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Journal

Proceedings of the National Academy of Sciences of the United States of America, 109(40)

ISSN

0027-8424

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Publication Date

2012-10-02

DOI

10.1073/pnas.1214596109

Peer reviewed

Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction

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Mucous cell hyperplasia and airway smooth muscle (ASM) hyper-responsiveness are hallmark features of inflammatory airway diseases, including asthma. Here, we show that the recently identified calcium-activated chloride channel (CaCC) TMEM16A is expressed in the adult airway surface epithelium and ASM. The epithelial expression is increased in asthmatics, particularly in secretory cells. Based on this and the proposed functions of CaCC, we hypothesized that TMEM16A inhibitors would negatively regulate both epithelial mucin secretion and ASM contraction. We used a high-throughput screen to identify small-molecule blockers of TMEM16A-CaCC channels. We show that inhibition of TMEM16A-CaCC significantly impairs mucus secretion in primary human airway surface epithelial cells. Furthermore, inhibition of TMEM16A-CaCC significantly reduces mouse and human ASM contraction in response to cholinergic agonists. TMEM16A-CaCC blockers, including those identified here, may positively impact multiple causes of asthma symptoms.

Asthma is a significant cause of morbidity and mortality worldwide, and the prevalence of this disease is increasing among all age, sex, and racial groups. Characteristic features of asthma include inflammation, subepithelial fibrosis, hyperplasia of mucus-producing cells, accumulation of mucus within airway lumens, hyperplasia of airway smooth muscle (ASM), and ASM hyperresponsiveness. Together, these symptoms impair lung function by limiting the flow of gases to and from the alveoli in the distal lung.

The current standard of care for asthma involves inhaled corticosteroids for the management of inflammation combined with long-acting agonists of β_2 -adrenergic receptors. Despite this treatment, lung function is not improved in 30–45% of asthmatic patients, and exacerbations continue to be a major problem (reviewed in ref. 1). Asthma can be divided into at least two distinct molecular phenotypes defined by the degree of Th2 inflammation (2, 3). Cytokines, including IL-4 and IL-13, promote airway epithelial mucous cell metaplasia, subepithelial fibrosis, and hyperplasia of smooth muscle in Th2-high asthmatics, and these patients generally show improved lung function with inhaled corticosteroid therapy. A greater understanding of this heterogeneity and the molecular and physiological events that lead to airway remodeling might lead to improved diagnosis and treatment.

Calcium-activated chloride channels (CaCCs) have been ascribed numerous cellular functions (reviewed in refs. 4 and 5), among these are epithelial fluid secretion and smooth muscle contraction, both of which contribute to the progression and severity of asthma. Moreover, calcium-activated chloride currents in the airway epithelium are enhanced by the Th2 cytokines IL-4 and IL-13, as well as IFN- γ (6). For these reasons, CaCC is an attractive potential therapeutic target for asthma (7). However, the study of CaCC was impeded by lack of information about the gene(s) encoding this channel. It was only relatively recently that

TMEM16A (transmembrane protein with unknown function 16, Ano1) was identified as the long-sought CaCC (8–10), and this has enabled investigations of the involvement of CaCC at the molecular level in a variety of contexts. The role of TMEM16A in the airway surface epithelium remains controversial (11, 12).

We hypothesized that increased TMEM16A-CaCC channel abundance and activity might contribute to mucus secretion and airway hyperresponsiveness in asthmatics. Here, we have used transcriptional profiling of primary human cells, immunohistochemistry, and mouse models to demonstrate that TMEM16A is expressed in healthy and asthmatic airway surface epithelial cells, particularly in secretory cells, and in smooth muscle cells. Moreover, the epithelial expression of TMEM16A is increased in asthmatic patients. We describe the identification of small-molecule blockers of TMEM16A-CaCC channels and demonstrate their ability to negatively regulate mucin secretion and ASM contraction. Our data suggest that TMEM16A could be a unique therapeutic target for asthma, with TMEM16A-CaCC channel blockers potentially serving as dual-acting agents to treat the two major causes of symptoms in asthma: mucus hypersecretion and ASM hyperresponsiveness.

Results

Increased Expression of TMEM16A in Epithelial Cells from Asthmatic Human Patients and Asthma Models. Mice sensitized and then challenged with ovalbumin (OVA) reproduce many key features of clinical asthma, including elevated levels of IgE, airway inflammation, mucous cell hyperplasia, and airway hyperresponsiveness (13). To determine whether asthma influences the abundance and distribution of TMEM16A protein in vivo, we sensitized C57BL/6 mice on days 0, 7, and 14 via i.p. injection of 50 μ g OVA adsorbed in 2 mg alum gel in 200 μ L PBS, and then challenged these mice with intranasal instillation of OVA (100 μ g in 40 μ L of saline) on days 21, 22, and 23 (14). Immunofluorescence with polyclonal antibodies against mouse TMEM16A (15) reveals a significant increase of TMEM16A protein in the airway epithelial cells of OVA-challenged mice ($n = 5$) compared with saline controls (Fig. 1*A–D*). We did not detect a signifi-

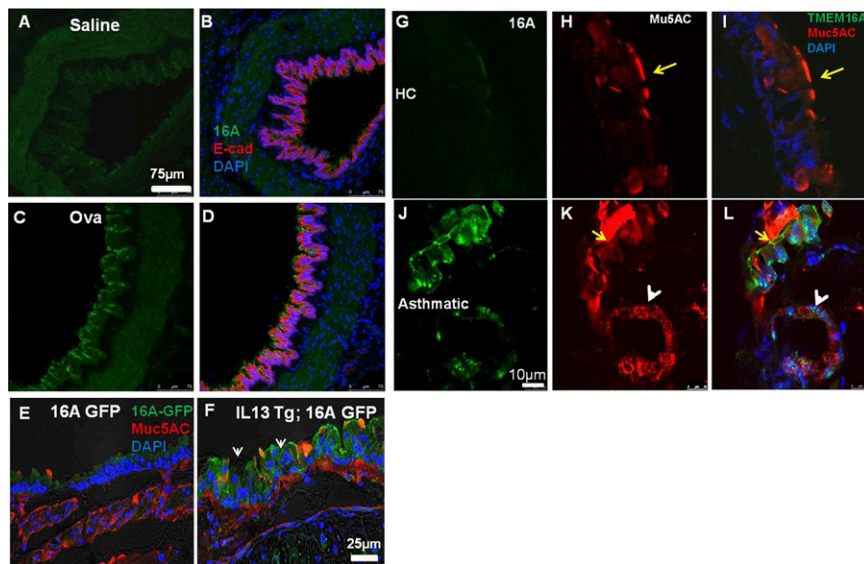
Author contributions: F.H., H.Z., M.W., H.Y., M.K., C.J.P., P.G.W., O.D.S., X.H., D.S., J.V.F., W.F., M.L., Y.-N.J., L.Y.J., and J.R.R. designed research; F.H., H.Z., M.W., H.Y., M.K., C.J.P., P.G.W., O.D.S., M.L.D., X.H., J.V.F., M.L., Y.-N.J., L.Y.J., and J.R.R. performed research; F.H., H.Z., M.W., H.Y., P.G.W., O.D.S., X.H., J.V.F., P.J.W., B.L.M.H., W.F., M.L., Y.-N.J., L.Y.J., and J.R.R. contributed new reagents/analytic tools; F.H., H.Z., M.W., H.Y., M.K., C.J.P., P.G.W., O.D.S., M.L.D., X.H., D.S., J.V.F., M.L., Y.-N.J., L.Y.J., and J.R.R. analyzed data; and F.H., C.J.P., Y.-N.J., L.Y.J., and J.R.R. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1214596109/-DCSupplemental.

Fig. 1. TMEM16A is up-regulated in epithelial cells of asthmatic human patients and asthma models. (A–D) The abundance of TMEM16A protein is increased in airway epithelial cells of the mouse asthma model of ovalbumin sensitization and challenge. (A and B) TMEM16A antibody staining in the airway epithelium of control (saline-treated) mice. (C and D) TMEM16A staining in the airway of OVA-treated asthmatic mice. Epithelial cells are marked with E-cadherin (red, overlay images in B and D). (E and F) The abundance and distribution of TMEM16A were visualized using TMEM16A:GFP fusion protein knock-in mice in which a functional and properly localized fusion protein of TMEM16A and GFP is under control of the endogenous TMEM16A regulatory elements. (E) Relatively little TMEM16A (green, anti-GFP) is observed in control airways. (F) When crossed onto a transgenic line that expresses high levels of IL-13 in the respiratory epithelium, the abundance of apical TMEM16A is increased, particularly in mucous cells (red, anti-Muc5AC). Multiciliated epithelial cells are visualized with differential interference contrast (arrowheads). (G–I) Immunostaining for TMEM16A and Muc5AC in healthy human bronchial biopsy tissue. Arrows point to the weak signal for TMEM16A in the Muc5AC-positive cells. HC, healthy control. (J–L) Immunostaining for TMEM16A and Muc5AC in asthmatic human bronchial epithelial cells. Arrows indicate TMEM16A staining in Muc5AC-positive cells. Arrowheads indicate secretory cells in submucosal glands.



cant change in staining intensity in the ASM (Fig. S1) that also expresses TMEM16A (15). To confirm the specificity of antibody staining, we generated knock-in mice with the cDNA encoding GFP fused to the final translated exon of *Tmem16a*, so that the functional TMEM16A:GFP protein is expressed under the control of the endogenous *Tmem16a* regulatory elements and is properly localized. As shown in Fig. 1E, immunofluorescence with a GFP antibody reveals minimal expression of TMEM16A in normal airway epithelial cells; however, transgenic overexpression of IL-13 in respiratory epithelial cells (16) greatly increases the abundance of apical TMEM16A in airway epithelial cells (Fig. 1F).

To determine if TMEM16A expression is altered in asthmatic patients, we used microarrays to examine the genome-wide expression profiles of epithelial cells from normal and asthmatic human airways harvested by bronchoscopic endobronchial brushing (17). We found TMEM16A mRNA is significantly increased in the epithelium from Th2-high human asthma patients, but not in Th2-low asthmatics, compared with healthy subjects (Fig. S2A; $P < 0.05$). Next, we performed quantitative real-time PCR (qPCR) on mRNA harvested by bronchial brush biopsy from healthy controls, Th2-high asthmatics, and Th2-low asthmatics. Using this method, there is a trend toward increased TMEM16A mRNA abundance in Th2 asthmatic patients, but this did not reach statistical significance (Fig. S2B). To determine whether TMEM16A expression is increased at the protein level in human asthmatics as in animal models, we performed immunocytochemical staining on human biopsy samples with an antibody against human TMEM16A. As shown in Fig. 1, only weak staining signal is detected in Muc5AC-positive secretory cells in the healthy human bronchial biopsy tissue (Fig. 1G–I). In contrast, TMEM16A abundance is increased in the epithelial cells, particularly Muc5AC+ secretory cells, in Th2-high asthmatics. It is noteworthy that the mucous secretory cells in asthmatic airways seem to be degranulated, and secreted mucins are localized within the lumen (Fig. 1J–L).

Normal human bronchial epithelial (NHBE) cells grown at the air-liquid interface (ALI) on transwell inserts develop a well-differentiated mucociliary phenotype and acquire the physiological properties of normal human bronchiolar epithelium (18, 19). This in vitro model of airway epithelium has been used for studies of ion transport (11, 20); cellular and molecular pathways (21); and viral infection (22). Previous work has shown that the Th2 cytokine IL-13 is necessary and sufficient to induce the full

range of asthma phenotypes in experimental models (16, 23). IL-13 acts directly on epithelial cells to cause mucus overproduction and hyperresponsiveness of the ASM (24). IL-13 treatment of NHBE cells grown at the ALI causes mucous cell hyperplasia similar to that seen in Th2-high asthmatic patients (Fig. S3) (25, 26). Consistent with previous studies (9), IL-13 also dramatically increases CaCC currents, as measured by the short circuit current (Isc) in Ussing chamber (Fig. S2C), and the abundance of TMEM16A mRNA (Fig. S2D).

TMEM16A Is Preferentially Expressed in Secretory Cells in Asthma Models. Obstruction of the airways with mucus is a potentially fatal feature of asthma. Therefore, factors that regulate the abundance of mucin-secreting cells and mucus secretion have been proposed as potential targets for treating asthma (25–27). Interestingly, immunofluorescence shows that TMEM16A is strongly expressed in Muc5AC-positive mucous cells in the OVA asthmatic mouse models (Fig. 2A–C). To further investigate the distribution of TMEM16A in the airway epithelium, we generated well-differentiated ALI cultures from FoxJ1-GFP transgenic mice in which cytoplasmic GFP is expressed in multiciliated airway epithelial cells. IL-13 treatment of these cultures caused an increase in TMEM16A detected by immunofluorescence, predominantly at the apical surfaces of Muc5AC-positive secretory cells (Fig. 2D–G). Little signal is detected in the GFP-positive multiciliated cells (Fig. 2H). Data from TMEM16A:GFP knock-in mice further confirm our observation that TMEM16A is preferentially expressed in secretory cells that also express SCGB1A1, Muc5AC, and/or Muc5B (Fig. 1F and Fig. S4).

High-Throughput Screening Identifies TMEM16A Blockers. Chloride channel blockers, such as niflumic acid (NFA) and its derivatives, reduce mucin secretion and improve airway function in asthma models (28–36); however, further drug development has been hampered by several factors, including a lack of knowledge about the molecular identity of CaCC (37–39). Our findings that TMEM16A-CaCC is up-regulated in the airway epithelium of asthmatics and preferentially in secretory cells in several asthma models support the relevance of this molecule as a target for the treatment of this disease.

We used a high-throughput screen for TMEM16A-CaCC blockers using a strategy similar to one recently reported (11). This assay is based on a genetically encoded YFP I^- ion flux assay

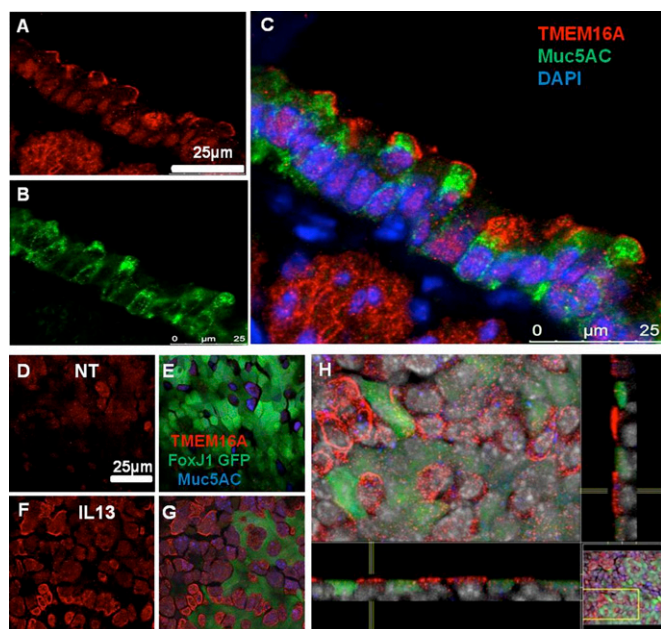


Fig. 2. TMEM16A is strongly expressed in the mucin-secreting cells in asthma models. (A–C) TMEM16A (red) is expressed in the mucin-secreting cells (green, MUC5AC) in the airway epithelium of OVA-challenged asthma mice. Note the expression of TMEM16A in ASM as well. (D–H) Bronchial epithelial cells from FoxJ1-GFP mice were cultured at the ALI and treated with IL-13 (10 ng/mL). (D and E) Antibody staining for TMEM16A (red) is stronger in the rare mucous cells (MUC5AC, blue staining), compared with the abundant multiciliated cells (FoxJ1-GFP, green) in control, nontreated (NT) cultures. (F and G) IL-13 treatment increases the number of mucous cells (blue, MUC5AC), and the abundance of TMEM16A (red) is increased at the apical surfaces of these cells. (H) High-magnification picture with the orthogonal views to show the apical localization of TMEM16A in the Muc5AC-positive cells.

(40–42) (Fig. 3A). I^- ions are normally found in low intracellular concentrations and have relatively few intrinsic exchangers or cotransporters in HEK293 cells. Therefore, the ability of I^- ions to be transported through Cl^- channels into the cell allows the entry of I^- ions to serve as a surrogate for Cl^- ion flux. The effect of putative channel blockers can be quantified via a genetically encoded fluorescent I^- ion biosensor (YFP-H148Q/I152L). HEK293 cells with stably expressed TMEM16A (mCherry tagged) and the YFP I^- ion biosensor show a bright basal YFP fluorescence. When exposed to ionomycin to increase intracellular calcium concentration, elevated TMEM16A-CaCC activity leads to I^- influx, resulting in YFP fluorescence quenching (Fig. 3A). From the initial screen of the validation set library of 2,000 compounds (Z' factor ~ 0.6), we identified three compounds that reduced I^- influx mediated by TMEM16A-CaCC (Fig. 3B); these did not affect ionomycin-induced calcium increase. NFA and NPPB are known CaCC blockers with IC_{50} of 140 μM and 150 μM , respectively, in this type of assay. The molecules identified in this screen, dichlorophen, benzobromarone, and hexachlorophene, are more potent with IC_{50} of 5.49 μM , 9.97 μM , and 10 μM , respectively, and Hill coefficients ~ 1 (Fig. 3B). We further assessed the ability of these compounds to block CaCC activity with electrophysiological recordings. Benzobromarone blocks TMEM16A-CaCC channels assessed by whole-cell patch clamp of HEK293 cells (Fig. 3C) and inside-out patch of *Axolotl* oocyte membrane (Fig. 3D) with Hill coefficient ~ 1 , indicative of pore block. This approach was also used to validate the inhibition of TMEM16A-CaCC with dichlorophen and hexachlorophene (Fig. S5). We further tested the activities of dichlorophen and benzobromarone on the epithelial sodium channel (ENaC), TMEM16B-CaCC, and cystic fibrosis transmembrane conductance regulator (CFTR) (Figs. S5 and S6). Both also blocked the CaCC activity encoded by the closely related TMEM16B, which was

expressed in HEK293 cells and assessed by whole-cell patch clamp. Dichlorophen did not block ENaC or CFTR currents (Fig. S6). Ussing chamber recordings show that benzobromarone did not significantly affect IBMX and forskolin-induced CFTR currents in NHBE cells (Fig. S7), despite a modest effect on both ENaC and CFTR detectable by whole-cell patch clamp (Fig. S6).

To determine the reversibility of the inhibitory effects of benzobromarone, we performed Ussing chamber recordings of NHBE cells cultured at the ALI and treated with benzobromarone and washed for 5 min. These cultures showed 82.3% recovery of the CaCC current compared with untreated controls (Fig. S8A). In whole-cell patch clamp of TMEM16A-expressing HEK cells, we calculated a time constant of benzobromarone offset at +60 mV as 15.6 ± 2.3 s ($n = 6$; Fig. S8B).

TMEM16A Modulates Mucin Secretion. Mucus overabundance is a feature of inflammatory airway diseases, including asthma and chronic obstructive pulmonary disease (COPD). The most important secretagogue for airway surface epithelial cells appears to be ATP, which acts on P2Y2 receptors on the apical membrane (26). The continuous presence of low levels of ATP in airway surface liquid causes continuous low activity of the secretory machinery, resulting in the steady release of mucins that provide a physical and biochemical barrier. When mucin secretion is increased, mucus can accumulate within airway lumens. Although still somewhat controversial (11), studies of TMEM16A-null mutant mice, as well as siRNA knockdown, suggest that TMEM16A mediates purinergic receptor-induced chloride transport across airway epithelia (9, 12, 43) and various exocrine cells (10, 11, 43, 44). To determine if TMEM16A-CaCC mediates mucin secretion in asthma models, we tested the blockers we identified in our high-throughput screening for their effects on ATP-induced mucin depletion in IL-13-treated NHBE cells. As shown in Fig. 4A and B, benzobromarone (10 μM) applied on the apical side of IL-13-treated NHBE cells effectively blocked the ATP (100 μM)-induced CaCC current in Ussing chamber recordings (1.137 ± 0.1301 vs. 3.099 ± 0.4721 , $n = 4$, $P = 0.0071$). Similar to previous studies (45, 46), we observed a depletion of the mucin stores in mucous cells after exposing the apical side of NHBE cells to ATP for 30 min (Fig. 4C and E). In contrast, concurrent application of benzobromarone and ATP prevents the mucin store depletion so that mucin granules, revealed by Muc5AC staining, remain in mucous cells (Fig. 4D). Quantification of Muc5AC staining intensity reveals a significant effect of the TMEM16A-CaCC blocker on ATP-induced mucin secretion (Fig. 4F). Similar effects on mucin secretion were seen with dichlorophen (10 μM ; Fig. S9).

CaCC Blockers Inhibit Methacholine-Induced ASM Contraction. ASM plays a multifaceted role in the pathogenesis of asthma. Excessive contraction (airway hyperresponsiveness) of ASM contributes to airway obstruction along with luminal mucus accumulation. Furthermore, excess smooth muscle mass (hyperplasia) has been reported in patients with fatal asthma (47). CaCC currents have been documented in respiratory smooth muscle and have been suggested to mediate depolarization, contributing to agonist-induced tracheobronchial constriction (48, 49). Indeed, we found TMEM16A is highly expressed in ASM cells (15) (Fig. 5A).

Methacholine (MCh) is a synthetic choline ester that acts as a muscarinic receptor agonist in the parasympathetic nervous system and is commonly used in a bronchial challenge test to diagnose bronchial hyperreactivity in asthma and COPD (50). To determine if TMEM16A plays a role in the contraction of airway smooth muscle, we tested the effect of the CaCC blockers on the contractile force generated by human ASM stimulated with MCh as described (50). As shown in Fig. 5B, 10 μM benzobromarone inhibits the contraction of isolated human bronchi in response to MCh, but not KCl (Fig. 5B and C; $P < 0.001$, $n = 5$). These data suggest that TMEM16A-CaCC is specifically activated by the intracellular calcium increases caused by the stimulation of the G protein-coupled muscarinic receptor. Significant block of

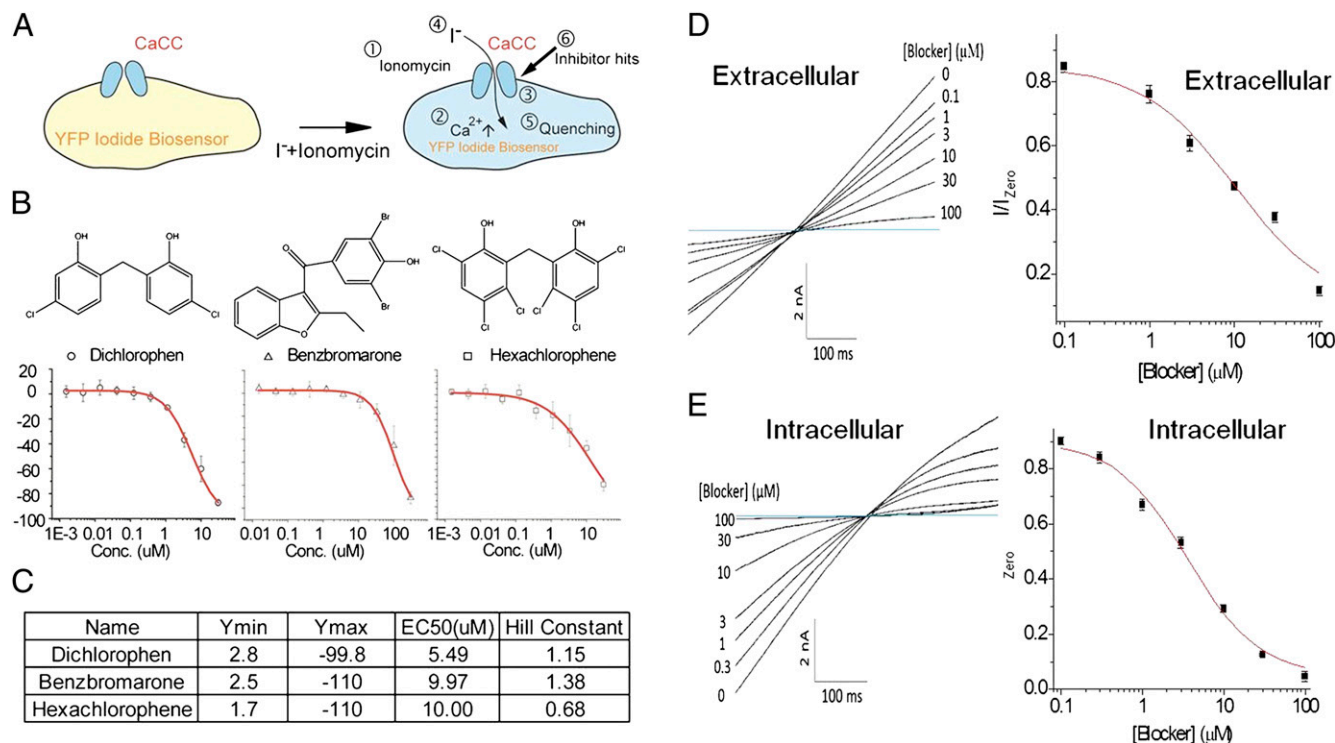


Fig. 3. High-throughput screening yields three blockers of TMEM16A. (A and B) High-throughput screening yields small molecules that block TMEM16A-CaCC. (A) The assay design. HEK293 cells stably express TMEM16A-CaCC and the genetically encoded fluorescent YFP I⁻ ion biosensor. Upon the addition of agonists of calcium motility ionomycin to cells, the increased Ca²⁺ will activate CaCC and, consequently, the increase of iodine ion flux into the cytoplasm through the open CaCC. The increased I⁻ ion levels in the cytoplasm will quench the fluorescence of YFP, which is monitored in real time by a fluorescence imaging plate reader. The validation set library of 2,000 compounds was screened for molecules that block TMEM16A-CaCC (inhibit the ability of ionomycin to induce quenching of the YFP biosensor). (B and C) Structure and properties of selected hits from the screen: dichlorophen, benzbromarone, and hexachlorophene. (D) Benzbromarone blocks CaCC currents from HEK293 cells expressing mouse TMEM16A. The patch was voltage-clamped from the holding potential of -80 mV with a 500-ms voltage ramp to +80 mV. The pipette solution contained 2 μM free Ca²⁺. Extracellular benzbromarone exhibits concentration-dependent block of CaCC current at +80 mV. The data were fitted to $I/I_{zero} = I_{min} + (I_{max} - I_{min}) / (1 + ([blocker]/K_i)^P)$, where $K_i = 10.4 \mu\text{M}$ and $P = 0.8$; $n = 5-13$. (E) An inside-out patch from an Axolotl oocyte expressing mouse TMEM16A was exposed to intracellular benzbromarone. The patch was voltage-clamped from the holding potential of -80 mV with a 500-ms voltage ramp to +80 mV. All benzbromarone solutions contained 100 μM free Ca²⁺. Intracellular benzbromarone also exhibits concentration-dependent block of CaCC current at -80 mV. The data were fitted to $I/I_{zero} = I_{min} + (I_{max} - I_{min}) / (1 + ([blocker]/K_i)^P)$, where $K_i = 3.6 \mu\text{M}$ and $P = 1.0$; $n = 4-11$.

agonist-induced mouse tracheal ASM contraction is also seen with dichlorophen (10 μM; Fig. S10).

Discussion

Because CaCC has been implicated in a number of physiological processes, this channel is an attractive potential therapeutic target for diseases such as asthma, cystic fibrosis, hypertension, gastric motility disorders, and diarrhea. However, because the molecular identity of this channel was not known until recently, progress toward the development of CaCC-targeted therapies has been severely limited. We previously showed that TMEM16A is expressed in the airway epithelium and mediates the bulk of calcium-activated chloride conductance in neonatal mouse airways (12, 15). Consistent with previous studies (9, 51), we report here that the CaCC activity and TMEM16A protein abundance are increased in airway surface epithelial cells from the asthmatic mouse models and human asthmatics. Agonists of TMEM16A activity were previously shown to increase the secretion by submucosal glands and IL-4-stimulated bronchial epithelial cells in vitro (52). These effects were blocked by the previously identified blocker T16A_{inh}-A01. Increased CaCC activity has been reported in mouse models of allergic airway disease (53, 54). These currents were blocked by the nonspecific chloride channel blocker NFA (54), but these studies did not detect an increase in the abundance of TMEM16A mRNA. In this study, we detected increased apical TMEM16A protein in two murine models of allergic airway disease: ovalbumin sensitization and transgenic overexpression of IL-13. These data raise the possibility that TMEM16A could be posttranscriptionally

regulated by inflammatory stimuli in the airway epithelium. A better understanding of the molecular regulation of TMEM16A abundance, localization, and activity will likely lead to the development of additional therapeutic approaches in a number of pathological contexts.

Using an in vitro model of well-differentiated primary normal airway epithelial cells, we showed that TMEM16A-CaCC modulates mucin secretion in response to purinergic stimulation. We currently do not know the mechanism behind this regulation and whether this function is coupled to the proposed function of TMEM16A-CaCC in the maintenance of airway hydration. Using immunofluorescence and confocal microscopy, we detected TMEM16A (and TMEM16A:GFP) at the apical membranes of secretory airway epithelial cells. Consistent with a previous report (6), we did not detect TMEM16A in association with mucins in secretory vesicles. A recent study showed that the nonspecific chloride channel blocker NFA inhibits the secretion of mucus granules in guinea pig asthma models, further supporting our hypothesis that CaCC may be associated with goblet cell degranulation, and CaCC inhibitors may be useful for the treatment of hypersecretion in asthma (55). In addition to the respiratory epithelium, we detected TMEM16A expression in ASM. Here, CaCC has been proposed to mediate agonist-induced depolarization and contraction (48, 49).

Given the expression pattern and proposed functions of TMEM16A, we hypothesized that TMEM16A-CaCC would be an ideal target to manage two features of asthma: mucin hypersecretion and ASM hyperresponsiveness. With this goal in mind,

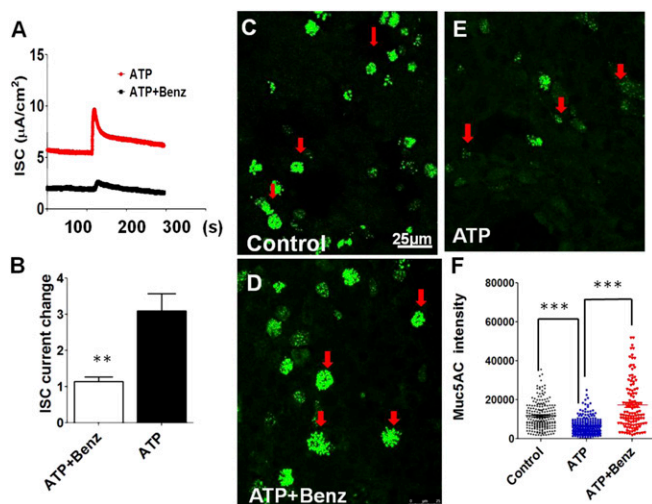


Fig. 4. TMEM16A blockers negatively regulate agonist-stimulated mucin secretion in IL-13-treated NHBE cells. (A and B) NHBE cells were grown at the ALI and treated with IL-13. Benzbromarone blocks the ATP-induced CaCC current measured by short circuit current (Isc) in Ussing chamber. Currents (A), normalized to baseline before ATP, are plotted as mean \pm SEM (B), $n = 4$, $***P < 0.001$. (C–F) Benzbromarone impairs ATP-induced mucin depletion in IL-13-treated NHBE cells as shown by Muc5AC staining of the mucin stores. (C) Control. (D) 100 μ M ATP with 10 μ M benzbromarone. (E) 100 μ M ATP. (F) Quantification and statistical analysis of Muc5AC staining intensity, $***P < 0.0001$. Statistical significance was determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

we used a high-throughput screening approach to identify three small-molecule inhibitors of CaCC that might be exploited to treat asthma. The recent identification of TMEM16A as CaCC allowed us to design the screen using cells stably expressing TMEM16A and also to confirm specificity and efficiency of the blockers by electrophysiology.

The CaCC antagonists used in earlier studies, such as niflumic acid (NFA) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid

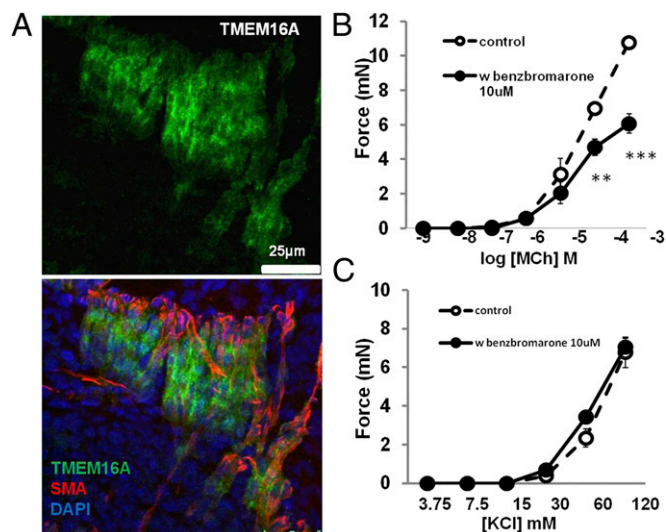


Fig. 5. CaCC blockers inhibit MCh-induced ASM contraction. (A) Sections of mouse airways were stained with antibodies against TMEM16A (green) and α -SMA (red). TMEM16A is expressed in the ASM cells. (B and C) Benzbromarone inhibits the MCh-induced contraction of human ASM (B) but not KCl-induced contraction, as shown in C. $**P < 0.01$, $n = 5$. (Note that all five bronchial rings are from the same human lung.)

(NPPB), are nonspecific and relatively inefficient ($IC_{50} \sim 150 \mu$ M). Therefore, these molecules are met with concerns about off-target effects. Data from more potent and specific blockers of TMEM16A-CaCC, including T16Ainh-A01 (11), recently suggested that TMEM16A does not constitute a major CaCC component in the normal airway epithelium. This finding is consistent with our study that shows limited expression of TMEM16A in normal airway epithelium. However, the three blockers we identified here significantly block CaCC in Th2 cytokine-treated primary human airway epithelial cells at micromolar concentrations, underscoring the importance of this channel in the asthmatic airway epithelium. Dichlorophen was previously identified as a TMEM16A blocker, but was not pursued because this molecule did not apparently block CaCC currents in an intestinal epithelial cancer cell line (11). One possible explanation is that TMEM16A is not the intestinal CaCC. Benzbromarone was previously identified as a CFTR blocker in Fischer rat thyroid cells (56). Here, in NHBE cells, we found that benzbromarone does not significantly affect CFTR current.

The TMEM16A-CaCC blockers identified here have $IC_{50} \sim 5$ – 10μ M, far more potent than NFA and NPPB. Moreover, dichlorophen is used as a veterinary anticestodal, and benzbromarone has been used clinically to treat gout (57–59). Although some safety concerns were raised regarding benzbromarone, this remains a matter of debate (57) and should not preclude preclinical trials for the management of asthma. The similarity of dichlorophen and hexachlorophene in structure might inform efforts in synthetic chemistry for drug design. Importantly, given the widespread expression and many proposed functions of TMEM16A, the delivery, metabolism, and safety of these blockers will need to be carefully tested.

In conclusion, we have shown that TMEM16A is increased in the airways of asthmatics, particularly in secretory cells. We identified three inhibitors of TMEM16A-CaCC and demonstrated their effectiveness in the modulation of mucin secretion and ASM contraction, two debilitating features of chronic asthma. Together, these data should establish the importance of TMEM16A-CaCC in airway physiology and facilitate the development of better strategies for the management of asthma.

Materials and Methods

NHBE and Mouse Tracheal Epithelial Cell Culture. Primary cultures of normal human bronchial epithelial cells were grown at the ALI as described previously (19). In brief, human bronchial tissues were obtained from patients following lung transplantation or from postmortem examinations performed within 24 h after death. The Committee on Human Research at the University of California, San Francisco, approved the use of human tissues for these studies, and informed consent from patients was obtained. Dissociated epithelial cells were plated at a density of $5 \times 10^5/cm^2$ onto 12-mm diameter, 0.4- μ m pore polycarbonate cell culture inserts (Snapwell; Corning) precoated with human placental collagen (15 μ g/ cm^2 ; Sigma). Once confluent, the apical medium was removed and cells were grown at an air-liquid interface in ALI medium at 37 $^{\circ}C$ in 5% $CO_2/95\%$ O_2 (vol/vol) (18). Medium was changed every 2–3 d. Cultures were used 21–30 d after plating, when cells are fully differentiated and an airway surface liquid film was seen. For IL-13 treatment, cells were stimulated by addition of recombinant human IL-13 (10 ng/mL; Pepro-Tech) to the medium for 7 d starting from day 14 in ALI culture, and the IL-13-containing medium was replaced every 2 d.

Similar procedures were used for culture of mouse tracheal epithelial cells, except that the cells were plated at a density of 1 – $2 \times 10^5/cm^2$ on 6.5-mm cell culture inserts.

Immunocytochemistry Mouse lungs were dissected and fresh frozen on a dry ice/ethanol mixture. Tissues from OVA-challenged mice and saline controls were embedded in the same block in optimal cutting temperature embedding medium (Tissue-Tek), and 16- μ m cryostat sections were mounted on the same slide. Slides were air-dried at room temperature for 10 min, then fixed with cold 4% paraformaldehyde (PFA) for 10 min, followed by prechilled methanol at $-20^{\circ}C$ for 5 min. Sections were stained with the following antibodies: rabbit anti-TMEM16A polyclonal antibody (1:1,000) (15), rat anti-E-cadherin (Invitrogen, 1:2,000), mouse anti-SMA (Sigma; 1:1,000), and biotinylated MUC5AC (clone 45M1, 1:1,000; NeoMarkers). Secondary antibodies conjugated with Alexa Fluor 555, 488, or 647 (1:1,000; Invitrogen) were used, as well as DAPI as a nuclear counterstain. Images were taken with

Leica SP5 confocal microscope and processed with ImageJ (National Institutes of Health). ALI-cultured NHBE and mouse tracheal epithelial cells were fixed with 4% PFA for 15 min and then permeabilized with 0.25% TX-100/PBS for 5 min. Immunocytochemistry was performed on the inserts that were subsequently excised and mounted on slides for imaging. Primary antibodies used were chicken anti-GFP (Aves Labs; 1:500), mouse anti-acetylated tubulin (Sigma; 1:1,000), rabbit anti-SCGB1A1 (1:10,000; kind gift from Barry Stripp, Durham, NC), mouse anti-MUC5AC (LabVision; 1:200), and rabbit anti-MUC5B (Santa Cruz Biotechnology; 1:100). Lungs from TMEM16A GFP knock-in mice were dissected and fixed with 4% PFA. Cryostat sections were immunostained with antibodies against GFP (Aves Laboratories; 1:500) and Muc5AC (LabVision; 1:200). Human bronchial biopsy tissue from healthy controls and Th2-high asthmatics were frozen on dry ice. Cryostat sections 7 μ m thick were fixed for 10 min

with 4% PFA and stained with monoclonal antibody against human TMEM16A (Novus; NB110-90609) and Muc5AC (clone 45M1, 1:1,000; NeoMarkers).

ACKNOWLEDGMENTS. We thank Lorna Zlock for technical assistance with cell culture; Denan Wang and Marena Tynan La Fontaine for assistance with mouse husbandry; Shaopeng (Leo) Yuang and Nirav Bhakta for assistance with qPCR; and David Erle for helpful discussions. This work was supported by an award from the Strategic Program for Asthma Research; departmental start-up funds and Research Development Program R613-CR11 from the Cystic Fibrosis Foundation (J.R.R.); National Institutes of Health Grants R03DA031670 and U54MH084691 (to M.L.); and National Institute of Child Health and Human Development Grant T32HD007470 (to M.L.D.). Y.-N.J. and L.Y.J. are Howard Hughes Medical Institute Investigators.

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