone Blocks AHR in OVA-sensitized Mice

Our findings that TMEM16A is the only $\text{Cl}_{(\text{Ca})}$ channel being expressed in ASM cells, and that TMEM16A is up-regulated in AHR in the mouse model of chronic asthma, prompted us to address whether TMEM16A is required for AHR. To this end, we examined the effect of benzbromarone, a TMEM16A-specific blocker (22), on AHR and augmented airway contraction in the mouse model of chronic asthma. We first determined the effect of benzbromarone on Mch-induced contraction. We found that benzbromarone can inhibit this contraction dose-dependently, extending the observation by Huang and colleagues (22). We next assessed the effect of benzbromarone on *in vivo* airway resistance in response to Mch. As shown in Figure 5, benzbromarone can fully prevent AHR in OVA-sensitized chronic asthma mice. Similar to niflumic acid, benzbromarone did not change the IL-17 level in bronchoalveolar

lavage fluid in OVA-sensitized mice (Figure E3). To confirm the role of TMEM16A in ASM cells in AHR, we studied the effect of benzbromarone on Mch-induced hypercontraction. Figure 5C shows that benzbromarone can inhibit this contraction in a dose-dependent manner. Notably, benzbromarone reduced the contraction by more in OVA-sensitized mice than in the control mice.

DISCUSSION

In the present study we demonstrated that *Tmem16a* encodes $\text{Cl}_{(\text{Ca})}$ channels in ASM cells, and that its activation contributes to agonist-induced contraction. Also, TMEM16A expression in ASM cells is up-regulated on OVA sensitization and challenge. Moreover, niflumic acid and benzbromarone, a specific blocker of TMEM16A, fully prevent *in vivo* AHR and *in vitro* augmented ASM contraction in this mouse model of chronic asthma. Because *Tmem16a* and *Tmem16b* are the only established genes that encode $\text{Cl}_{(\text{Ca})}$ channels, and TMEM16B is not expressed in airways, we suggest that up-regulation of TMEM16A channels in ASM cells is a major biological event contributing to AHR in OVA-sensitized mice.

Pharmacological and biophysical studies have long indicated the presence of $\text{Cl}_{(\text{Ca})}$ channels in ASM cells (38), and functional studies have also demonstrated that this channel plays a significant role in mediating agonist-induced Ca^{2+} signaling and contraction in ASM (28, 33, 43–45). But, as in other cell types, the gene for this channel in ASM cells has remained elusive. In this study, we found that (1) cloned TMEM16A from ASM cells generates $\text{Cl}_{(\text{Ca})}$ currents with kinetics that resemble those of native smooth muscle cells from airways and pulmonary arteries (41) and (2) genetic deletion of TMEM16A prevents Ca^{2+} sparks from activating STICs, a phenotypical electrical signal in ASM cells resulting from the opening of approximately 300 $\text{Cl}_{(\text{Ca})}$ channels near the Ca^{2+} spark sites (29). Together with the findings that TMEM16A is highly expressed in ASM cells from both neonatal mice and mature mice (20, 21) (Figure 2), and that $\text{Cl}_{(\text{Ca})}$ channels in ASM cells have Ca^{2+} sensitivity almost identical to that of TMEM16A (19, 29), it is safe to conclude that *Tmem16a* encodes $\text{Cl}_{(\text{Ca})}$ channels in ASM cells. Because these cells do not express TMEM16B, another isoform in the TMEM16 family that encodes $\text{Cl}_{(\text{Ca})}$ channels (18), it is highly likely that *Tmem16a* is the sole gene for $\text{Cl}_{(\text{Ca})}$ channels in ASM cells.

We further observed that both Mch and caffeine cause less contraction in TMEM16A $^{-/-}$ ASM cells. This suggests that contractile agonists activate these channel proteins as part of their mechanisms to generate the contraction. This can be achieved because the reversal potential for Cl^- in smooth muscle is more positive than the resting membrane potential. Thus Ca^{2+} agonists caffeine and Mch activate TMEM16A

Figure 4. Transmembrane protein 16A (TMEM16A) deletion causes failure of Ca^{2+} sparks to activate spontaneous transient inward currents (STICs) and impairment in Ca^{2+} agonist-induced airway smooth muscle (ASM) cell shortening. (A) Spatial and temporal evolution of spontaneous Ca^{2+} sparks in ASM cells from neonatal wild-type (WT) and TMEM16A^{-/-} mice. The deletion of *Tmem16a* was confirmed by a genotyping protocol (23). The cells were loaded with 50 μM Fluo-3 via patch pipette and held at -80 mV. Images with a pixel size of 333 × 333 nm were acquired at a rate of 67 Hz (WT) and 100 Hz (knockout; KO) with an exposure time of 5 milliseconds. Change in Ca^{2+} concentration is expressed as $\Delta\text{F}/\text{F}_0$ and displayed on a pseudocolor scale. Traces in the top row display the time course of $\Delta\text{F}/\text{F}_0$ (at the pixel with the peak fluorescence) during the course of Ca^{2+} sparks shown above. Numbers and arrows around the traces correspond to the time when the images above were acquired. Traces in the bottom row show membrane currents associated with Ca^{2+} sparks. (B) Average results indicate that Ca^{2+} sparks were the same in both KO cells and WT cells, but no STICs were detected in KO cells. Data represent means ± SEM; $n = 9$ cells for KO cells and 12 cells for WT cells. (C) Agonist-induced cell shortening is impaired in ASM cells from TMEM16A^{-/-} mice. Isolated single ASM cells were imaged with a digital microscope as described previously (34). Bar charts show average results in response to 100 μM methacholine (Mch) (left) and 20 mM caffeine (right) in WT cells and TMEM16A^{-/-} cells. Data represent means ± SEM; for Mch, $n = 18$ for WT cells and 15 for KO cells, $*P < 0.05$ by unpaired Student *t* test; for caffeine, $n = 14$ for WT cells and $n = 12$ for TMEM16A^{-/-} cells, $*P < 0.05$ by unpaired Student *t* test.

Cl^- channels to depolarize the membrane, which in turn activates voltage-dependent Ca^{2+} channels, as demonstrated previously in several studies (28). It is worth noting that systemic

TMEM16A knockout mice die neonatally, at least partially due to congenital airway cartilage malformation and airway closure (23). Therefore, TMEM16A is not only critical for the development of airways but also important in mediating the contraction of airways. It would be interesting to determine whether the impairment in cartilage is attributed to the lack of Cl^- currents, due to deletion of TMEM16A, in ASM cells and/or airway epithelial cells.

We noticed a significant difference in contraction in response to Ca^{2+} agonists between TMEM16A knockout and $\text{Cl}_{(\text{Ca})}$ channel inhibitor treatments, that is, TMEM16A deletion caused an approximately 50% decrease in agonist-induced cell shortening (Figure 4C), whereas niflumic acid and benzbramarone fully inhibited agonist-induced contraction (Figure 1C and Figures 5A and 5C). The reason for this difference is unclear. In vascular smooth muscle cells, niflumic acid activates BK channels (36), but two lines of evidence indicate this does not occur in ASM cells. First, electrophysiological recordings showed that niflumic acid does not activate BK channels in ASM cells from mouse and guinea pig (29, 38), and second, in the present study, paxilline, a specific blocker of BK channels (46, 47), did not affect niflumic acid-induced relaxation of airways precontracted by Mch. Interestingly, there have been studies showing that smooth muscle expresses niflumic acid-sensitive Cl^- channels in sarcoplasmic reticulum, and Cl^- flux via these channels could facilitate Ca^{2+} release by maintaining the driving force for Ca^{2+} across the membrane (48, 49). As such, blocking these Cl^- channels by applying niflumic acid could suppress the Ca^{2+} release induced by Ca^{2+} agonists. Although the nature of these Cl^- channels is yet to be determined, TMEM16A is not a likely candidate because TMEM16A appears to localize predominantly on the plasma membrane (Figure 3), and the properties of Ca^{2+} sparks are not altered by TMEM16A deletion (Figure 4). Therefore a plausible reason for the difference in contraction between niflumic acid treatment and TMEM16A deletion could be that the former blocks both TMEM16A in the plasma membrane and Ca^{2+} -independent Cl^- channels in the sarcoplasmic reticulum whereas TMEM16A deletion affects only $\text{Cl}_{(\text{Ca})}$ channels in the plasma membrane.

$\text{Cl}_{(\text{Ca})}$ channels have long been suspected in mediating AHR (5). In the present study, we confirmed this suspicion as niflumic acid and benzbramarone can prevent AHR to Mch stimulation in OVA-sensitized mice. By directly studying isolated airways without epithelium, we provide further evidence that it is the augmented ASM contraction per se that contributes to AHR in this mouse model of chronic asthma. Moreover, because niflumic acid and benzbramarone can eliminate the augmented contraction in OVA-sensitized mice, enhanced activity of $\text{Cl}_{(\text{Ca})}$ channels in ASM cells appears to be the determining factor in AHR. This is further supported by our observation that TMEM16A in ASM was up-regulated in OVA-sensitized mice. Because systemic deletion of TMEM16A leads the mice to die young, it prevents us from studying whether up-regulation of TMEM16A in ASM alone is sufficient to induce AHR. Smooth muscle-specific TMEM16A knockout mice, if they survive to maturity, would be needed to address this important and critical question. As elaborated in the Introduction, niflumic acid reverses OVA- or Th2 cytokine-induced AHR, and mCLCA3/hCLCA1 in airway epithelial cells was considered to be its target. But the finding that mCLCA3 is a secreted protein rather than a membrane protein, and that an mCLCA3 knockout displays no change in AHR, indicates this protein is not the $\text{Cl}_{(\text{Ca})}$ channel mediating AHR. With our findings in this study, it would be interesting to examine the relationship between mCLCA3 and TMEM16A in airways. It is possible that mCLCA3 could serve as an upstream signal that increases TMEM16A expression and activity.

Figure 5. Transmembrane protein 16A (TMEM16A) antagonist benzbromarone (Benz) prevents airway hyperresponsiveness (AHR) and augmented airway contraction in an ovalbumin (OVA)-sensitized mouse model of chronic asthma. (A) Benzbromarone dose-dependently inhibited methacholine (Mch)-induced airway contraction in C57BL/6 mice. The isometric force was measured by cumulative addition of Mch in concentrations between 10^{-8} and 10^{-4} M in the presence or absence of benzbromarone. Symbols and error bars represent means $\pm$ SEM, $n = 4$ –19. (B) Compared with phosphate-buffered saline (PBS)-challenged control mice, airway response to Mch was significantly increased 24 hours after the last allergen challenge in OVA-sensitized mice ($n = 5$ for the PBS group and the OVA group). Moreover, benzbromarone essentially blocked the airway responses to Mch in both groups ($n = 6$ for the OVA group; $n = 5$ for the control group). Mch concentrations are the values in the nebulizer. (C) Benzbromarone dose-dependently inhibited contractile responses to Mch in both PBS controls and OVA-sensitized mice. Benzbromarone was cumulatively added after the contraction was completely elicited by 30 μ M Mch. Note that the inhibition in absolute force was greater in OVA-sensitized mice than in control mice. Results represent means $\pm$ SEM ($n = 6$, $*P < 0.05$).

In summary, our data reveal genetically that *Tmem16a* encodes $Cl_{(Ca)}$ channels in ASM cells; it contributes to contractile agonist-mediated contraction and its up-regulation most likely underlies the pathogenesis of AHR in asthma. Given that TMEM16A is at least partially responsible for $Cl_{(Ca)}$ channel currents in response to Ca^{2+} -dependent secretagogues in airway epithelial cells, that its expression is elevated in the airway epithelial cells in patients with asthma and in a mouse model of acute asthma, and that proinflammatory cytokine IL-4 highly up-regulates the expression of TMEM16A in human airway epithelial cells, our results further highlight the importance of TMEM16A in normal airway function and the potential of targeting this protein as a therapeutic strategy for treating asthma and other airway obstructive diseases with AHR.

Author disclosures are available with the text of this article at www.atsjournals.org.

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