TMEM16A Inhibitors Reveal TMEM16A as a Minor Component of Calcium-activated Chloride Channel Conductance in Airway and Intestinal Epithelial Cells*

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TMEM16A (ANO1) functions as a calcium-activated chloride channel (CaCC). We developed pharmacological tools to investigate the contribution of TMEM16A to CaCC conductance in human airway and intestinal epithelial cells. A screen of ~110,000 compounds revealed four novel chemical classes of small molecule TMEM16A inhibitors that fully blocked TMEM16A chloride current with an IC50 < 10 μM, without interfering with calcium signaling. Following structure-activity analysis, the most potent inhibitor, an aminophenylthiazole (T16Ainh-A01), had an IC50 of ~1 μM. Two distinct types of inhibitors were identified. Some compounds, such as tannic acid and the arylaminothiophene CaCCinh-A01, fully inhibited CaCC current in human bronchial and intestinal cells. Other compounds, including T16Ainh-A01 and digallic acid, inhibited total CaCC current in these cells poorly, but blocked mainly an initial, agonist-stimulated transient chloride current. TMEM16A RNAi knockdown also inhibited mainly the transient chloride current. In contrast to the airway and intestinal cells, all TMEM16A inhibitors fully blocked CaCC current in salivary gland cells. We conclude that TMEM16A carries nearly all CaCC current in salivary gland epithelium, but is a minor contributor to total CaCC current in airway and intestinal epithelium. The small molecule inhibitors identified here permit pharmacological dissection of TMEM16A/CaCC function and are potential development candidates for drug therapy of hypertension, pain, diarrhea, and excessive mucus production.

Calcium-activated chloride channels (CaCCs) are ubiquitously expressed in epithelial and nonepithelial cells, where they are involved in epithelial fluid secretion, sensory signal transduction, smooth muscle contraction, oocyte fertilization, and other functions (1–3). CaCCs are potential drug targets for hypertension, asthma, secretory diarrheas, and pain (3).

Three groups reported that the TMEM16A (anoctamin-1, ANO1) gene encodes a CaCC (4–6), showing calcium-activated Cl− currents following heterologous expression. TMEM16A is expressed broadly in mammalian tissues, including tracheal, intestinal, and glandular epithelia, smooth muscle cells, and interstitial cells of Cajal in the gastrointestinal tract (4, 7–9). TMEM16A is also expressed in various tumors, where it has been proposed to play a role in tumor cell proliferation (4, 10). TMEM16A knock-out mice die soon after birth because of tracheomalacia (11). CaCC current measurements in these mice suggested a major role of TMEM16A in epithelial chloride secretion in the airways (12) and salivary gland (4, 13). However, the contribution of TMEM16A to CaCC conductance in adult tissues is not known because of the neonatal lethality of knock-out mice, the lack of potent and selective inhibitors, and the presence of multiple TMEM16 isoforms, some of which, including TMEM16B, also have CaCC activity (6, 14). Knowledge of the contribution of TMEM16A to CaCC activity is important in human disease pathogenesis and for development of new therapies, such as CaCC activators for cystic fibrosis (CF), and CaCC inhibitors for hypertension, asthma, and non-CFTR-dependent secretory diarrheas.

Here, we developed small molecule pharmacological tools to investigate TMEM16A involvement in CaCC conductance in various human epithelial cell cultures. TMEM16A inhibitors were identified by high throughput screening using a cell-based plate reader assay involving measurement of calcium agonist-induced iodide influx in FRT cells co-expressing human TMEM16A and the fluorescent iodide-sensing protein YFP-H148Q/I152L/F46L. Analysis of inhibitor effects on CaCC currents in human epithelial cell cultures indicated, contrary to expectation, only a minor role of TMEM16A to total CaCC current in airway and intestinal epithelium.

EXPERIMENTAL PROCEDURES

Materials and Solutions—Amiloride, ATP, UTP, ionomycin, and other chemicals, unless otherwise indicated, were purchased from Sigma. CFTRinh-172 and CaCCinh-A01 were synthesized as described (15, 16). T16Ainh-A01 was purchased from Asinex (San Diego, CA). The compound collections used for screening included ~100,000 synthetic small molecules from ChemDiv (San Diego, CA) and Asinex, and ~7500 purified natural products from Analyticon (Potsdam, Germany), Timtek (Newark, NJ), and Biomol (Plymouth Meeting, PA). Compounds were maintained as dimethyl sulfoxide stock solutions. Structure-activity analysis was done on analogs purchased from ChemDiv and Asinex. The HCO3−-buffered solu-
Small Molecule TMEM16A Inhibitors

...of apical chloride conductance, the basolateral membrane was permeabilized with nystatin (360 μg/ml), and a chloride gradient was applied in which the basolateral membrane was bathed with the HCO₃⁻-buffered solution, and in the apical solution 120 mM NaCl was replaced by sodium gluconate.

Short circuit current was measured using an EVC4000 Multi-Channel V/I Clamp (World Precision Instruments, Sarasota, FL) and recorded using PowerLab/8sp (AD Instruments, Castle Hill, Australia).

Patch Clamp—Whole cell recordings were made at room temperature on TMEM16A-expressing FRT cells and human submandibular A253 cells. The pipette solution contained 130 mM CsCl, 0.5 mM EGTA, 1 mM MgCl₂, 1 mM Tris-ATP, and 10 mM HEPES (pH 7.2). The bath solution contained 140 mM N-methyl-D-glucamine-Cl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4). Pipettes were pulled from borosilicate glass and had resistances of 3–5 megohms after fire polishing. Seal resistances were between 3 and 10 gigohms. After establishing the whole cell configuration, TMEM16A was activated by 100 μM ATP or by 275 nM free calcium in the pipette solution (1 mM CaCl₂ added to pipette solution). Whole cell currents were elicited by applying hyperpolarizing and depolarizing voltage pulses from a holding potential of 0 mV to potentials between −100 mV and +100 mV in steps of 20 mV. Recordings were made at room temperature using an Axopatch-200B (Axon Instruments). Currents were digitized with a Digidata 1440A converter (Axon Instruments), filtered at 5 kHz, and sampled at 1 kHz.

Cytoplasmic Calcium Measurements—FRT cells in 96-well black-walled microplates were loaded with Fluo-4 NW at 48 h after plating per the manufacturer’s protocol (Invitrogen). Fluo-4 fluorescence was measured with a FLUOSTar Optima fluorescence plate reader (BMG Labtechnologies) equipped with syringe pumps and custom excitation/emission filters (485/538 nm).

Immunoblot Analysis—FRT, A253, T84, and CFTR-expressing human bronchial epithelial cells were lysed with cell lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, and protease inhibitor mixture (Roche Applied Science)). Cell debris was removed by centrifugation, and proteins in the supernatant were resolved by SDS-PAGE and immunoblotted using standard procedures (transfer to polyvinylidene difluoride membrane, 1-h blocking in 5% nonfat dry milk, primary TMEM16A antibody (1:1000 dilution, ab16293; Abcam Inc., Cambridge, MA) and secondary antibody (1:3000 dilution) incubations, and enhanced chemiluminescence detection).

RESULTS

Identification of Small Molecule TMEM16A Inhibitors—High throughput screening was done using FRT cells that were stably transfected with human TMEM16A and the iodoide-sensitive fluorescent protein YFP-H148Q/I152L/F46L. FRT cells were chosen because of their low basal halide transport, rapid growth on uncoated plastic, high and stable expression of transfected cDNAs, and formation a tight epithelium (for short circuit current analysis), as well as our prior...
success in chloride channel drug discovery using these cells (15, 18). Fig. 1A shows cytoplasmic YFP fluorescence in the transfected cells and immunoblot verification of TMEM16A protein expression. Fig. 1B shows robust CaCC current in the TMEM16A-expressing cells in response to the calcium agonists ATP and ionomycin. Agonist-stimulated current was absent in nontransfected FRT cells (data not shown).

As diagrammed in Fig. 1C, screening to identify TMEM16A inhibitors was done in 96-well plates in which test compounds (at 25 μM) were added 10 min before the assay to a physiological chloride-containing solution bathing the cells. The assay involved addition of an iodide solution containing ATP. Iodide influx was measured in individual wells from the initial time course of decreasing YFP fluorescence. Examples of inactive and active compounds are shown in Fig. 1D, along with negative (dimethyl sulfoxide vehicle) control. Active compounds reduced iodide influx, as seen by a reduced rate of decreasing fluorescence.

Screening of ~110,000 drug-like small molecules and natural products yielded 13 compounds that inhibited TMEM16A-mediated iodide influx by >50% at 10 μM. The compounds fell into six distinct chemical classes, one of which (CaCCinh-A01) was identified previously in a phenotype screen for CaCC inhibitors done using human intestinal HT-29 cells (16). Another compound, digallic acid, is related chemically to tannic acid, which we identified previously as a CaCC inhibitor (19). Following verification by measurements of short circuit current and cytoplasmic calcium (see below) and structure-activity studies, chemical structures of the most potent compound of each class are shown in Fig. 2A. Except for digallic acid and CaCCinh-A01, the structures of the new inhibitor classes (named T16Ainh-Xxx, where X refers to classes A, B, C, or D, and xx is the compound identifying number) are unrelated chemically to previously reported CaCC inhibitors or to known CFTR inhibitors including CFTRinh-172, GlyH-101, and PPQ (structures not shown).

Analysis of structure-activity relationships (SARs) was done by screening of more than 800 chemical analogs of class A, B, C, and D compounds identified in the primary screen. Activity data for the most active TMEM16A inhibitors of class A are summarized in Table 1. Activity data and a summary of SAR analysis for class B, C, and D compounds are provided in supplemental Figs. S1–S3, respectively. Fig. 2B summarizes the SAR analysis of class A compounds, which consist of a 2-amino,4-phenythiazole core coupled to a second heterocycle (R1) via a thio-acetyl linker. For the second heterocycle, pyrimidine and 2-aminobenzene (T16Ainh-A04) gave the most potent inhibition. Other heterocycles such as quinoline (T16Ainh-A13) and 2-pyridine (T16Ainh-A14, A15) were inactive. Substitution on the pyrimidine ring reduced inhibition potency. 3,4,5-Trisubstituted analogs (T16Ainh-A01, A02, A03) were among the most potent inhibitors, with IC50 of 1.5–1.8 μM. A bulky group such as phenyl at the 3-position reduced inhibition (T16Ainh-A12, IC50 >100 μM), although smaller substituents including amine, hydroxy, and alkyl groups were tolerated. Substitutions (R2) on the phenyl ring of the thiazole with electron-withdrawing (chloride, fluoride) and donating groups (methoxy) had minimal effect on inhibition potency.

Characterization of TMEM16A Inhibitors—Inhibitors were characterized by electrophysiological and intracellular calcium measurements. Fig. 3A shows a short circuit current in
TMEM16A-expressing FRT cells in which the basolateral membrane was permeabilized with amphotericin B, and a transepithelial chloride gradient was applied, such that the observed current is a direct, quantitative measure of apical membrane TMEM16A chloride conductance. Test compounds were added 5 min prior to TMEM16A activation by 100 μM ATP. Compounds T16Ainh-A01 and digallic acid fully inhibited an ATP-induced short circuit current. Concentration-inhibition data for four inhibitors, which will be used further below, are shown in Fig. 3 (right), with fitted IC50 values: T16Ainh-A01 (1.1 μM), digallic acid (3.6 μM), CaCCinh-A01 (2.1 μM), and tannic acid (6.4 μM).

Fig. 3B shows Fluo-4 fluorescence measurement of ATP and ionomycin-stimulated cytoplasmic calcium elevation. Cytoplasmic calcium was not altered by 10 μM T16Ainh-A01 or 100 μM digallic acid, as shown, or by the other TMEM16A inhibitors in Fig. 2A (data not shown). 10 μM T16Ainh-A01 and 100 μM digallic acid had little effect on CFTR Cl− conductance (inhibited by <10%; Fig. 3C). Fig. 3D shows that T16Ainh-A01, digallic acid, CaCCinh-A01, and tannic acid each inhibited the TMEM16 isoform TMEM16B, which has been reported to have CaCC activity (6, 14). Whole cell patch clamp analysis was done to determine inhibition mechanisms of T16Ainh-A01 and digallic acid (Fig. 3E). 10 μM T16Ainh-A01 and 100 μM digallic acid inhibited nearly completely TMEM16A chloride current (induced by 275 nM free calcium in the pipette) at all voltages, indicating a voltage-independent block mechanism.

**TMEM16A Inhibitors Block Calcium-activated Chloride Conductance in Salivary Gland Cells**—We studied three types of human epithelia-derived cell cultures to determine the contribution of TMEM16A to calcium-stimulated chloride currents: A253 cells (salivary gland-derived), T84 cells (colon-derived), and human bronchial epithelial cells (primary cultures of bronchial epithelium). Fig. 4A shows immunoblot analysis of TMEM16A protein in each cell type, in TMEM16A siRNA-treated A253 cells, and in interleukin-4 (IL-4)-treated human bronchial epithelial cells. Although several nonspecific bands were seen using available antibodies, bands were seen at the correct molecular size of TMEM16A,
TABLE 1
TMEM16A inhibition by class A compounds
Structure-activity relationship of class A inhibitors is shown. IC_{50} was determined from a fluorescence plate reader assay.

| Cmpd | R1 | R2 | IC_{50} (µM) | Cmpd | R1 | R2 | IC_{50} (µM) |
|------|----|----|-------------|------|----|----|-------------|
| A01  | H_3C | N | OCH_3 | 1.8 | A09 | N | H | 3.9 |
| A02  | H_3C | N | Cl | 1.5 | A10 | N | OCH_3 | 5.2 |
| A03  | H_3C | N | F | 1.6 | A11 | N | NH_2 | 7.8 |
| A04  | H_3C | N | CH_3 | 1.8 | A12 | N | H | >100 |
| A05  | H_3C | N | OCH_3 | 1.9 | A13 | N | OCH_3 | >100 |
| A06  | H_3C | N | CH_3 | 2.2 | A14 | N | H | >100 |
| A07  | H_3C | N | H | 2.4 | A15 | N | CF_3 | >100 |
| A08  | H_3C | N | OCH_3 | 2.8 | | | | |

which were reduced by siRNA knockdown and increased by IL-4 treatment. Fig. 4B shows whole cell patch clamp recordings of A253 cells in the presence of 10 µM CFTR_{inh-172} in the bath solution to inhibit CFTR. Characteristic outwardly rectifying CaCC currents were seen. 10 µM T16A_{inh-A01} and 100 µM digallic acid strongly inhibited chloride current (induced by 275 nM free calcium in the pipette).

**TMEM16A Inhibitors Block Calcium-activated Chloride Conductance Poorly in Intestinal and Airway Epithelial Cells**—Short circuit current measurements in T84 cells showed strong CaCC current following calcium elevation by 100 µM ATP (Fig. 5A). As above, in these studies CFTR_{inh-172} was present to ensure that chloride current is CaCC-dependent rather than CFTR-dependent because calcium elevation in some cell types can activate CFTR through calcium-sensitive adenylyl cyclases (17). We found that 10 µM T16A_{inh-A01} and 100 µM digallic acid had little effect on total CaCC current, but selectively blocked the early, transient current elevation following ATP addition (Fig. 5A). In contrast, 30 µM CaCC_{inh-A01} and 100 µM tannic acid strongly inhibited CaCC current following ATP stimulation (Fig. 5C). These findings suggest that TMEM16A accounts for only a small fraction of total CaCC current in T84 cells. To confirm this interpretation, siRNA knockdown was done. TMEM16A knockdown by siRNA in T84 cells reduced TMEM16A protein expression by 42 ± 5% (Fig. 5B). Short circuit current measurements
showed an effect of TMEM16A knockdown qualitatively similar to that seen with T16Ainh-A01 and digallic acid. Knockdown reduced the early, transient current elevation following ATP addition, but had little effect on total CaCC current. Inhibitor effects were also studied in a different human intestinal cell line, HT-29. Fig. 5D shows that 10 μM T16Ainh-A01 and 100 μM digallic acid had little effect on CaCC activity, whereas 30 μM CaCCinh-A01 and 100 μM tannic acid strongly...
inhibited CaCC in YFP-expressing HT29 cells, supporting the interpretation of data in T84 cells.

Experiments were also done in primary cultures of human bronchial epithelial cells from CF subjects, which lack functional CFTR and thus avoid the potentially confounding issue of calcium-induced CFTR activation. Fig. 6A shows large CaCC currents in these cells following UTP stimulation. As found in the intestinal cells, T16Ainh-A01 and digallic acid inhibited initial peak current by ~50%, whereas these compounds had relatively little effect on the CaCC current measured at 5 min after UTP stimulation. Similar results were found for non-CF bronchial epithelial cell cultures studied in the presence of CFTRinh-172 (data not shown). These data support the conclusion that TMEM16A is responsible mainly for the early, transient CaCC current following agonist stimulation in airway and intestinal epithelial cells. Fig. 6B shows UTP-stimulated CaCC current in which T16Ainh-A01 was added before versus at three different times after UTP. The reduced T16Ainh-A01 inhibition with time after UTP addition supports the interpretation that TMEM16A contributes mainly to the early, transient CaCC current. Finally, to confirm the effect of T16Ainh-A01 and digallic acid on apical CaCC current, we measured apical membrane chloride conductance in CF bronchial epithelial cells following basolateral membrane permeabilization by 360 μM nystatin and in the presence of a basolateral-to-apical chloride gradient. 100 μM T16Ainh-A01 and 100 μM digallic acid each reduced UTP-stimulated peak current by ~25% (Fig. 6C).

Last, experiments were done in cells treated with IL-4, which strongly increases TMEM16A expression in human airway epithelium (5). As mentioned above, Fig. 4A confirmed a marked increase in TMEM16A expression after 24-h incubation with IL-4 in primary cultures of CF human bronchial epithelium. In CF human bronchial epithelial cells and Calu-3
FIGURE 6. TMEM16A inhibitors poorly block CaCC chloride current in human bronchial epithelial cells. A, short circuit current measured in primary cultures of CF human bronchial epithelial cells. CaCC was activated by 100 μM UTP after 3-min pretreatment with 10 μM T16Ainh-A01 (left) or 100 μM digallic acid (right). Bar graphs summarize inhibition of UTP-induced initial peak current (0 min) and current at 5 min (mean ± S.E., n = 7–9). Remaining UTP-induced current was completely blocked by 100 μM tannic acid as indicated. Epithelial sodium channel was inhibited by 10 μM amiloride. B, short circuit current showing UTP-induced CaCC current in CF human bronchial epithelial cells with 10 μM T16Ainh-A01 added at four different time points (−2 min, 1 min, 3 min, 5 min) as indicated. Bar graph summarizes inhibition of CaCC at each time point (mean ± S.E., n = 7–9). C, apical chloride conductance measured after basolateral membrane permeabilization by nystatin (360 μg/ml) and with indicated apical and basolateral solution (Cl−). Cells were pretreated with 10 μM T16Ainh-A01 (red curve) or 100 μM digallic acid (blue curve), and CaCC was activated by 100 μM UTP. Bar graph summarizes inhibition of UTP-induced initial peak current (mean ± S.E. (error bars), n = 3).

cells, IL-4 treatment produced ≈10-fold increases in UTP-induced initial peak current (Fig. 7). T16Ainh-A01-sensitive current increased by ≈8-fold in IL-4-treated CF human bronchial epithelial cells compared with untreated cells (Fig. 7C). These results support the interpretation that TMEM16A is a minor contributor to total CaCC current in unstimulated airway epithelial cells.

DISCUSSION

The discovery of TMEM16A as a CaCC is an important advance, as the molecular identity of CaCCs has been unclear, with conflicting data reported for various CaCC candidates including bestropins, ClCs and tweety (reviewed in refs. 20, 21). TMEM16A is ubiquitously expressed in epithelia (8, 22) and has been proposed as a major CaCC in epithelial fluid transport. One goal of our study here was to develop small molecule tools to quantify the contribution of TMEM16A to CaCC in major epithelial tissues where it is expressed, includ-
IL-4 strongly increased TMEM16A expression (Fig. 4A) and calcium agonist-induced short circuit current in CF human bronchial epithelial cells and Calu-3 cells (Fig. 7), but not in T84 cells (data not shown). IL-4 treatment strongly increased the ATP-induced initial peak current, but did not inhibit the subsequent sustained current. T16Ainh-A01 reduced the initial peak current but not the sustained current. These results support the conclusion that TMEM16A comprises, in part, the initial CaCC current following agonist stimulation but is of minor importance in the time-integrated, total CaCC current in normal airway and intestinal epithelium.

Our findings have several implications for chloride transport in the airways and intestine. CFTR is considered to be the major chloride channel in human airways, whose deficiency in CF is associated with altered airway function leading to chronic infection and deterioration of lung function (23). Pharmacological activation of alternative chloride channels, such as CaCCs, is considered to have therapeutic values in CF, with two compounds currently in clinical trials that elevate cytoplasmic calcium (24, 25). However, little or no significant CaCC current has been found for these isoforms (30), at least in studies involving heterologous expression. It is interesting that some compounds, such as CaCCinh-A01 and tannic acid, act as nonselective CaCC inhibitors, suggesting common structural features of CaCCs in airway and intestinal epithelia.

In conclusion, we have identified small molecule pharmacological tools to dissect the physiological roles of TMEM16A and CaCCs in airway and intestinal epithelia. Our data suggest that direct activators of TMEM16A are unlikely to be useful for CF therapy because of its minor contribution to total CaCC in airway epithelium. Indeed, we recently identified small molecule TMEM16A activators that strongly activate CaCC conductance in TMEM16A-transfected cells but have little effect in human bronchial epithelial cells. However, the activators are effective in IL-4-treated human bronchial epithelial cells, where the activator-induced current is blocked completely by T16Ainh-A01.3 Without clear identification of the major airway CaCC(s), a phenotype-based screen is needed to identify CaCC activators for potential CF therapy. In the intestine, there is evidence for involvement of enterocyte CaCCs in non-CFTR-dependent secretory diarrheas caused by certain drugs and microorganisms (26–29). As in airway epithelium, our findings here suggest that TMEM16A is a minor contributor to intestinal fluid secretion, mandating the need to identify the major enterocyte CaCC(s) and to evaluate potential antidiarrheal therapeutics, such as gallotannins (19), that are nonselective in their CaCC inhibition action.

The identity remains unknown of the CaCC(s) responsible for the majority of agonist-stimulated CaCC current in airway and intestinal epithelia. Our results rule out TMEM16B as an important contributor because the TMEM16B blockers strongly inhibited TMEM16B. Other TMEM16 isoforms may be responsible for CaCC currents, as isoforms TMEM16F, TMEM16H, and TMEM16J have been identified in airway epithelial and intestinal epithelia (12, 22, 30). However, little or no significant CaCC current has been found for these isoforms (30), at least in studies involving heterologous expression. It is interesting that some compounds, such as CaCCinh-A01 and tannic acid, act as nonselective CaCC inhibitors, suggesting common structural features of CaCCs in airway and intestinal epithelia.

In conclusion, we have identified small molecule pharmacological tools to dissect the physiological roles of TMEM16A and CaCCs in airway and intestinal epithelia.

3 W. Namkung, P.-W. Phuan, and A. S. Verkman, unpublished data.
TMEM16A. Functional studies using these compounds indicate that although TMEM16A is responsible for essentially all CaCC in salivary gland epithelium, it is a minor contributor to total, time-integrated CaCC current in airway and intestinal epithelia, mandating the need to establish the molecular identity of remaining CaCC(s). The nonselective CaCC inhibitors identified here and in our prior screening studies have potential utility for therapy of various human diseases associated with CaCC-dependent functions, including hypertension (smooth muscle contraction), pain (nociceptive neuron function), excessive airway mucus production, and certain tumors.

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