TMEM16F is a component of a Ca\(^{2+}\)-activated Cl\(^{-}\) channel but not a volume-sensitive outwardly rectifying Cl\(^{-}\) channel

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TMEM16F is a component of a Ca\(^{2+}\)-activated Cl\(^{-}\) channel but not a volume-sensitive outwardly rectifying Cl\(^{-}\) channel. Am J Physiol Cell Physiol 304: C748–C759, 2013. First published February 20, 2013; doi:10.1152/ajpcell.00228.2012.—TMEM16 (transmembrane protein 16) proteins, which possess eight putative transmembrane domains with intracellular NH2- and COOH-terminal tails, are thought to comprise a Cl\(^{-}\) channel family. The function of TMEM16F, a member of the TMEM16 family, has been greatly controversial. In the present study, we performed whole cell patch-clamp recordings to investigate the function of human TMEM16F. In TMEM16F-transfected HEK293T cells but not TMEM16K- and mock-transfected cells, activation of membrane currents with strong outward rectification was found to be induced by application of a Ca\(^{2+}\) ionophore, ionomycin, or by an increase in the intracellular free Ca\(^{2+}\) concentration. The free Ca\(^{2+}\) concentration for half-maximal activation of TMEM16F currents was 9.6 μM, which is distinctly higher than that for TMEM16A/B currents. The outwardly rectifying current-voltage relationship for TMEM16F currents was not changed by an increase in the intracellular Ca\(^{2+}\) level, in contrast to TMEM16A/B currents. The Ca\(^{2+}\)-activated TMEM16F currents were anion selective, because replacing Cl\(^{-}\) with aspartate in the bathing solution without changing cation concentrations caused a positive shift of the reversal potential. The anion selectivity sequence of the TMEM16F channel was I\(^{-}\) > Br\(^{-}\) > Cl\(^{-}\) > F\(^{-}\) > aspartate. Niflumic acid, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel blocker, inhibited the TMEM16F-dependent Cl\(^{-}\) currents. Neither overexpression nor knockdown of TMEM16F affected volume-sensitive outwardly rectifying Cl\(^{-}\) channel (VSOR) currents activated by osmotic swelling or apoptotic stimulation. These results demonstrate that human TMEM16F is an essential component of a Ca\(^{2+}\)-activated Cl\(^{-}\) channel with a Ca\(^{2+}\) sensitivity that is distinct from that of TMEM16A/B and that it is not related to VSOR activity.

anoctamin 6; anoctamin 10; Ca\(^{2+}\)-activated Cl\(^{-}\) channel; volume-sensitive Cl\(^{-}\) channel

Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs) play important physiological roles in membrane excitability, transepithelial transport, smooth muscle contraction, and other functions (9, 11). Although the molecular identity of CaCCs has been unclear for a long time, it has recently been demonstrated that human and mouse transmembrane protein 16A [TMEM16A, also called anoctamin 1 (ANO1)] functions as a major component of CaCCs (5, 23, 26, 37). The TMEM16 protein family consists of 10 different proteins that possess 8 putative transmembrane domains with intracellular NH2- and COOH-terminal tails (37). In addition to TMEM16A, human and mouse TMEM16B has been reported to generate CaCC currents in mammalian cell expression systems (22, 24, 29, 31). On the other hand, the functions of other TMEM16 proteins are poorly understood. Considering that TMEM16B has relatively high primary sequence identity with TMEM16A (~60%) but that the identity with other TMEM16 proteins is low (~20–40%) in humans, it is of great interest to determine whether TMEM16 members other than TMEM16A and TMEM16B function as CaCCs or other types of anion channel.

In a recent study (1), functional expression of a volume-sensitive outwardly rectifying Cl\(^{-}\) channel (VSOR), the molecular identity of which is unknown (17, 18, 20), was found to be suppressed by knockdown of human TMEM16A, TMEM16F, TMEM16H, and TMEM16J proteins. This suggests that some TMEM16 member(s) might therefore form a component(s) of a hetero-oligomeric VSOR. In addition, it has recently been demonstrated that human TMEM16F is an essential component of an outwardly rectifying Cl\(^{-}\) channel the function of which is associated with apoptotic cell death (16). In contrast, human TMEM16A, TMEM16B, TMEM16F, TMEM16G, and TMEM16K were reported to show CaCC activity based on observations made by fluorescence-based assays and whole cell patch-clamp recordings (25); except for TMEM16A, the Cl\(^{-}\) channel activity is very small. Surprisingly, mouse TMEM16F has recently been demonstrated to function as a Ca\(^{2+}\)-dependent phospholipid scramblase (27, 32). Since the function of TMEM16F is controversial, research on it is one of the most exciting areas in the field of TMEM16 study.

In the present study, we performed whole cell patch-clamp recordings to examine the Cl\(^{-}\) channel activity of TMEM16F and TMEM16K, which are both expressed in three different human cell lines that endogenously express VSOR activity. We focused on the question of whether TMEM16F or TMEM16K is a component of VSOR or a specific type of CaCC.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney HEK293T cells were grown in DMEM (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidity-controlled incubator with 5% CO2 (MCO-96; Sanyo, Tokyo, Japan). Human cervix HeLa cells were cultured in minimum essential medium (Sigma, St. Louis, MO) containing 10% FBS as well as penicillin and streptomycin at the same concentrations as above. Human epithelial Intestine 407 cells were cultured as previously reported (13).

Expression analysis for mRNAs of the TMEM16 family. Expression of all TMEM16 family members except for TMEM16A was examined by RT-PCR in HEK293T, HeLa, and Intestine 407 cells. Total RNA was extracted from these cells using Sepasol-RNA I reagent (Nacalai Tesque, Kyoto, Japan) and reverse transcribed with an oligo(dT)12–18 primer

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mRNA expression of TMEM16f and TMEM16K channels was normalized using these mRNAs in mock-transfected cells as an internal standard. The primers used for quantitative PCR were as follows (name: forward and reverse primers, product size): hTMEM16F: 5'-AGAAAGATAAACCCGAGT-3', 5'-GACGAAACCCGAGT-3'; hTMEM16K: 5'-GACGAACTGGCCATTGTTG-3', 5'-AAGGATGCGGAAAGAC-3', 189 bp.

Patch-clamp experiments. In patch-clamp experiments to measure CaCC currents and VSOR currents, HEK293T or HeLa cells cultured on coverslips (Matsunami Glass, Osaka, Japan) and in suspension with agitation for 15–30 min, respectively, were used. For patch clamping in cells transfected with cDNA or siRNA, cells exhibiting GiP or Alexa Fluor 488 fluorescence were selected under a fluorescence microscope. Whole cell recordings were performed with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) at room temperature. pClamp 9.2 software (Molecular Devices) was used for control pulse control and data acquisition, and pClamp 9.2 and WinASCD (kindly provided by Dr. G. Droogmans, KU Leuven; ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip) software was utilized for data analysis. Currents were filtered at 1 kHz in an Axopatch 200B amplifier and digitized at 5 kHz. Patch electrodes had a resistance of 2–4 MΩ when filled with pipette solution. The access resistance (<10 MΩ) was electrically compensated by 70% to minimize voltage errors. The time course of current activation and that of current recovery were monitored by repetitively applying alternating pulses (500-ms duration) from a holding potential of 0 to ±40 and ±80 mV every 15 s and 10 s, respectively. To observe the voltage dependence of currents, step pulses (1 s or 500 ms duration) were applied from −100 to +100 mV in 20-mV increments with or without a prepotential of −100 mV. The amplitudes of the instantaneous VSOR and steady-state peak CaCC currents were measured 10–20 and 450–500 ms, respectively, after step pulse onset.

The standard bathing solution contained the following (in mM): 110 CsCl, 5 MgSO4, 2 CaSO4, 12 HER, 7 Tris, and 100 mannitol (pH 7.5, 300 mosmol/kgH2O). To make the hypotonic bathing solution, the concentration of mannitol was reduced to 40 mM (pH 7.5, 270 mosmol/kgH2O). The standard pipette (intracellular) solution was containing the following (in mM): 110 CsCl, 2 MgSO4, 1 NaF, 15 Tris, 10 HER, 100 HEPES, 100 EGTA, and 50 mannitol (pH 7.3, 300 mosmol/kgH2O). The osmolality of solutions was measured using an Osm802 freezing-point depression osmometer (Vogel, Giessen, Germany). When ion permeability was examined, the bathing solution contained 110 mm NaCl instead of 110 mM CsCl, and NaCl was equally replaced by NaF, NaBr, NaI, or Na-aspartate, or N-methyl-D-glucamine (NMDG)-Cl. In experiments to investigate the Ca2+-dependency of TMEM16f channels, an appropriate amount of CaSO4 was added to pipette solutions to obtain a free Ca2+ concentration of 1, 3, 6, 10, 30, 100, 300, or 1,000 μM. The free Ca2+ concentration was calculated with Cabuf software (kindly provided by G. Droogmans, KU Leuven, Belgium).

Cell volume measurements. Changes in cell volume were quantified by an electronic sizing technique using a Coulter-type cell size analyzer (CDA-500: Sysmex, Kobe, Japan). The isotonic solution was serum-free minimum essential medium containing 26 mM NaHCO3 and 20 mM HEPS (pH 7.4, 300 mosmol/kgH2O).

Statistics. Data are presented as means ± SE of n observations. Statistical differences of the data were evaluated by Student’s t-test or one-way ANOVA and were considered significant at P < 0.05.

RESULTS

Expression of TMEM16f and TMEM16K did not affect VSOR activity. It has recently been demonstrated that several TMEM16 proteins are components of a volume-sensitive Cl− channel (1). To test this, we investigated the mRNA expression of all TMEM16 family members except for TMEM16A in three different cell lines that exhibit high endogenous functional

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Leukemia Virus Transcriptase (Invitro) or with the PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Osaka, Japan) according to the manufacturer’s instructions. TMEM16 cDNAs were amplified by standard PCR using Blend Taq (Toyobo, Osaka, Japan) or ExTaq (Takara Bio). The primers designed for PCR were as follows (name: forward and reverse primers, product size): hTMEM16B: 5'-GGGGGAGTGGTTGGAGGAGAGG-3', 397 bp; hTMEM16C: 5'-CTTTCATGATATTAATACTC-3' and 5'-TCCCTGCCCTACTTG-3', 488 bp; hTMEM16D: 5'-CATCCTGGACACGACCAAGC-3' and 5'-TTTAAATGTTGAAATCTC-3', 472 bp; hTMEM16E: 5'-GGCTCTGAGTATCCCTG-3' and 5'-CACCCTGCTCCTTACCTA-3', 219 bp; hTMEM16F: 5'-CAGA-3' and 5'-ACAGAAGACCCGAGAAGAAG-3', 231 bp.

Genec supergene of TMEM16F and TMEM16K. TMEM16F and TMEM16K expression was suppressed by RNA interference (RNAi) in HEK293T and HeLa cells. Alexa Fluor 488-conjugated small interfering (si)RNAs specific to human TMEM16F (antisense, 5'-UAUGUCUCGAGGAAGGAC-3'; sense, 5'-GGCUCAAUCUGGAA-3') and human TMEM16K (antisense, 5'-UGUAGCAUCAGUUGA-3'; sense, 5'-GGCAAGCAACUGUAAC-3') were designed and synthesized by Qiagen (Valencia, CA). Each siRNA was transfected into HEK293T or HeLa cells using HiperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. A negative control siRNA modified with Alexa Fluor 488 (Allstars, Qiagen) was utilized in control transfections. RT-PCR and whole cell patch-clamp experiments were performed 36–48 h after transfection. The primers designed for PCR were as follows (name: forward and reverse primers, product size): hTMEM16F: 5'-CACAAGGAACCCGAGAAGAAG-3', 269 bp; 5'-TGGGAGTGGAAGAAGCTGAG-3', 332 bp; hTMEM16F: 5'-ACAGAAGACCCGAGAAGAAG-3', 641 bp; hTMEM16K: 5'-CATGTTGGAGCCTACTGAGC-3', 417 bp; hGAPDH: 5'-GCCCAGTGTGAA-3', 106 bp; hTMEM16F: 5'-CAAAAGCAGGGAAAGTTA-3', 219 bp; hTMEM16F: 5'-GCAAAGGAAGTCCAGA-3', 270 mosmol/kgH2O). The standard pipette (intracellular) solution, the concentration of mannitol was reduced to 40 mM (pH 7.5, 270 mosmol/kgH2O). The standard pipette (intracellular) solution was containing the following (in mM): 110 CsCl, 5 MgSO4, 2 CaSO4, 12 HER, 7 Tris, and 100 mannitol (pH 7.5, 300 mosmol/kgH2O). To make the hypertonic bathing solution, the concentration of mannitol was reduced to 40 mM (pH 7.5, 270 mosmol/kgH2O). The standard pipette (intracellular) solution was containing the following (in mM): 110 CsCl, 2 MgSO4, 1 NaF, 15 Tris, 10 HER, 10 HEPES, 100 EGTA, and 50 mannitol (pH 7.3, 300 mosmol/kgH2O). The osmolality of solutions was measured using an Osm802 freezing-point depression osmometer (Vogel, Giessen, Germany). When ion permeability was examined, the bathing solution contained 110 mm NaCl instead of 110 mM CsCl, and NaCl was equally replaced by NaF, NaBr, NaI, or Na-aspartate, or N-methyl-D-glucamine (NMDG)-Cl. In experiments to investigate the Ca2+-dependency of TMEM16f channels, an appropriate amount of CaSO4 was added to pipette solutions to obtain a free Ca2+ concentration of 1, 3, 6, 10, 30, 100, 300, or 1,000 μM. The free Ca2+ concentration was calculated with Cabuf software (kindly provided by G. Droogmans, KU Leuven, Belgium).

Cell volume measurements. Changes in cell volume were quantified by an electronic sizing technique using a Coulter-type cell size analyzer (CDA-500: Sysmex, Kobe, Japan). The isotonic solution was serum-free minimum essential medium containing 26 mM NaHCO3 and 20 mM HEPES (pH 7.4, 300 mosmol/kgH2O).
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VSOR activity, HeLa, HEK293T, and Intestine 407 (3, 13, 28, 30). RT-PCR experiments showed that the TMEM16F and TMEM16K genes have salient expression in all cell types (Fig. 1). In the present study, we focused on TMEM16F and TMEM16K.

To investigate whether TMEM16F and TMEM16K contribute to the function of VSOR, we first knocked down these TMEM16 proteins with appropriate siRNAs in HEK293T cells. As shown in Fig. 2A, siRNAs against TMEM16F and TMEM16K in contrast to control siRNAs, effectively suppressed TMEM16F and TMEM16K expression, respectively, without affecting GAPDH expression. Quantitative PCR analysis showed that the expression level of TMEM16F mRNA in TMEM16F-deficient cells was 4.3 ± 0.6% of control (n = 3) and that the expression level of TMEM16K mRNA in TMEM16K-deficient cells was 11.8 ± 3.0% of control (n = 4). However, VSOR currents exhibiting mild outward rectification and time-dependent inactivation at positive potentials underwent increases upon exposure to hypotonic bathing solution that were no different in control and TMEM16F- or TMEM16K-deficient HEK293T cells (Fig. 2, B and C). In addition, the times for half-activation of the currents in control, TMEM16F-deficient, and TMEM16K-deficient cells were similar to each other (11.4 ± 1.1, 11.3 ± 1.0, 11.3 ± 1.0 min, respectively; n = 8–17). In HeLa cells treated with TMEM16F and TMEM16K siRNAs, we also observed significant reductions in TMEM16F and TMEM16K mRNA expression, respectively, without any changes in VSOR activity (Fig. 2D).

Next, we observed the effects of exogenous overexpression of TMEM16F and TMEM16K. As shown in Fig. 3A, RT-PCR experiments indicated that mRNA expression of TMEM16F and TMEM16K was increased in HEK293T cells 24 h after transfection with each respective gene. Quantitative PCR analysis showed that the expression level of TMEM16F mRNA in TMEM16F-overexpressing cells was 168-fold higher than that in mock-transfected cells (168 ± 74; n = 4) and that the expression level of TMEM16K mRNA in TMEM16K-overexpressing cells was 370-fold higher than that in mock-transfected cells (377 ± 160; n = 4). In TMEM16F- and TMEM16K-overexpressing HEK293T cells, however, the amplitude of VSOR currents induced by hypotonic stress was indistinguishable from that in mock-transfected cells (Fig. 3, B and C). The currents in mock-, TMEM16F-, and TMEM16K-transfected cells exhibited half-activation times

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Fig. 1. Lanes B–K: mRNA expression for transmembrane protein 16 (TMEM16) family members in human HeLa (A), HEK293T (B), and Intestine 407 (C) cells. RT-PCR analysis with 35 PCR cycles indicated that TMEM16F, TMEM16H, and TMEM16K are expressed in all these types of cells. Salient expression of TMEM16F and TMEM16K was observed in triplicate experiments. + and − represent lanes with and without reverse transcriptase (RT), respectively. Data in each panel are representative of 2 to 3 separate experiments.
of 14.7 ± 1.9, 12.5 ± 1.2, and 12.8 ± 2.1 min, respectively, and those were not significantly different from each other. To confirm whether the current was conveyed by VSOR, we investigated the effects of 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (DCPIB), which is known to be a selective VSOR blocker (6), on swelling-activated currents in HEK293T cells. As shown in Fig. 3D, DCPIB at 10 μM inhibited most of the currents activated in TMEM16F-transfected cells under hypotonic conditions. These results suggest that neither TMEM16F nor TMEM16K contributes to VSOR activity.

Since it has recently been reported that TMEM16F is a component of the outwardly rectifying Cl− channel activated by apoptotic inducers such as Fas ligand and staurosporine (16), we next investigated this possibility. In HEK293T cells, bath application of staurosporine at 4 μM induced outwardly rectifying Cl− currents similar to VSOR currents (Fig. 4), as
previously observed in HeLa cells (28). However, knockdown of TMEM16F in HEK293T cells failed to suppress the staurosporine-induced activation of VSOR currents (Fig. 4, A and B). Moreover, the half-activation time of the staurosporine-induced currents in TMEM16F-deficient cells (5.8 ± 0.8 min; n = 6) was not significantly different from that in control cells (5.5 ± 0.7 min; n = 7). In TMEM16F-overexpressing cells, staurosporine also induced similar VSOR currents (Fig. 4C) with the half-activation time of 5.4 ± 1.4 min (n = 3), which is not significantly different from that in mock-transfected cells (7.1 ± 1.9 min; n = 4). Since staurosporine-induced activation of VSOR currents is known to be involved in the induction of apoptotic volume decrease (AVD) in HeLa cells, we next investigated whether TMEM16F gene silencing affects AVD induction in HeLa cells treated with staurosporine (15, 19, 28). Staurosporine-induced AVD in HeLa cells transfected with control siRNA was indistinguishable from that in cells transfected with TMEM16F siRNA: The cell volumes decreased to 81.5 ± 1.9% (n = 6) and
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80.2 ± 1.5% (n = 6; P > 0.05), respectively, of the original volume 120 min after application of staurosporine.

**TMEM16F, but not TMEM16K, is a component of a CaCC.** We next investigated whether TMEM16F and TMEM16K function as CaCCs. Exposure to a Ca\(^{2+}\) ionophore, ionomycin, sometimes triggered very small currents in mock-transfected HEK293T cells (Fig. 5A, top), indicating the presence of endogenous CaCC currents. In TMEM16F-transfected HEK293T cells, in contrast, application of ionomycin led to more marked activation of membrane currents with a large transient peak followed by a sustained current, as shown in Fig. 5A (middle). The TMEM16F-dependent currents exhibited strong outward rectification and showed time-dependent activation and deactivation at positive and negative potentials, respectively. On the other hand, membrane currents recorded in TMEM16K-transfected cells were comparable to those in mock-transfected cells (Fig. 5A, bottom). Current-voltage (I-V) relationships shown in Fig. 5B indicate that ionomycin-induced outwardly rectifying currents in TMEM16F-transfected cells are much greater than those in mock-transfected and TMEM16K-transfected cells. As shown in Fig. 5C, outwardly rectifying currents triggered by raising the intracellular free Ca\(^{2+}\) concentration to 100 μM in TMEM16F-transfected cells are also much larger than those in mock-transfected and TMEM16K-transfected cells. These results indicate that TMEM16F, but not TMEM16K, functions as a component of a CaCC with strong outward rectification.

To examine whether the TMEM16F-dependent current is mainly carried by Cl\(^{-}\), we completely replaced Cl\(^{-}\) in the bathing solution with aspartate, without changing the concentration of any cations. In TMEM16F-overexpressing HEK293T cells, the replacement of extracellular Cl\(^{-}\) with aspartate decreased the outward current triggered by an increase in the intracellular free Ca\(^{2+}\) concentration to 100 μM (Fig. 6, A and B) and significantly shifted the reversal potential to a positive value (Fig. 6, B and C). On the other hand, no significant change of the reversal potential was observed by replacement of extracellular Na\(^{+}\) with NMDG\(^{+}\) (Fig. 6, D and E). These results demonstrate that TMEM16F-dependent currents are anion selective.

**Fig. 4. Lack of effects of TMEM16F gene knockdown and overexpression on staurosporine-induced VSOR currents in HEK293T cells. A**: representative whole cell VSOR currents induced by 4 μM staurosporine (applied during the time designated by bars) in cells treated with control siRNA or TMEM16F siRNA (ΔTMEM16F), elicited by application of alternating pulses of ± 40 mV (left) and step pulses from −100 to +100 mV in 20-mV increments (right). †Time points at which step pulses were applied. **B**: Instantaneous I-V relationships for VSOR currents measured after staurosporine application in cells treated with siRNAs. Each data point represents the means ± SE (vertical bar) of 7 and 6 experiments for control and siRNA-treated cells, respectively. **C**: instantaneous I-V relationships for VSOR currents measured after staurosporine application in mock- and TMEM16F-transfected cells. Each data point represents the means ± SE (vertical bar) of 4 and 3 experiments for mock- and TMEM16F-transfected cells, respectively.
Katz equation yielded a permeability ratio, $P_{\text{aspartate}}/P_{\text{Cl}}$, of 0.47 ± 0.08 ($n = 9$). Next, we investigated the anion selectivity of the TMEM16F-dependent channel. Substitution of extracellular Cl prior to F shifted the reversal potential from 4.7 ± 0.2 to 10.9 ± 0.6, -2.7 ± 0.9, and 14.0 ± 3.2 mV ($n = 5–6$), respectively (Fig. 6F). The anion selectivity sequence for the TMEM16F channel, determined from the reversal potentials, was $\Gamma > \text{Br} > \text{Cl} > F$ (Fig. 6G).

Since niflumic acid (NFA) has been reported to block Ca$^{2+}$-activated TMEM16A and TMEM16B Cl$^-$ channels (5, 22, 26, 37), we examined the effect of NFA on TMEM16F-dependent Cl$^-$ currents. Application of NFA at 300 µM significantly inhibited TMEM16F-dependent Cl$^-$ currents activated by increasing the intracellular free Ca$^{2+}$ concentration to 100 µM (Fig. 7, A and B). The NFA effect on TMEM16F currents was reversible (data not shown) and concentration dependent (Fig. 7C). The estimated half-maximal inhibitory concentration (IC$_{50}$) was 213 µM.

Ca$^{2+}$ sensitivity of the TMEM16F Cl$^-$ channel is low. To assess the sensitivity of TMEM16F Cl$^-$ channel activation to Ca$^{2+}$, we applied different concentrations of Ca$^{2+}$ in the pipette (intracellular) solution. As shown in Fig. 8A, TMEM16F Cl$^-$ currents increased with rising intracellular free Ca$^{2+}$ in a concentration-dependent manner and showed essentially no changes in time-dependent activation at positive voltages or deactivation at negative voltages. The peak steady-state $I$-$V$ relationship exhibited strong outward rectification at free Ca$^{2+}$ concentrations of 10 to 100 µM (Fig. 8B). The concentration-response curve for outward TMEM16F currents observed at +100 mV shows that channel
activation requires a rather high concentration of intracellular free Ca$^{2+}$, with a half-maximal concentration (EC$_{50}$) of 9.6 ± 0.4 µM (n = 4–23; Fig. 8C). Endogenous CaCC induced by increase in intracellular Ca$^{2+}$ concentration in mock-transfected cells exhibited similar current profiles (Fig. 8A, last trace) and an indistinguishable concentration-dependence with EC$_{50}$ of 8.7 ± 1.4 µM (n = 3–14; Fig. 8C), although the maximal current amplitude was much smaller than that in TMEM16F-transfected cells.

Since Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) was reported to regulate Ca$^{2+}$-dependent activation of endogenous CaCC (2, 11), we investigated effects of KN-93, a membrane-permeable CaMKII inhibitor, on TMEM16F Cl$^{-}$ channels. Even when cells were treated with KN-93 added to the bathing solution at 10 µM, TMEM16F Cl$^{-}$ currents activated by raising the intracellular free Ca$^{2+}$ concentration to 100 µM were not essentially affected (Fig. 9).

**DISCUSSION**

**TMEM16F and TMEM16K do not underlie VSOR activity.** Based on the observations that knockdown of endogenous TMEM16 proteins including TMEM16A, TMEM16F,
TMEM16H, and TMEM16J with corresponding siRNAs decreased swelling-activated outwardly rectifying Cl\(^{-}\) currents in HEK293 cells, it was proposed that some TMEM16 proteins are crucial components of the VSOR hetero-oligomer (1). In the present study, we observed the actual robust endogenous expression of TMEM16F and TMEM16K in three types of human epithelial cells exhibiting high functional VSOR activity (Fig. 1). We demonstrated, however, that siRNA-induced
knockdown of TMEM16F and TMEM16K failed to suppress endogenous VSOR currents in HEK293T and HeLa cells (Fig. 2). Furthermore, we confirmed that even in TMEM16F- and TMEM16K-overexpressing HEK293T cells, VSOR currents were activated to the same extent as in control cells upon hypotonic swelling (Fig. 3). Our results therefore indicate that neither TMEM16F nor TMEM16K is a component of VSOR in human epithelial cells.

We previously demonstrated that an outwardly rectifying Cl\(^{-}\) current is activated by apoptotic stimuli, including staurosporine, Fas ligand, TNF-\(\alpha\), and H\(_2\)O\(_2\), and that the current is conveyed by VSOR in HeLa cells (28). More recently, TMEM16F has been proposed to function as an outwardly rectifying Cl\(^{-}\) channel activated by apoptotic stimuli such as staurosporine and Fas ligand in several cell lines (16). In the present study, however, staurosporine-induced outwardly rectifying Cl\(^{-}\) currents were indistinguishable regardless of the TMEM16F expression (Fig. 4). In addition, staurosporine-induced AVD observed in HeLa cells transfected with TMEM16F siRNA was equivalent to that in control cells. These results demonstrate that TMEM16F does not contribute to the VSOR activity induced by staurosporine. Future studies will clarify whether other members of the TMEM16 family are components of the VSOR activated by osmotic cell swelling or apoptotic stimuli.

**TMEM16F, but not TMEM16K, is a component of a CaCC.**

In this study we demonstrated that the transfection of TMEM16F gives rise to activity of a Ca\(^{2+}\)-activated channel in HEK293T cells. The TMEM16F-dependent currents recorded by whole cell patch-clamp under symmetrical CsCl conditions exhibited strong outward rectification (Fig. 5). Time-dependent activation and deactivation were observed at +100 mV and −100 mV, respectively. The current profile was similar to the profiles for TMEM16A and TMEM16B Cl\(^{-}\) channels (4, 22, 33, 37). In fact, the TMEM16F-dependent current was found to be anion-selective, because reduction of the extracellular Cl\(^{-}\) concentration without an alteration in the concentration of any cations in the bathing solution shifted the reversal potential to a positive voltage (Fig. 6, A-C) and substitution of Na\(^{+}\) with NMDG\(^{+}\) in the bathing solution did not significantly change the reversal potential (Fig. 6, D and E). The anion selectivity sequence for TMEM16F channels was I\(^{-}\) > Br\(^{-}\) > Cl\(^{-}\) > F\(^{-}\) > aspartate\(^{-}\) (Fig. 6, B, C, F, and G), which corresponds to the selectivity sequences for TMEM16A and TMEM16B. Taken together, these results show that TMEM16F is a component of a CaCC. Also, TMEM16F Cl\(^{-}\) currents were inhibited by NFA, a known CaCC blocker, in a concentration-dependent manner with an IC\(_{50}\) of 213 \(\mu\)M (Fig. 7). Although TMEM16A-dependent Cl\(^{-}\) currents were found to be significantly inhibited at the concentration as low as 10 \(\mu\)M (37), significant inhibition of TMEM16F Cl\(^{-}\) currents was observed by NFA only at \(\geq 100 \mu\)M (Fig. 7C). These results indicate that the TMEM16F Cl\(^{-}\) channel is less sensitive to NFA compared with the TMEM16A Cl\(^{-}\) channel.

It has recently been reported that some members of the TMEM16 family, including TMEM16F, are intracellularly localized when transfected in several cell lines (7). However, Schreiber et al. (25) demonstrated by immunocytochemistry that TMEM16F is expressed on the plasma membrane; furthermore, by iodide quenching and whole cell patch-clamp experiments, they showed that it produces a very small Ca\(^{2+}\)-activated Cl\(^{-}\) conductance. Here, we observed large outwardly rectifying Cl\(^{-}\) currents in TMEM16F-expressing HEK293T cells when the intracellular free Ca\(^{2+}\) concentration was increased, indicating plasma membrane expression of TMEM16F.

It was previously reported that TMEM16K produces small CaCC currents in whole cell patch-clamp recordings (25). The present study, however, demonstrated that in TMEM16K-overexpressing HEK293T cells, ionomycin induced only small
CaCC currents that are comparable to endogenous CaCC currents in mock-transfected cells (Fig. 5, A and B). In addition, we could not observe TMEM16K-dependent Cl\(^{-}\) currents even when the intracellular free Ca\(^{2+}\) concentration was increased to 100 \(\mu\)M (Fig. 5C). The reason for the discrepancy between our results and the previously reported results is at present unknown. However, it must be noted that 25 amino acids in the putative pore region of TMEM16K are missing (14). Thus a reasonable assumption might be that TMEM16K has no channel function, although the possibility of intracellular expression of TMEM16K in our expression system cannot be ruled out.

**Ca\(^{2+}\) sensitivity of TMEM16F-dependent CaCC is distinct from that of TMEM16A/B.** The voltage- and time-dependence of Ca\(^{2+}\)-induced activation of TMEM16A and TMEM16B Cl\(^{-}\) channel currents is known to be Ca\(^{2+}\)-dependent (4, 22, 35, 37): at nanomolar levels of intracellular free Ca\(^{2+}\), the TMEM16A and TMEM16B currents exhibit outward rectification and time-dependent activation at positive potentials, but their \(I-V\) relationships become linear and the fraction of time-dependent currents was reduced when the intracellular free Ca\(^{2+}\) concentration was increased to micromolar levels. In the present study, however, TMEM16F-dependent Cl\(^{-}\) currents exhibited outward rectification and time-dependent activation at positive potentials even at a free Ca\(^{2+}\) concentration as high as 100 \(\mu\)M (Fig. 8A), and the pattern of outwardly rectifying \(I-V\) relationships was essentially independent of the intracellular free Ca\(^{2+}\) level between 10 and 100 \(\mu\)M (Fig. 8B). Although activation of the TMEM16F Cl\(^{-}\) current was Ca\(^{2+}\)-dependent, the Ca\(^{2+}\) sensitivity of the TMEM16F-dependent Cl\(^{-}\) channel is lower than that of the TMEM16A- and TMEM16B-dependent Cl\(^{-}\) channels: the EC\(_{50}\) for Ca\(^{2+}\)-dependent activation of TMEM16F currents was 9.6 \(\mu\)M at +100 mV (Fig. 8C), whereas for TMEM16A and TMEM16B currents, it has been reported to be 0.4 \(\mu\)M at +60 mV and 1.2–4.0 \(\mu\)M at +40 to +60 mV, respectively (22, 29, 37). In the present study, we observed endogenous CaCC currents in mock-transfected cells. The EC\(_{50}\) for Ca\(^{2+}\)-dependent activation of endogenous CaCC currents recorded at +100 mV was 8.7 \(\mu\)M, which is similar to that for TMEM16F-dependent CaCC currents (Fig. 8C). Thus it appears that the TMEM16F-dependent CaCC is distinct from TMEM16A- and TMEM16B-dependent CaCCs in its Ca\(^{2+}\) sensitivity.

Native CaCCs in a variety of cells have been reported to show different sensitivities to intracellular Ca\(^{2+}\) that fall in the nanomolar to micromolar range (10): in skeletal muscle cells, myocytes, epithelial cells, and endothelial cells, for example, half-maximal activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels occurs at submicromolar Ca\(^{2+}\) levels. In contrast, CaCCs in olfactory neurons show lower Ca\(^{2+}\) sensitivity. The differences in Ca\(^{2+}\) sensitivity might reflect the different TMEM16 protein subtypes specifically expressed in each tissue. Actually, TMEM16A and TMEM16B have been demonstrated to constitute epithelial and olfactory Ca\(^{2+}\)-activated Cl\(^{-}\) channels, respectively (8, 29). Considering that TMEM16F is ubiquitously expressed (25), it would be interesting to investigate how the level of TMEM16F expression affects the Ca\(^{2+}\) sensitivity of endogenous CaCCs in different cell types.

In the present study, we observed slow time course of activation of TMEM16F Cl\(^{-}\) channels induced by ionomycin (Fig. 5B) and an elevated Ca\(^{2+}\) concentration (Fig. 9A), suggesting an involvement of some indirect activation mechanism. Activation of some types of CaCCs is known to be mediated by CaMKII (2, 11). In the present study, however, a CaMKII inhibitor (KN-93) had no effects on the Ca\(^{2+}\)-induced activation of TMEM16F Cl\(^{-}\) channels (Fig. 9). The direct interaction of TMEM16A with a SNARE protein complex and an ezrin-radixin-moesin network suggested an involvement of trafficking to the plasma membrane in the TMEM16A activation mechanism (21). However, the membrane capacitance was constant during a series of experiments in the present study (data not shown). Thus it appears that Ca\(^{2+}\)-induced activation of TMEM16F Cl\(^{-}\) channels is not mediated by CaMKII or membrane trafficking. Further investigation is needed, in future, to clarify whether the TMEM16F Cl\(^{-}\) channel is indirectly activated via other Ca\(^{2+}\)-dependent pathways.

**Conclusions.** Of the members of the TMEM16 family, TMEM16F has been especially controversial in regards to its function. TMEM16F has been reported to produce small CaCC currents (25); it has also been reported to contribute to VSOR currents (1), outwardly rectifying Cl\(^{-}\) currents involved in apoptosis (16), and a Ca\(^{2+}\)-dependent phospholipid scramblase activity (27, 32). We demonstrate here that human TMEM16F is a component of a CaCC that has strong outward rectification independent of the intracellular free Ca\(^{2+}\) level and has a Ca\(^{2+}\) sensitivity lower than that of TMEM16A or TMEM16B; however, it is unrelated to the VSOR activity which is activated upon osmotic cell swelling or apoptotic stimulation.

**NOTE ADDED IN PROOF**

Since this manuscript was submitted, three articles reporting possible functions of TMEM16F have appeared. Szteyn et al. (33) reported that endogenous TMEM16F functions as a CaCC in mouse dendritic cells on the basis of siRNA-mediated knockdown. Tian et al. (34) also recently reported that Cl\(^{-}\)-current activation was induced in human TMEM16F-overexpressing HEK293 cells when the intracellular Ca\(^{2+}\) concentration was increased to more than 10 \(\mu\)M. These observations are in agreement with our present study. In contrast, Yang et al. (36) reported that TMEM16F forms a Ca\(^{2+}\)-activated cation channel based on the data obtained by knockout mice and heterologous expression. Tian et al. (34) also observed sizable cation permeability through TMEM16F channels. In the present study, however, human TMEM16F-mediated channel was found to be anion-selective (Fig. 6D, E).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: T.S., T.F., H.S., and Y.O. conception and design of research; T.S., T.I., and K.S. performed experiments; T.S., T.I., and K.S. analyzed data; T.S., T.I., K.S., T.F., H.S., and Y.O. interpreted results of experiments; T.S., T.I., and K.S. prepared figures; T.S., K.S., T.F., H.S., and...
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