

# TMEM16A $\text{Ca}^{2+}$ -Activated $\text{Cl}^-$ Channel Regulates the Proliferation and Migration of Brain Capillary Endothelial Cells

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## ABSTRACT

The blood-brain barrier (BBB) is essential for the maintenance of homeostasis in the brain. Brain capillary endothelial cells (BCECs) comprise the BBB, and thus a delicate balance between their proliferation and death is required. Although the activity of ion channels in BCECs is involved in BBB functions, the underlying molecular mechanisms remain unclear. In the present study, the molecular components of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  ( $\text{Cl}_{\text{Ca}}$ ) channels and their physiological roles were examined using mouse BCECs (mBCECs) and a cell line derived from bovine BCECs, t-BBEC117. Expression analyses revealed that TMEM16A was strongly expressed in mBCECs and t-BBEC117 cells. In t-BBEC117 cells, whole-cell  $\text{Cl}^-$  currents were sensitive to the  $\text{Cl}_{\text{Ca}}$  channel blockers, 100  $\mu\text{M}$  niflumic acid and 10  $\mu\text{M}$  T16A<sub>inh</sub>-A01, and were also reduced markedly by small-interfering RNA (siRNA) knockdown of TMEM16A. Importantly, block of  $\text{Cl}_{\text{Ca}}$  currents with  $\text{Cl}_{\text{Ca}}$  channel blockers or TMEM16A siRNA induced membrane hyperpolarization. Moreover, treatment with TMEM16A siRNA caused an increase in resting cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ). T16A<sub>inh</sub>-A01 reduced cell viability in a concentration-dependent manner. Either  $\text{Cl}_{\text{Ca}}$  channel blockers or TMEM16A

siRNA also curtailed cell proliferation and migration. Furthermore,  $\text{Cl}_{\text{Ca}}$  channel blockers attenuated the trans-endothelial permeability. In combination, these results strongly suggest that TMEM16A contributes to  $\text{Cl}_{\text{Ca}}$  channel conductance and can regulate both the resting membrane potential and  $[\text{Ca}^{2+}]_{\text{cyt}}$  in BCECs. Our data also reveal how these BCECs may be involved in the maintenance of BBB functions, as both the proliferation and migration are altered following changes in channel activity.

## SIGNIFICANCE STATEMENT

In brain capillary endothelial cells (BCECs) of the blood-brain barrier (BBB), TMEM16A is responsible for  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels and can regulate both the resting membrane potential and cytosolic  $\text{Ca}^{2+}$  concentration, contributing to the proliferation and migration of BCECs. The present study provides novel information on the molecular mechanisms underlying the physiological functions of BCECs in the BBB and a novel target for therapeutic drugs for disorders associated with dysfunctions in the BBB.

## Introduction

The blood-brain barrier (BBB) plays a critical role in the regulation of homeostasis in the brain microenvironment. Under physiological conditions, the BBB restricts the movement of substances between the circulation and the brain and protects neurons from the invasion of peripheral noxious substances. The BBB is formed by brain capillary endothelial

cells (BCECs). BCECs are structurally characterized by intercellular tight junctions, relatively low transcellular transport activity, and surrounding cell types, including pericytes, astrocytes, neurons, and microglia. These surrounding cells form a neurovascular unit, which contributes to the maintenance and enhancement of the BBB (Abbott et al., 2006; Nakagawa et al., 2009). The barrier function of the BBB is maintained by the turnover of BCECs, and this involves a dynamic balance between the production of new cells by cell proliferation and their degradation by cell death (Abbott et al., 2006; Sweeney et al., 2019).

In vascular endothelial cells, an increase in cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) can influence cell fate, including cell viability and rates of proliferation, migration, and death. Endothelial  $\text{Ca}^{2+}$  signaling is modulated by the activity of ion channels (Nilius and Droogmans, 2001). BCECs have been

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**ABBREVIATIONS:**  $\tau_{\text{act}}$ , time constant for current activation; BBB, blood-brain barrier; BCEC, brain capillary endothelial cell; BrdU, bromodeoxyuridine;  $[\text{Ca}^{2+}]_{\text{cyt}}$ , cytosolic  $\text{Ca}^{2+}$  concentration;  $\text{Cl}_{\text{Ca}}$ ,  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$ ; DMEM, Dulbecco's modified Eagle's medium; fura-2/AM, fura-2 acetoxymethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glia fibrillary acidic protein; HEK, human embryonic kidney; ICC, interstitial cells of Cajal;  $\text{K}_{\text{IR}}$ , inward-rectifying  $\text{K}^+$ ; mBCEC, mouse brain capillary endothelial cell; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NFA, niflumic acid; PCR, polymerase chain reaction; PDGFR- $\beta$ , platelet-derived growth factor receptor- $\beta$ ; siRNA, small-interfering RNA;  $\text{SK}_{\text{Ca}}$ , small-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ ; TEER, trans-endothelial electrical resistance; TRP, transient receptor potential; TRPC, transient receptor potential canonical subfamily.

reported to express several types of ion channels, such as voltage-dependent  $K^+$  channels, inward-rectifying  $K^+$  ( $K_{IR}$ ) channels,  $Ca^{2+}$ -activated  $K^+$  channels, and transient receptor potential (TRP) channels. Their combined activity contributes to the regulation of brain blood flow (Longden et al., 2016; Sweeney et al., 2019). Previously we have found that small-conductance  $Ca^{2+}$ -activated  $K^+$  ( $SK_{Ca}$ ) channels ( $SK_{Ca2}$ ) (Yamazaki et al., 2006),  $K_{IR2.1}$  channels (Yamazaki et al., 2011), TRP canonical subfamily (TRPC) channels (TRPC1/3) (Yamazaki et al., 2007), and store-operated  $Ca^{2+}$  channels (Orai1/2 and stromal interaction molecule 1 (STIM1)) (Kito et al., 2014, 2015) are expressed in BCECs and can facilitate cell proliferation and death. Although the physiological significance of  $Ca^{2+}$  and  $K^+$  channels in BCECs has been examined in detail, that of the functional expression of  $Cl^-$  channels remains unclear.

$Cl^-$  channels contribute to a number of physiological processes, such as epithelial fluid secretion, cell volume regulation, osmolarity sensing, smooth muscle contraction, and neuroexcitation. Among  $Cl^-$  channels,  $Ca^{2+}$ -activated  $Cl^-$  ( $Cl_{Ca}$ ) channels are widely distributed and activated by an increase in  $[Ca^{2+}]_{cyt}$ .  $Cl_{Ca}$  conductance regulates epithelial fluid secretion, smooth muscle contraction, and neurotransmission (Verkman and Galiotta, 2009). Two TMEM16 family proteins, TMEM16A and TMEM16B, were recently identified as functional  $Cl_{Ca}$  channels. TMEM16A channels are expressed in many cell types, including epithelial cells, smooth muscle cells, the interstitial cells of Cajal (ICC), and nociceptive neurons (Pedemonte and Galiotta, 2014). On the other hand, the expression of TMEM16B channels is localized in olfactory, retinal, and hippocampal neurons (Pedemonte and Galiotta, 2014). In the TMEM16 research field, the majority of studies focus on physiological and pathological functions in the epithelium, smooth muscles, neurons, and ICC. In contrast, relatively little information has been obtained from the endothelium.

In the present study, the functional expression and physiological significance of the TMEM16 family were examined in mouse BCECs (mBCECs) and a cell line derived from bovine BCECs, t-BBEC117, using  $Cl_{Ca}$  channel blockers and TMEM16A small-interfering RNA (siRNA) by quantitative real-time polymerase chain reaction (PCR), Western blotting, whole-cell patch-clamp, membrane potential and  $[Ca^{2+}]_{cyt}$  measurements by fluorescent dyes, cell viability, proliferation, migration assays, and trans-endothelial permeability measurements. We have found that TMEM16A formed  $Cl_{Ca}$  channels in BCECs and strongly modulated both their resting membrane potential and  $[Ca^{2+}]_{cyt}$ . These changes result in the regulation of the proliferation and migration of these cells.

## Materials and Methods

**Ethical Approval.** All experiments were approved by the Ethics Committee of Nagoya City University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society.

**Cell Isolation.** mBCECs were isolated as reported previously (Perrière et al., 2005). In brief, the cerebral cortices of male mice (C57BL/6N, 6–8 weeks; Japan SLC, Hamamatsu, Japan) were removed free of outer blood vessels and meninges and immersed in ice-cold PBS. The dissected cortices were homogenized and incubated in Dulbecco's modified Eagle's medium (DMEM, low glucose; Wako Pure Chemical Industries, Osaka, Japan) containing 255 U/ml collagenase (type 2; Worthington Biochemical, Lakewood, NJ), 0.1% dispase II

(neutral protease, grade II; Roche Diagnostics, Mannheim, Germany), and 10 U/ml deoxyribonuclease I (from bovine pancreas, type IV; Sigma-Aldrich, St. Louis, MO) at 37°C for 90 minutes. The digested tissues were centrifuged (1000 rpm, 4°C, 10 minutes), and the supernatant was removed. The tissue pellet was suspended in DMEM (low glucose) containing 20% bovine serum albumin (fraction V, pH 7.0; Seikagaku, Tokyo, Japan) and centrifuged (1000 rpm, 4°C, 5 minutes). After the supernatant was removed, the tissue pellet was suspended in DMEM (low glucose) containing 0.1% dispase II and 10 U/ml deoxyribonuclease I, incubated at 37°C for 60 minutes, and dispersed mechanically. After the supernatant was removed by centrifugation (1000 rpm, 4°C, 5 minutes), the cell pellet was suspended in DMEM (high glucose; Wako Pure Chemical Industries) containing 15% plasma-derived serum (from platelet poor human plasma; Sigma-Aldrich), 100 U/ml penicillin G (Wako Pure Chemical Industries), 200 µg/ml streptomycin (Meiji Seika Pharma, Tokyo, Japan), and 4 µg/ml puromycin (InvivoGen, San Diego, CA) and then filtered using the Cell Strainers (40 µm; Falcon/Corning, Corning, NY) to obtain the capillary fraction. The capillary cells were incubated on a cell culture dish coated with 0.3 mg/ml collagen (Cellmatrix type IV; Nitta Gelatin, Osaka, Japan) in DMEM (high glucose) supplemented with plasma-derived serum, penicillin G, streptomycin, and puromycin for 48 hours. Finally, mBCECs were cultured on a collagen-coated cell culture dish in DMEM (high glucose) supplemented with 15% FBS (Nichirei Biosciences, Tokyo, Japan), penicillin G, and streptomycin.

**Cell Culture.** The immortalized cell line of bovine BCECs, t-BBEC117, was established by transfection of SV40 large T antigen and then by isolating a single clone. In vitro culture of these cells has been accompanied by characterization of the BBB phenotypes based on the following criteria: 1) spindle-shape morphology, 2) rapid uptake of acetylated-low density lipoprotein, 3) formation of tight junction-like structures, 4) high alkaline phosphatase activity, and 5) expression of multidrug resistance and glucose transporter-1 mRNA (Sobue et al., 1999). t-BBEC117 cells were cultured on a noncoating cell culture dish in DMEM (high glucose) supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Human embryonic kidney (HEK) 293 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank at the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). Normal and TMEM16-expressing HEK293 cells (Saeki et al., 2019) were cultured in DMEM (high glucose) supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin.

**Quantitative Real-Time PCR.** Total RNA extraction from t-BBEC117 cell homogenates, reverse transcription, and quantitative real-time PCR, using the ABI PRISM 7000 (Applied Biosystems, Foster City, CA) and LightCycler 96 (Roche Diagnostics) real-time PCR systems, were performed as previously reported (Yamazaki et al., 2011). Specific primers for bovine TMEM16 genes were designed as follows: TMEM16A (GenBank Accession number, NM\_001192717), (+) CTG GCC TTT GTC ATC GTC TTC, (–) GTG GAT CTG CTG GCT GAT GTC; TMEM16B (XM\_024992694), (+) GCC TGG CTG GGA TTA TAT ACA TC, (–) CTC TCT GCT GGG AAT ATC TTC TTC; TMEM16C (NM\_001191315), (+) ATT TGC AAG GCC ACT GAA GTC, (–) CAG ATA TGT CAC CTT GGC GTA GA; TMEM16D (NM\_001102050), (+) TCC CTC GCT TGG TAT ATG CTT A, (–) TAC AGA CAA GCT GGC ATT CAC A; TMEM16E (NM\_001168406), (+) CGT TAT TTG TGG CCT CTT TTC C, (–) CCT GTA CTG AGT CGT AAG CTT CCA; TMEM16F (XM\_005206409), (+) CCT GGA CAC CCA CAA GAG TAT AAA, (–) CAC AGA ATA GAT GAC GTG CTC CAT; TMEM16G (XM\_024989870), (+) GGT CCA TGG TGC ACT TCA TCT T, (–) AAG GTG GTA ACG AAG CCG AAC T; TMEM16H (XM\_024994806), (+) CTG CAG GTA GGT CGG TAT CTG, (–) GTG TGG TCA TCG GTC GTA TCT; TMEM16J (XM\_010821300), (+) GGC CCT GGT TCA TGT GAT, (–) GCT CTC TCG CTC TGA AAA GGT; TMEM16K (NM\_001102169), (+) CTA TGC CAT CGT GAT CGA GAT, (–) GAT TCT GGT AGG CAG ATT CCA A; and GAPDH (NM\_001034034), (+) TTC TGG CAA AGT GGA CAT CGT, (–) CTT GAC TGT GCC GTT GAA CTT G.

**Western Blot.** Western blot experiments were performed as previously described (Yamamura et al., 2018b). In brief, the protein fraction was extracted from mBCECs and t-BBEC117 cell homogenates by using RIPA buffer (Cell Signaling Technology, Danvers, MA). The extracted protein (40  $\mu$ g/lane) was subjected to 7.5% SDS-PAGE. After the resulting blots were treated with 5% fat-free milk (Megmilk Snow Brand, Tokyo, Japan), these were incubated with a polyclonal anti-TMEM16A antibody (1:100; ab53212; Abcam, Cambridge, MA) (Yamamura et al., 2018a) at 4°C for 24 hours, treated with an anti-rabbit horseradish peroxidase-conjugated IgG antibody (1:1000; AP132P; Chemicon International, Temecula, CA) at 4°C for 1 hour, and then exposed to an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Protein expression was normalized using monoclonal anti- $\beta$ -actin (1:5000; A1978; Sigma-Aldrich) and anti-mouse horseradish peroxidase-conjugated IgG (1:2000; AP124P; Chemicon International) antibodies. BCECs, pericytes, and astrocytes were identified by immunoblotting, using monoclonal anti-ZO-1 (1:100; sc-33725; Santa Cruz Biotechnology, Dallas, TX), anti-platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ) (1:1000; #3162; Cell Signaling Technology), and anti-glia fibrillary acidic protein (GFAP) (1:100; sc-33673; Santa Cruz Biotechnology) antibodies, respectively. The luminescence images were analyzed by using a LAS-3000 system (Fujifilm, Tokyo, Japan).

**siRNA Knockdown.** t-BBEC117 cells were cultured to a confluent monolayer (~80%) on a noncoating 60-mm dish.

Cultured t-BBEC117 cells were electroporated with 200 nM of control (Medium GC Duplex #3; Invitrogen) or TMEM16A (Stealth RNAi, Tmem16aRSS317801; Invitrogen, Carlsbad, CA) siRNA, using a Cell Line Nucleofector Kit V (Lonza, Walkersville, MD) by the Nucleofector 2b electroporation system (Lonza). Experiments using siRNA-treated cells were performed 72 hours after electroporation.

**Electrophysiological Recordings.** Electrophysiological recordings were performed on single t-BBEC117 cells, using a whole-cell patch clamp technique with a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan), analog digital converter (Digidata 1440A; Axon/Molecular Devices, Foster City, CA), and pCLAMP software (version 10; Axon/Molecular Devices), as previously reported (Yamamura et al., 2012). A 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-buffered solution was used as an extracellular solution as follows: 137 mM NaCl, 5.9 mM KCl, 2.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH. The pipette solution had the following ionic composition: 120 mM CsCl, 20 mM tetraethylammonium (TEA)-Cl, 2.8 mM  $\text{MgCl}_2$ , 2 mM  $\text{ATPNa}_2$ , 10 mM HEPES, 5 mM  $O,O'$ -bis(2-aminoethyl)ethyleneglycol- $N,N,N',N'$ -tetraacetic acid (EGTA), and 4.25 mM  $\text{CaCl}_2$  (pCa 6.0, 1  $\mu$ M). The pH was adjusted to 7.2 with CsOH. Electrophysiological recordings were performed at room temperature (23–25°C).

**Measurement of the Membrane Potential.** Fluorescent changes in the membrane potential were monitored using the ARGUS/HISCA imaging system (Hamamatsu Photonics, Hamamatsu, Japan). Single t-BBEC117 cells were loaded with the voltage-sensitive fluorescent dye, 100 nM bis(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC<sub>4</sub>(3); Dojin, Kumamoto, Japan], at room temperature for 30 minutes. DiBAC<sub>4</sub>(3) was added to the extracellular solution during measurements. t-BBEC117 cells were illuminated at a 488-nm wavelength, and the fluorescent emissions (>520 nm) were captured. Fluorescent intensity was normalized by the maximum fluorescence intensity in a 140-mM  $\text{K}^+$  solution ( $F/F_{140\text{K}}$ ). Standard HEPES-buffered solution was used as an extracellular solution. The 140-mM  $\text{K}^+$  HEPES-buffered solution had the following ionic composition: 5.9 mM NaCl, 140 mM KCl, 2.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH.

**[ $\text{Ca}^{2+}$ ]<sub>cyt</sub> Measurements.** [ $\text{Ca}^{2+}$ ]<sub>cyt</sub> measurements were performed using the Argus/HISCA imaging system. Single t-BBEC117 cells were loaded with 10  $\mu$ M of fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes/Invitrogen, Eugene, OR) at room temperature for 30 minutes. The fura-2 signal was converted to [ $\text{Ca}^{2+}$ ]<sub>cyt</sub> as follows (Yamazaki et al., 2006): [ $\text{Ca}^{2+}$ ]<sub>cyt</sub> =  $K_d \times B \times [(R - R_{\min})/(R_{\max} - R)]$ , where  $K_d$  is the

dissociation constant of fura-2 (224 nM);  $R$  is the fluorescence ratio ( $F_{340}/F_{380}$ );  $R_{\min}$  and  $R_{\max}$  are the fluorescence ratios in the absence of and with the saturation of  $\text{Ca}^{2+}$ , respectively; and  $B$  is the average fluorescence proportionality coefficient obtained at 380 nm under  $R_{\min}$  and  $R_{\max}$  conditions ( $F_{\min}^{380}/F_{\max}^{380}$ ). Standard HEPES-buffered solution was used as an extracellular solution.  $\text{Ca}^{2+}$ -free HEPES-buffered solution was prepared by adding 1 mM of EGTA instead of 2.2 mM of  $\text{CaCl}_2$ .

**Cell Viability Assay.** t-BBEC117 cells ( $5 \times 10^3$  cells/well) were subcultured in a noncoating 96-well plate and incubated at 37°C for 12 hours. Cellular viability was evaluated based on the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; 5 mg/ml; Sigma-Aldrich) assay, as previously described (Yamazaki et al., 2006). The results obtained were quantified colorimetrically as absorbance at 595 nm, using the Multiskan JX (ver.1.1; Thermo Fisher Scientific, Waltham, MA).

**Cell Proliferation Assay.** Cell preparation was performed in the same manner as that described for the MTT assay. The proliferation of t-BBEC117 cells was evaluated using the Cell Proliferation ELISA BrdU (colorimetric) kit (Roche Diagnostics) based on the bromodeoxyuridine (BrdU) incorporation assay. Colorimetric quantification as absorbance at 450 nm was measured by using a similar method to that for the MTT assay.

**Wound-Healing Assay.** Cell migration was evaluated by a scratch wound assay. t-BBEC117 cells were cultured to a confluent monolayer on a noncoating 24-well plate and then wounded by a sterile 200- $\mu$ l tip. After the pretreatment with the drug or siRNA for 72 hours in DMEM (high glucose) containing 2% FBS, these cells were stained with Hoechst 33342 (1:2000; Molecular Probes/Invitrogen) at room temperature for 10 minutes. The number of cells that had migrated into the scratch zone (1  $\times$  0.75 mm) for 72 hours was counted by the high-content imaging system Operetta (Perkin Elmer Life Sciences, Boston, MA).

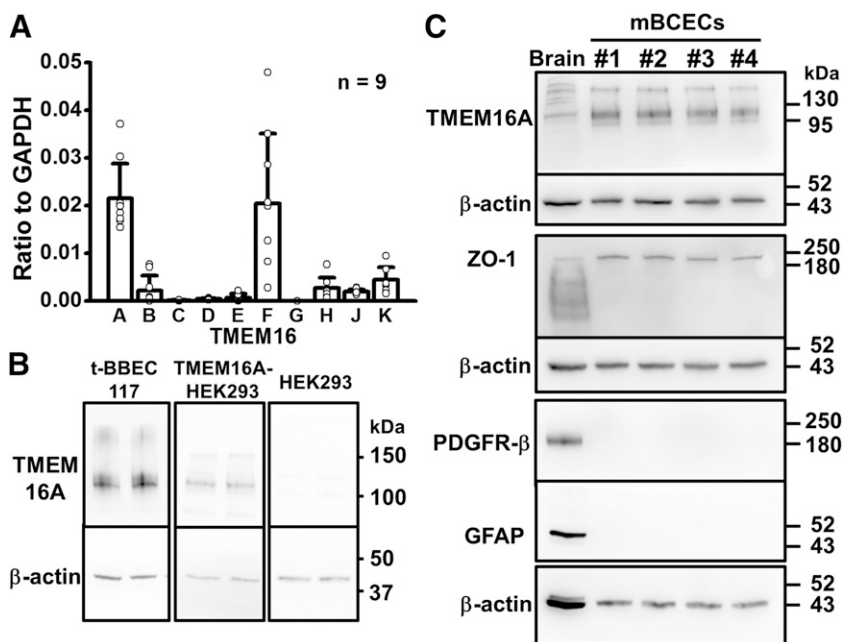
**Trans-Endothelial Electrical Resistance Measurements.** t-BBEC117 cells ( $8 \times 10^4$  cells/well) were cultured to a confluent monolayer on the Cell Culture Inserts (1.0- $\mu$ m pore size) and Companion Plates (12-well; Falcon/Corning) for 96 hours and then exposed to the drug for 72 hours. The trans-endothelial electrical resistance (TEER) of the confluent monolayer of t-BBEC117 cells was measured by the Millicell ERS-2 electrical resistance system (EMD Millipore, Billerica, MA).

**Drugs.** Pharmacological reagents were obtained from Sigma-Aldrich, except for EGTA and HEPES (Dojin). Niflumic acid (NFA) and T16A<sub>inh</sub>-A01 (2-[5-ethyl-1,6-dihydro-4-methyl-6-oxo-2-pyrimidinylthio]- $N$ -[4-(4-methoxyphenyl)-2-thiazolyl]-acetamide) were dissolved in dimethyl sulfoxide at a concentration of 100 and 10 mM, respectively, as a stock solution.

**Statistical Analysis.** Pooled data are shown as the mean  $\pm$  S.D. The sample size was determined in advance. The significance of differences between two groups was assessed by the Student's  $t$  test using the BellCurve for Excel software (version 3.10; Social Survey Research Information, Tokyo, Japan). The significance of differences among groups was assessed by Tukey's test after a one-way analysis of variance using the same software.

## Results

**Expression of TMEM16A in BCECs.** Expression profile of the selected TMEM16 family member in a cell line of bovine BCECs, t-BBEC117, was examined by quantitative real-time PCR and Western blotting. In these t-BBEC117 cells, TMEM16A ( $0.022 \pm 0.007$  of GAPDH,  $n = 9$ ) and TMEM16F ( $0.020 \pm 0.015$ ,  $n = 9$ ) were strongly expressed at the mRNA level (Fig. 1A). Because TMEM16A is a confirmed molecular component of  $\text{Cl}_{\text{Ca}}$  channels in various cells (Pedemonte and Galletta, 2014), we have focused on the expression and function of TMEM16A in t-BBEC117 cells. Western blot analysis showed the expression of



**Fig. 1.** Expression analyses of the TMEM16 family in BCECs. Expression profile of TMEM16 genes (TMEM16A to K except for I) in a cell line of bovine BCECs, t-BBEC117, and mBCECs was examined by quantitative real-time PCR and Western blotting. (A) Quantitative real-time PCR analysis of TMEM16 candidates. Note that mRNA expression of TMEM16A and TMEM16F is abundant in t-BBEC117 cells ( $n = 9$ ). The threshold cycle (Ct) value of GAPDH was  $17.4 \pm 1.2$  ( $n = 9$ ). (B) Western blot analysis of TMEM16A (114 kDa) and  $\beta$ -actin (42 kDa) in t-BBEC117 cells. Normal and TMEM16A-expressing HEK293 cells were used as negative and positive controls for TMEM16A expression, respectively. Similar results were obtained from seven independent experiments. (C) Western blot analysis of TMEM16A with ZO-1 (BCEC marker), PDGFR- $\beta$  (pericyte marker), GFAP (astrocyte marker), and  $\beta$ -actin in mBCECs ( $n = 4$ ). Data are shown as mean  $\pm$  S.D.

the TMEM16A protein (114 kDa) in t-BBEC117 cells ( $n = 7$ ; Fig. 1B).

The expression of TMEM16A in native BCECs was examined by Western blotting. BCECs were identified by the presence of ZO-1 protein (BCEC marker) and the absence of PDGFR- $\beta$  (pericyte marker) and GFAP (astrocyte marker) proteins ( $n = 4$ ; Fig. 1C). TMEM16A was expressed at the protein level in mBCECs ( $n = 4$ ). Collectively, these results strongly suggest that TMEM16A is expressed in BCECs.

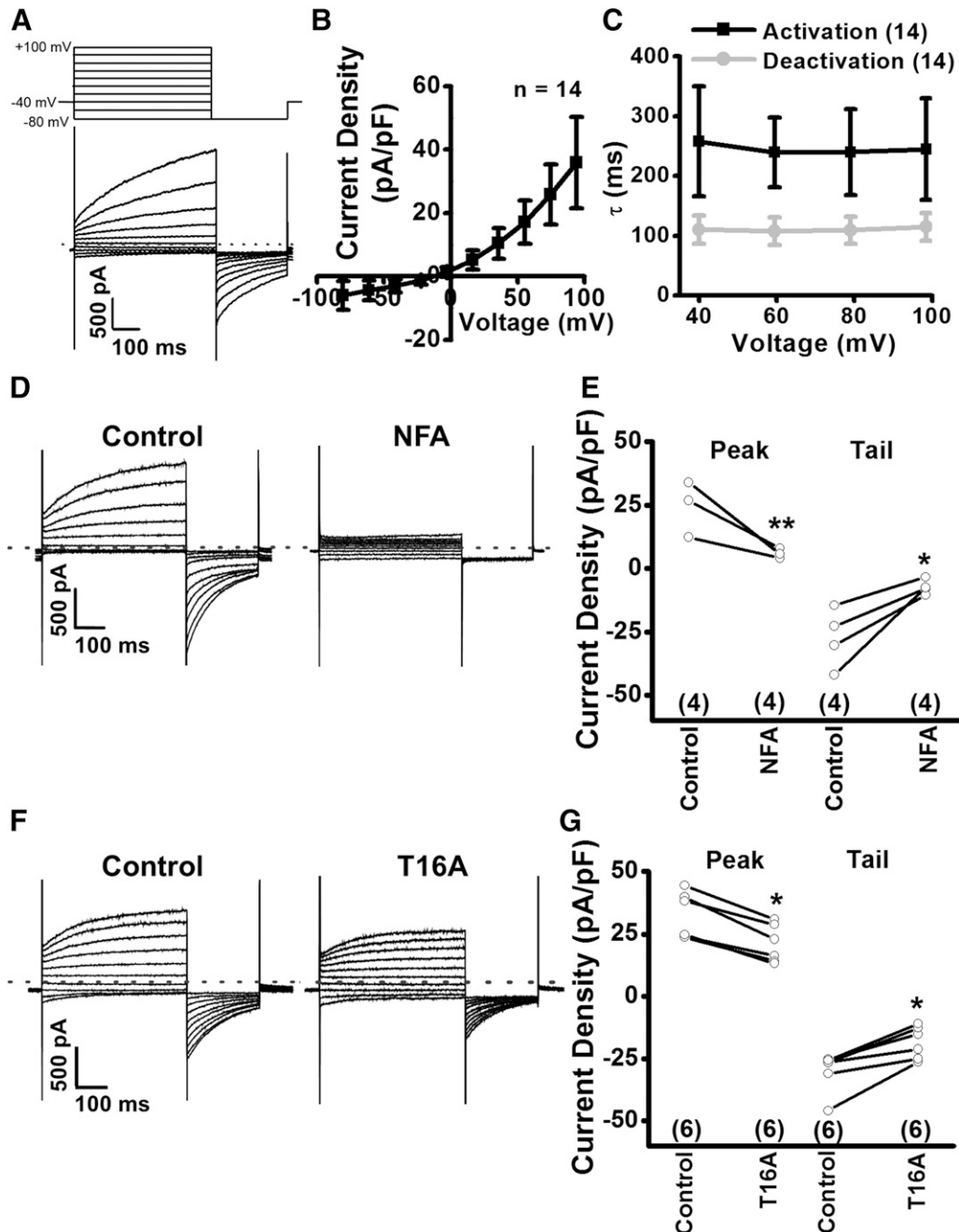
**Cl<sub>Ca</sub> Currents in t-BBEC117 Cells.** Whole-cell Cl<sub>Ca</sub> currents were recorded in t-BBEC117 cells under voltage-clamp conditions. K<sup>+</sup> currents were abolished by Cs<sup>+</sup> and TEA in the pipette solution. Ca<sup>2+</sup> concentration in the pipette solution was fixed at pCa 6.0 (1  $\mu$ M). Single cells were depolarized for 500 milliseconds from the holding potential of  $-40$  mV to the selected test potentials ( $-80$  to  $+100$  mV) in  $+20$ -mV increments and were then repolarized to  $-80$  mV for 250 milliseconds every 15 seconds. Cell capacitance averaged  $30.9 \pm 9.2$  pF ( $n = 14$ ). Depolarizations positive to  $+40$  mV evoked time-dependent outward currents ( $35.9 \pm 14.5$  pA/pF at  $+100$  mV,  $n = 14$ ; Fig. 2, A and B). The time constant for current activation ( $\tau_{act}$ ) at  $+100$  mV was  $244 \pm 85$  milliseconds ( $n = 14$ ; Fig. 2C). On the other hand, repolarizing stimuli caused characteristic inward tail currents ( $-33.4 \pm 11.3$  pA/pF at  $-80$  mV after the  $+100$ -mV stimulation,  $n = 14$ ; Fig. 2A). The time constant for tail current deactivation ( $\tau_{tail}$ ) at  $-80$  mV after the  $+100$ -mV stimulation was  $115 \pm 23$  milliseconds ( $n = 14$ ; Fig. 2C).

The effects of conventional and selective Cl<sub>Ca</sub> channel blockers, NFA and T16A<sub>inh</sub>-A01, respectively, on outward and tail currents were examined in t-BBEC117 cells. Both outward and tail currents were strongly inhibited by 100  $\mu$ M NFA ( $5.9 \pm 1.6$  and  $-7.3 \pm 2.9$  pA/pF, respectively,  $n = 4$ ,  $P < 0.05$  vs. control; Fig. 2, D and E). These currents were also blocked by 10  $\mu$ M T16A<sub>inh</sub>-A01 ( $21.1 \pm 7.7$  and  $-21.5 \pm 7.2$  pA/pF, respectively,  $n = 6$ ,  $P < 0.05$ ; Fig. 2, F and G).

To obtain more direct evidence that the Cl<sub>Ca</sub> currents in t-BBEC117 cells are mediated by TMEM16A channels, siRNA

knockdown experiments on TMEM16A were performed. The knockdown efficiency and selectivity of TMEM16A using a specific siRNA of TMEM16A in t-BBEC117 cells was confirmed by quantitative real-time PCR and Western blotting. The expression of TMEM16A mRNA was reduced markedly by TMEM16A siRNA (by 48%,  $n = 8$ ; Fig. 3A), whereas the mRNA expression of other TMEM16 genes was not changed by TMEM16A siRNA ( $n = 4$ ). However, the possibility of involvement of TMEM16F was unable to be completely excluded because the mRNA level was relatively variable in t-BBEC117 cells electroporated with control or TMEM16A siRNA. Expression of the TMEM16A protein was also decreased by TMEM16A siRNA (by 36%,  $n = 4$ ; Fig. 3B). In t-BBEC117 cells electroporated with TMEM16A siRNA, the outward and tail amplitudes of Cl<sub>Ca</sub> currents were significantly attenuated ( $14.3 \pm 3.9$  and  $-16.0 \pm 5.7$  pA/pF,  $n = 4$ ,  $P < 0.05$  vs. control siRNA of  $32.8 \pm 9.7$  and  $-37.7 \pm 9.9$  pA/pF,  $n = 4$ , respectively; Fig. 3, C and D). Taken together, these results strongly suggest that Cl<sub>Ca</sub> currents that are composed of the TMEM16A-encoding protein and sensitive to NFA and T16A<sub>inh</sub>-A01 are functionally expressed in t-BBEC117 cells.

**Contribution of Cl<sub>Ca</sub> Channels to the Resting Membrane Potential.** We next examined whether the activity of Cl<sub>Ca</sub> channels contributed to the resting membrane potential of t-BBEC117 cells. This was done by monitoring changes in signals from voltage-sensitive fluorescent dye, 100 nM DiBAC<sub>4</sub>(3). Membrane potential is presented as  $F/F_{140K}$ , where  $F$  was the fluorescence intensity and  $F_{140K}$  was the maximum fluorescence intensity in the 140 mM K<sup>+</sup> HEPES-buffered solution (theoretically  $\sim 0$  mV). The fluorescence intensity of DiBAC<sub>4</sub>(3) is increased by membrane depolarization and decreased by hyperpolarization. Furthermore, resting membrane potential was hyperpolarized by the application of 100  $\mu$ M NFA ( $0.66 \pm 0.15$ ,  $n = 45$ ,  $P < 0.05$  vs. control of  $0.73 \pm 0.12$ ; Fig. 4, A and B). Similarly, the resting membrane potential was hyperpolarized by 10  $\mu$ M T16A<sub>inh</sub>-A01 ( $0.67 \pm 0.15$ ,  $n = 56$ ,  $P < 0.01$  vs. control of  $0.81 \pm 0.19$ ).

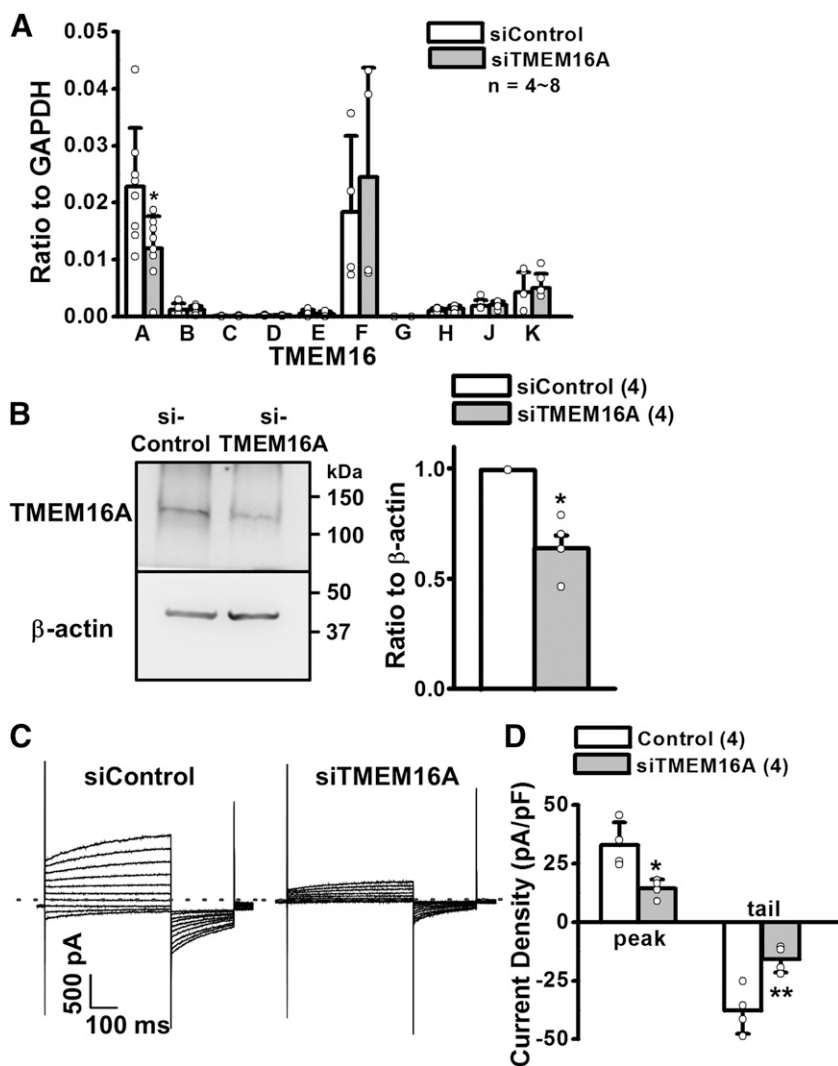


**Fig. 2.** Whole-cell  $\text{Cl}_{\text{Ca}}$  currents in t-BBEC117 cells.  $\text{Cl}_{\text{Ca}}$  currents were recorded in t-BBEC117 cells under the whole-cell voltage-clamp configuration.  $\text{K}^+$  currents were abolished by  $\text{Cs}^+$  and TEA, and  $[\text{Ca}^{2+}]_{\text{cyt}}$  was fixed at pCa 6.0 (1  $\mu\text{M}$ ) in the pipette solution. Single cells were depolarized for 500 milliseconds from the holding potential of  $-40$  mV to test potentials ( $-80$  to  $+100$  mV) in  $+20$ -mV increments and subsequently repolarized for 250 milliseconds to  $-80$  mV every 15 seconds. (A) A representative trace of whole-cell  $\text{Cl}_{\text{Ca}}$  currents in t-BBEC117 cells. Note that time-dependent outward currents and inward tail currents, which are characteristic of  $\text{Cl}_{\text{Ca}}$  currents, were observed. (B) Current density-voltage relationship ( $n = 14$ ). (C)  $\tau_{\text{act}}$  and  $\tau_{\text{tail}}$  of  $\text{Cl}_{\text{Ca}}$  currents ( $n = 14$ ). (D) Representative traces of  $\text{Cl}_{\text{Ca}}$  currents in the absence and presence of  $100 \mu\text{M}$  NFA. (E) Inhibitory effects of NFA on outward (at  $+100$  mV; peak) and inward (at  $-80$  mV following  $+100$  mV depolarization; tail) current densities ( $n = 4$ ). (F) Representative  $\text{Cl}_{\text{Ca}}$  currents in the absence and presence of  $10 \mu\text{M}$  T16A $_{\text{inh}}$ -A01 (T16A). (G) Inhibitory effects of T16A $_{\text{inh}}$ -A01 on outward and inward current densities ( $n = 6$ ). Data are shown as means  $\pm$  S.D. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control (paired  $t$  test).

The resting membrane potential was also shifted in the hyperpolarizing direction by the siRNA knockdown of TMEM16A ( $0.79 \pm 0.08$ ,  $n = 66$ ,  $P < 0.01$  vs. control siRNA of  $0.84 \pm 0.06$ ,  $n = 74$ ; Fig. 4, C and D). In t-BBEC117 cells electroporated with TMEM16A siRNA, hyperpolarizing changes in the membrane potential caused by the  $\text{Cl}_{\text{Ca}}$  channel blockers, NFA ( $0.077 \pm 0.048$ ,  $n = 22$ ,  $P < 0.01$  vs. control siRNA of  $0.139 \pm 0.050$ ,  $n = 44$ ) and T16A $_{\text{inh}}$ -A01 ( $0.016 \pm 0.029$ ,  $n = 44$ ,

$P < 0.01$  vs. control siRNA of  $0.092 \pm 0.033$ ,  $n = 30$ ), were smaller (Fig. 4, C and E). These results indicate that the activity of  $\text{Cl}_{\text{Ca}}$  channels formed by TMEM16A is importantly involved in regulating the resting membrane potential in t-BBEC117 cells.

**Effects of TMEM16A siRNA Knockdown on Resting  $[\text{Ca}^{2+}]_{\text{cyt}}$ .** Because membrane hyperpolarization often increases  $[\text{Ca}^{2+}]_{\text{cyt}}$  in nonexcitable cells, including BCECs (Guéguinou et al., 2014), the effects of the siRNA knockdown of TMEM16A on



**Fig. 3.** Reduced Cl<sub>Ca</sub> currents by the TMEM16A knockdown. Effects of siRNA knockdown of TMEM16A on Cl<sub>Ca</sub> currents in t-BBEC117 cells were examined. (A) Knockdown efficiency and selectivity of TMEM16A siRNA were confirmed in t-BBEC117 cells using quantitative real-time PCR method (n = 4–8). The threshold cycle (Ct) value of GAPDH was  $17.0 \pm 1.3$  (n = 8). (B) Knockdown efficiency of TMEM16A siRNA was confirmed in t-BBEC117 cells by Western blotting. Similar results were obtained from four independent experiments. (C) Representative Cl<sub>Ca</sub> current records from t-BBEC117 cells that had been electroporated with either control (siControl) or TMEM16A (siTMEM16A) siRNA. These single cells were depolarized for 500 milliseconds from the holding potential of -40 mV to test potentials (-80 to +100 mV) by +20-mV increments and subsequently repolarized to -80 mV for 250 milliseconds every 15 seconds. (D) Summarized data of outward (at +100 mV; peak) and inward (at -80 mV following +100-mV depolarization; tail) current densities in t-BBEC117 cells electroporated with control or TMEM16A siRNA (n = 4). Data are shown as means  $\pm$  S.D. \**P* < 0.05; \*\**P* < 0.01 vs. control siRNA (unpaired two-tailed *t* test).

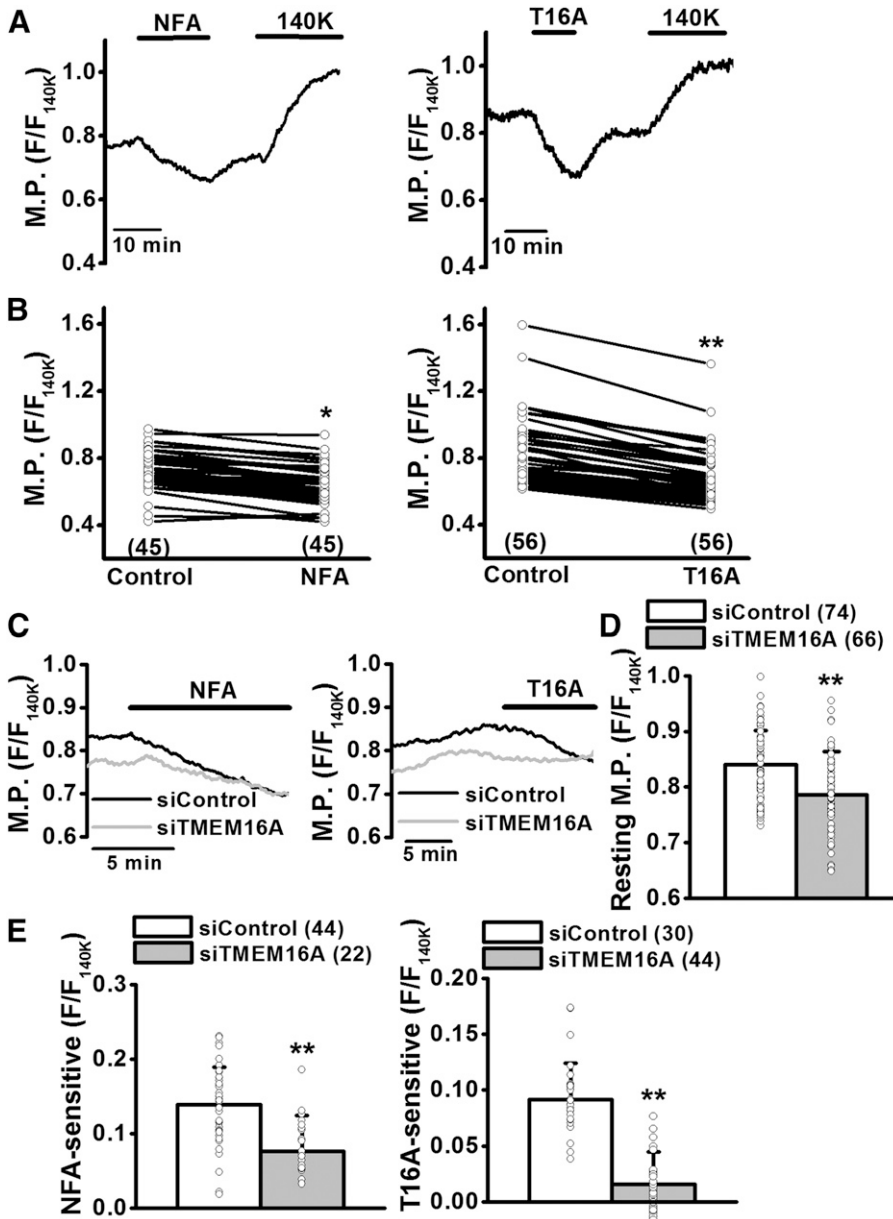
resting  $[Ca^{2+}]_{cyt}$  were examined in t-BBEC117 cells. After t-BBEC117 cells were loaded with the fluorescent  $Ca^{2+}$  indicator, 10  $\mu$ M fura-2/AM,  $[Ca^{2+}]_{cyt}$  was measured. TMEM16A siRNA-treated t-BBEC117 cells showed higher  $[Ca^{2+}]_{cyt}$  at the resting level ( $223 \pm 77$  nM, n = 32, *P* < 0.01 vs. control siRNA of  $144 \pm 47$  nM, n = 40) than control siRNA-electroporated cells (Fig. 5). In combination with the results obtained from membrane potential experiments, reduced TMEM16A channel activity appears to cause membrane hyperpolarization and subsequent  $[Ca^{2+}]_{cyt}$  increases in t-BBEC117 cells.

**Involvement of Cl<sub>Ca</sub> Channel Activity in Cell Viability and Proliferation.** Possible contributions of the activity of Cl<sub>Ca</sub> channels to the viability of t-BBEC117 cells were evaluated using the MTT assay. Cell viability was decreased markedly by the exposure to 100  $\mu$ M NFA for both 72 hours ( $2.23 \pm 0.26$ , n = 16, *P* < 0.05 vs. control of  $2.90 \pm 0.90$ , n = 8) and 96 hours ( $2.76 \pm 0.22$ , n = 16, *P* < 0.01 vs. control of  $4.04 \pm 0.65$ , n = 8; Fig. 6A). Similar results were obtained by the application of 30  $\mu$ M T16A<sub>inh</sub>-A01 for 72 hours ( $2.16 \pm 0.17$ , n = 16, *P* < 0.01 vs. control of  $3.09 \pm 1.11$ , n = 8; Fig. 6B). Cell viability was also attenuated concentration-dependently after the 96-hour treatment at lower concentrations of 3  $\mu$ M ( $2.96 \pm 0.36$ , n = 16, *P* < 0.05 vs. control of  $4.04 \pm 0.67$ , n = 8) and 10  $\mu$ M ( $3.26 \pm 0.59$ , n = 16, *P* < 0.05) as well as 30  $\mu$ M

( $2.25 \pm 0.30$ , n = 16, *P* < 0.01). In t-BBEC117 cells electroporated with TMEM16A siRNA, cell viability significantly decreased at 72 hours ( $1.58 \pm 0.04$ , n = 6, *P* < 0.01 vs. control siRNA of  $1.76 \pm 0.04$ , n = 6) and 96 hours ( $1.67 \pm 0.04$ , n = 6, *P* < 0.01 vs. control siRNA of  $2.04 \pm 0.09$ , n = 6; Fig. 6C).

The involvement of Cl<sub>Ca</sub> channels in the proliferation of t-BBEC117 cells was examined using the BrdU incorporation assay. These results revealed that cell proliferation was reduced by the treatment with 100  $\mu$ M of NFA ( $0.15 \pm 0.06$ , n = 9, *P* < 0.01 vs. control of  $0.25 \pm 0.05$ , n = 9) or 30  $\mu$ M of T16A<sub>inh</sub>-A01 ( $0.18 \pm 0.05$ , n = 9, *P* < 0.05) for 72 hour (Fig. 6D). Furthermore, the siRNA knockdown of TMEM16A also significantly reduced the proliferation of t-BBEC117 cells at 72 hours ( $0.13 \pm 0.03$ , n = 6, *P* < 0.05 vs. control siRNA of  $0.20 \pm 0.07$ , n = 6; Fig. 6E). Thus, the activity of TMEM16A Cl<sub>Ca</sub> channels contributes to regulating the proliferation of t-BBEC117 cells.

**Inhibitory Effects of Cl<sub>Ca</sub> Channel Blockade on Cell Migration.** To reveal the contributions of Cl<sub>Ca</sub> channels to cell migration, a wound-healing assay was performed under 2% FBS conditions in t-BBEC117 cells. After the treatment with Cl<sub>Ca</sub> channel blockers or TMEM16A siRNA, these cells were stained with Hoechst 33342. Then, the number of cells that had migrated into the scratch zone was counted by the high-content imaging system Operetta. Migrated cell numbers



**Fig. 4.** Contribution of  $Cl_{Ca}$  channels to the resting membrane potential. Effects of selected  $Cl_{Ca}$  channel blockers on the resting membrane potential were monitored in t-BBEC117 cells that had been loaded with the voltage-sensitive fluorescent indicator, 100 nM DiBAC<sub>4</sub>(3). Fluorescent intensity of DiBAC<sub>4</sub>(3) was increased and decreased by membrane depolarization and hyperpolarization, respectively. Fluorescent intensity signal was normalized by the maximum fluorescent intensity in the 140 mM K<sup>+</sup> HEPES-buffered solution (F/F<sub>140K</sub>). (A) Representative time courses of the membrane potential in t-BBEC117 cells. Note that block of  $Cl_{Ca}$  channels by 100  $\mu$ M NFA and 10  $\mu$ M T16A<sub>inh</sub>-A01 (T16A) caused membrane hyperpolarization. (B) Effects of NFA ( $n = 45$ ) and T16A<sub>inh</sub>-A01 ( $n = 56$ ) on the resting membrane potential. (C) Representative time courses of the membrane potential in the absence and presence of  $Cl_{Ca}$  channel blockers in t-BBEC117 cells that had been electroporated with control (siControl) or TMEM16A (siTMEM16A) siRNA. (D) Summarized data of the resting membrane potential in control ( $n = 74$ ) or TMEM16A ( $n = 66$ ) siRNA-electroporated t-BBEC117 cells. (E) Sensitivities to  $Cl_{Ca}$  channel blockers on the resting membrane potential in t-BBEC117 cells that had been electroporated with control or TMEM16A siRNA ( $n = 22-44$ ). Data are shown as means  $\pm$  S.D. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control or control siRNA [paired  $t$  test for (B); unpaired two-tailed  $t$  test for (D and E)].

were significantly decreased by the application of 100  $\mu$ M of NFA ( $51 \pm 3$  cells,  $n = 4$ ,  $P < 0.01$  vs. control of  $149 \pm 5$  cells,  $n = 4$ ; Fig. 7, A and B) or 10  $\mu$ M of T16A<sub>inh</sub>-A01 ( $59 \pm 5$  cells,  $n = 4$ ,  $P < 0.01$ ) for 72 hours. The number of migrated cells were also decreased significantly by TMEM16A siRNA for 72 hours ( $59 \pm 12$  cells,  $n = 4$ ,  $P < 0.01$  vs. control siRNA of  $167 \pm 14$  cells,  $n = 4$ ; Fig. 7, C and D). In combination, these results strongly suggest that the activity of TMEM16A  $Cl_{Ca}$  channels regulates the migration, as well as the proliferation, of t-BBEC117 cells.

#### Physiological Roles of TMEM16A in BBB Function.

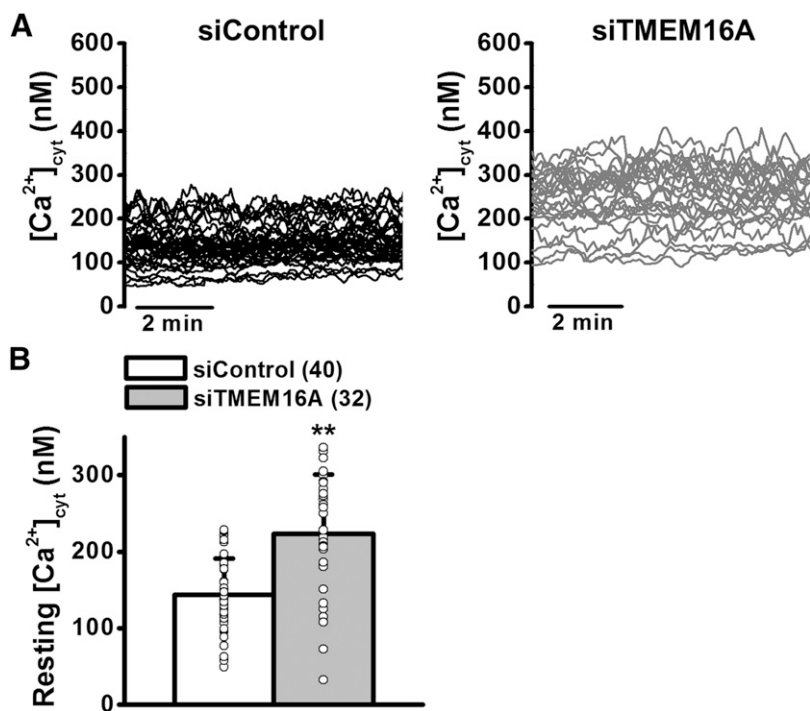
To reveal the involvement of  $Cl_{Ca}$  channels in the BBB function, the trans-endothelial permeability of t-BBEC117 cells was analyzed by the TEER measurements. The TEER of the confluent monolayer of t-BBEC117 cells was  $24.6 \pm 4.7 \Omega \times \text{cm}^2$  ( $n = 6$ ; Fig. 8), as previously reported in immortalized cell lines of BCECs (Weksler et al., 2005; Yang et al., 2017). The TEER was significantly decreased by the application of 100  $\mu$ M of

NFA ( $8.1 \pm 3.0 \Omega \times \text{cm}^2$ ,  $n = 6$ ,  $P < 0.01$ ) or 10  $\mu$ M of T16A<sub>inh</sub>-A01 ( $6.4 \pm 1.1 \Omega \times \text{cm}^2$ ,  $n = 6$ ,  $P < 0.01$ ) for 72 hours. This result suggests that the activity of TMEM16A  $Cl_{Ca}$  channels is involved in the regulation of the trans-endothelial permeability in the BBB.

## Discussion

In the BBB, BCECs are responsible for its lower permeable barrier function. Accordingly, a delicate balance between the proliferation and death of BCECs is required to maintain BBB functions (Abbott et al., 2006; Sweeney et al., 2019). In various cells, including endothelial cells, cell proliferation and death are modulated by changes in  $[Ca^{2+}]_{\text{cyt}}$  (Nilius and Droogmans, 2001). Although it is known that changes in  $[Ca^{2+}]_{\text{cyt}}$  levels in BCECs are modulated strongly by the activity of ion channels, the underlying mechanisms remain unclear. In the present study, we found that a specific type of  $Cl_{Ca}$  channels, TMEM16A,





**Fig. 5.** Effects of the TMEM16A siRNA knockdown on resting  $[Ca^{2+}]_{cyt}$ . The effects of the siRNA knockdown of TMEM16A on resting  $[Ca^{2+}]_{cyt}$  measured in t-BBEC117 cells loaded with the  $Ca^{2+}$ -sensitive fluorescent indicator, fura-2/AM. (A)  $[Ca^{2+}]_{cyt}$  levels in t-BBEC117 cells that had been electroporated with control (siControl;  $n = 40$ ) or TMEM16A (siTMEM16A;  $n = 32$ ) siRNA. (B) Summarized data of  $[Ca^{2+}]_{cyt}$  in control ( $n = 40$ ) or TMEM16A ( $n = 32$ ) siRNA-electroporated t-BBEC117 cells. Data are shown as mean  $\pm$  S.D. \*\* $P < 0.01$  vs. control siRNA (unpaired two-tailed  $t$  test).

are expressed in BCECs. These channels contribute to  $Cl_{Ca}$  conductance and participate in determining both the resting membrane potential and  $[Ca^{2+}]_{cyt}$ , which are involved in the proliferation and migration of BCECs.

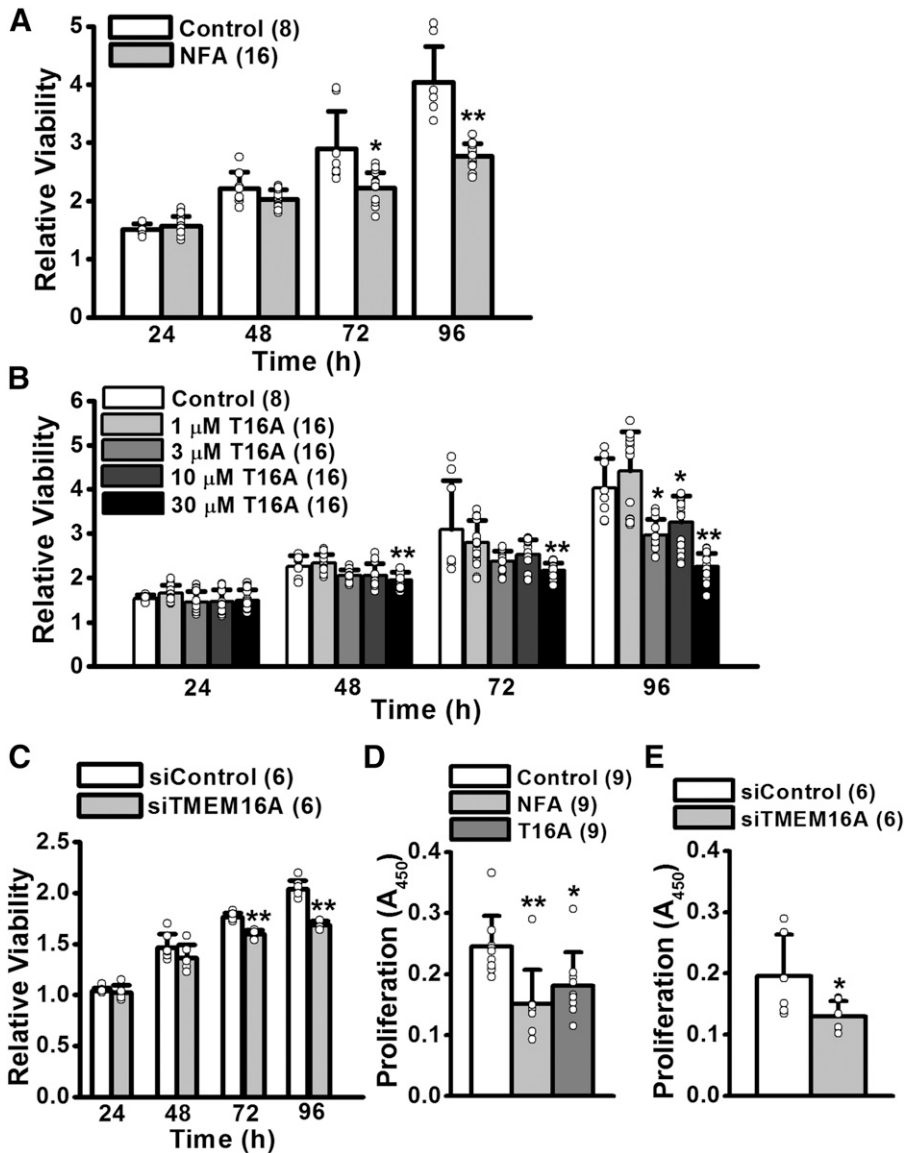
In the present study, expression analyses of the TMEM16 family revealed that TMEM16A can be detected at the mRNA and protein levels in mBCECs and t-BBEC117 cells. In addition, in t-BBEC117 cells, relatively large voltage-dependent currents can consistently be recorded under the whole-cell voltage-clamp configuration, after  $K^+$  currents were completely blocked by  $Cs^+$  and TEA, and  $[Ca^{2+}]_{cyt}$  was fixed at  $1 \mu M$  in the pipette solution. Specifically, depolarizing voltage steps to positive membrane potentials elicited a slow time-dependent outward current, and subsequent repolarization produced a characteristic inward tail current. The  $\tau_{act}$  and  $\tau_{tail}$  of TMEM16A currents ranged between 120–300 and 55–150 milliseconds, respectively (Adomaviciene et al., 2013; Scudieri et al., 2013; Ohshiro et al., 2014b; Yamamura et al., 2018a). In the present study, the kinetic parameters ( $\tau_{act}$  and  $\tau_{tail}$ ) of  $Cl_{Ca}$  currents in t-BBEC117 cells were 244 and 115 milliseconds, respectively, which are similar to those of TMEM16A currents. In addition, these currents were sensitive to a selective blocker of TMEM16A channels, T16A<sub>inh</sub>-A01, as well as to a conventional  $Cl_{Ca}$  channel blocker, NFA. The results obtained from the siRNA knockdown of TMEM16A strongly suggest TMEM16A-encoding  $Cl_{Ca}$  currents in t-BBEC117 cells.

$Cl_{Ca}$  channel blockers or TMEM16A siRNA caused membrane hyperpolarization and a subsequent increase in  $[Ca^{2+}]_{cyt}$  in BCECs. In nonexcitable cells including endothelial cells, membrane hyperpolarization promotes the electrochemical driving force for  $Ca^{2+}$  and, thus, facilitates  $Ca^{2+}$  influx through voltage-independent  $Ca^{2+}$  channels (Yamazaki et al., 2011; Guéguinou et al., 2014; Kito et al., 2014). Cytosolic  $Cl^-$  concentration varies widely, 10–60 mM, often in a tissue-dependent manner. Although the cytosolic  $Cl^-$  concentration

in BCECs is not known, it is estimated to be 30–40 mM in vascular endothelial cells (Kitamura and Yamazaki, 2001). Accordingly, the theoretical equilibrium potential of  $Cl^-$  in BCECs may be approximately  $-30$  mV under physiological conditions. The resting membrane potential of t-BBEC117 cells was between  $-30$  and  $-40$  mV, as previously reported (Yamamura et al., 2016). Block of  $Cl_{Ca}$  channels led to consistent hyperpolarization under our experimental conditions. We previously reported that membrane hyperpolarization following the activation of  $K^+$  channels promoted  $Ca^{2+}$  influx through nonselective cation channels in t-BBEC117 cells (Yamazaki et al., 2006, 2011). Similarly, membrane hyperpolarization following  $Cl_{Ca}$  channel inhibition also facilitated  $Ca^{2+}$  influx through the same signaling pathway as  $K^+$  channel activation.

We previously reported that membrane hyperpolarization and subsequent  $[Ca^{2+}]_{cyt}$  increases led to BCEC proliferation (Yamazaki et al., 2006), whereas excessive hyperpolarization and abnormal  $[Ca^{2+}]_{cyt}$  increases induced cell death (Kito et al., 2011; Yamazaki et al., 2011). These effects are mediated by  $SK_{Ca2}$  (Yamazaki et al., 2006),  $K_{IR2.1}$  (Kito et al., 2011; Yamazaki et al., 2011), TRPC1/3 (Yamazaki et al., 2007), and Orai1/2/STIM1 (Kito et al., 2014, 2015) channels. Furthermore, the present results showed that TMEM16A  $Cl_{Ca}$  channels were involved in regulating the proliferation and migration of BCECs. When  $Cl_{Ca}$  channel blockers and the TMEM16A knockdown attenuated the proliferation and migration of BCECs, the  $IC_{50}$  value of T16A<sub>inh</sub>-A01 for cell viability was  $\sim 10 \mu M$ . Because the  $IC_{50}$  value for TMEM16A channels was reported to be 1–10  $\mu M$  in reconstituted and native cells (Namkung et al., 2011; Davis et al., 2013; Ohshiro et al., 2014a; Yamamura et al., 2018a), the inhibitory effects of T16A<sub>inh</sub>-A01 on the proliferation and migration are potentially mediated by TMEM16A  $Cl_{Ca}$  channels. Similar inhibitory effects using T16A<sub>inh</sub>-A01 and TMEM16A knockdown were reported in ICC, pancreatic duct cells (Mazzone et al., 2012),



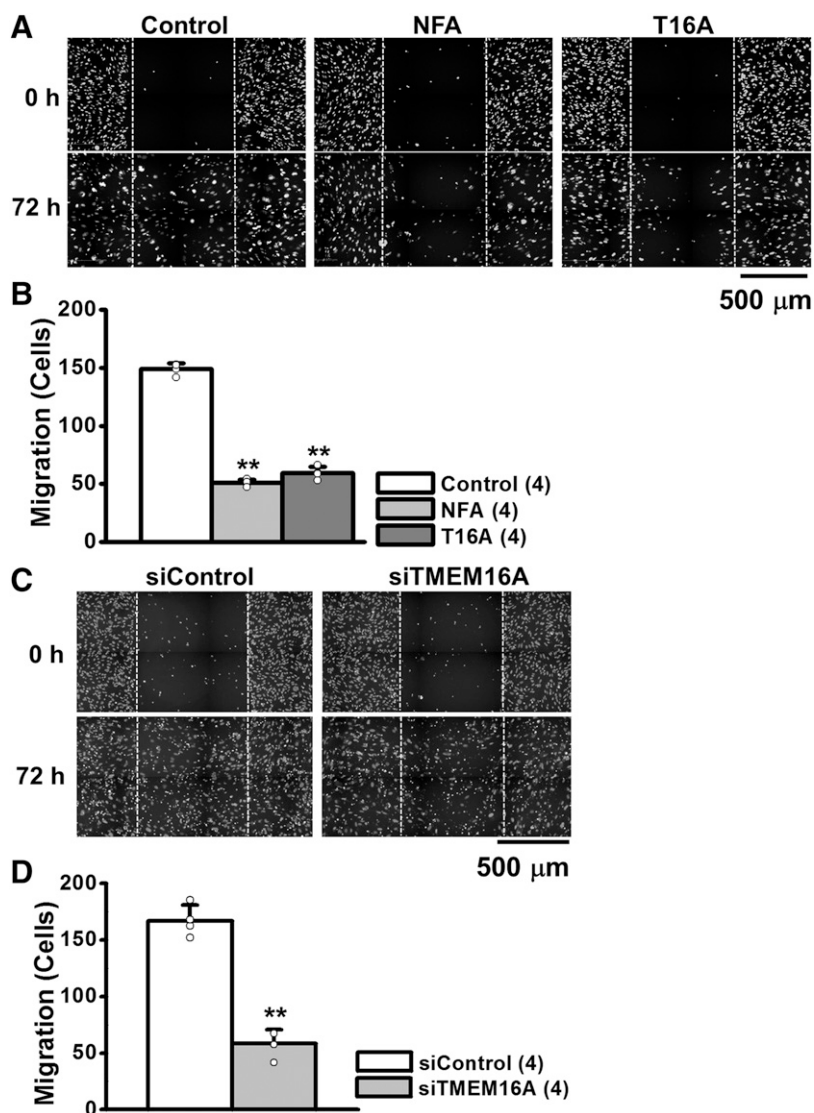


**Fig. 6.** Involvement of TMEM16A  $Cl_{Ca}$  channel activity on cell viability and proliferation. The involvement of the activity of  $Cl_{Ca}$  channels mediated by TMEM16A expression on the viability and proliferation of t-BBEC117 cells was examined by the MTT and BrdU assays, respectively. (A) Effects of a conventional  $Cl_{Ca}$  channel blocker, 100  $\mu$ M NFA, on the viability of t-BBEC117 cells ( $n = 8-16$ ). Data were normalized at 0 hours (1.0). (B) Effects of a selective  $Cl_{Ca}$  channel blocker, T16A<sub>inh</sub>-A01 (T16A), on cell viability ( $n = 8-16$ ). Note that the inhibitory effect on cell viability was concentration-dependent. (C) Inhibitory effects of TMEM16A siRNA on cell viability ( $n = 6$ ). (D) Effects of  $Cl_{Ca}$  channel blockers on the proliferation of t-BBEC117 cells ( $n = 9$ ). (E) Reduced cell proliferation by the treatment with TMEM16A siRNA ( $n = 6$ ). Data are shown as means  $\pm$  S.D. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. control or control siRNA [unpaired two-tailed  $t$  test for (A, C, and E); Tukey's test for (B and D)].

and some carcinoma cells, including head and neck squamous (Duvvuri et al., 2012), prostate (Liu et al., 2012), and colorectal (Sui et al., 2014) cancer cells. In contrast, downregulation of TMEM16A channels can promote the proliferation of rat basilar artery smooth muscle cells (Wang et al., 2012). These quite different effects of TMEM16A channels may be due to differences among cell types. Our results clearly showed that the activity of TMEM16A-encoding  $Cl_{Ca}$  channels is a positive regulator of the proliferation and migration of BCECs that maintains the balance between the proliferation and death of these cells in the BBB.

The roles of TMEM16A in cell survival and death, particularly cell proliferation, metastasis, invasiveness, and tumor development in cancer cells, are well established. TMEM16A can activate epidermal growth factor receptor,  $Ca^{2+}$ /calmodulin-dependent protein kinase II, mitogen-activated protein kinase, and nuclear factor  $\kappa$ B signaling (Wang et al., 2017). However, the signaling mechanisms of TMEM16A for the proliferation and migration of endothelial cells remain unclear. Further studies are needed

to elucidate the complete molecular mechanisms associated with TMEM16A for the proliferation and migration of BCECs. TMEM16A channels were reported to be functionally expressed in cardiac vascular endothelial cells for the prevention of ischemic damage (Wu et al., 2014), umbilical vein endothelial cells for facilitating reactive oxygen species generation associated in the setting of hypertension (Ma et al., 2017), and brain microvascular endothelial cells for regulating BBB integrity after ischemic stroke (Liu et al., 2019). However, many of physiological and pathological functions of TMEM16A in the endothelium remain unclear. In the present study, we found that the activity of TMEM16A channels partly but substantially contributed to the proliferation and migration of BCECs and the barrier function of the BBB. Because dysfunctions in the BBB can lead to the disruption of homeostasis in the brain, they have been associated with various diseases, such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, multiple sclerosis, stroke, epilepsy, and brain tumors (Abbott et al., 2006; Sweeney et al., 2019). For these



**Fig. 7.** Contribution of TMEM16A Cl<sub>Ca</sub> channels to cell migration. To examine the contributions of TMEM16A Cl<sub>Ca</sub> channels on the migration of t-BBEC117 cells, a wound-healing assay was performed in DMEM (high glucose) containing 2% FBS. After the treatment with Cl<sub>Ca</sub> channel blockers or TMEM16A siRNA, these cells were stained with Hoechst 33342. The number of cells that had migrated into the scratch zone was counted by the high-content imaging system Operetta. Dotted lines indicate the “scratch zone” at 0 hours, the start of this experiment. (A) Representative images showing the migration of t-BBEC117 cells before (0 hours) and after (72 hours) the treatment with the Cl<sub>Ca</sub> channel blockers, 100  $\mu$ M NFA and 10  $\mu$ M T16A<sub>inh</sub>-A01 (T16A). (B) Summarized data showing inhibitory effects of the Cl<sub>Ca</sub> channel blockers, NFA and T16A<sub>inh</sub>-A01, on cell migration ( $n = 4$ ). (C) Representative images of the migration of t-BBEC117 cells before (0 hours) and after (72 hours) treatment with TMEM16A siRNA. (D) Summarized data showing the migration of t-BBEC117 cells electroporated with control (siControl) or TMEM16A (siTMEM16A) siRNA ( $n = 4$ ). Data are shown as means  $\pm$  S.D. \*\* $P < 0.01$  vs. control or control siRNA [Tukey’s test for (A); unpaired two-tailed  $t$  test for (D)].

reasons, TMEM16A channels may be a novel target for therapeutic drugs for disorders associated with dysfunctions in the BBB.

In conclusion, we have demonstrated that TMEM16A is expressed in BCECs. The activity of TMEM16A Cl<sub>Ca</sub> channels contributes to regulation of the resting membrane

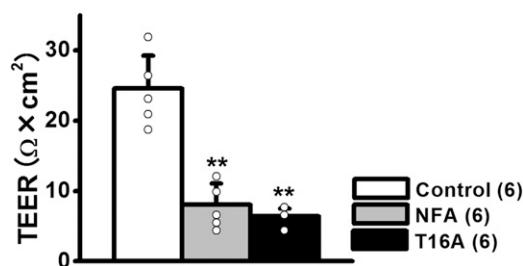
potential and [Ca<sup>2+</sup>]<sub>cyt</sub> in BCECs. Both of those signals are crucial for their proliferation and migration. Our results provide novel information on the molecular mechanisms underlying the physiological functions of BCECs in the BBB.

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#### Authorship Contributions

*Participated in research design:* T. Suzuki, Imaizumi, Yamamura.  
*Conducted experiments:* T. Suzuki, Yasumoto.  
*Contributed new reagents or analytic tools:* Asai.  
*Performed data analysis:* T. Suzuki, Yasumoto, Y. Suzuki, Imaizumi, Yamamura.  
*Wrote or contributed to the writing of the manuscript:* T. Suzuki, Yamamura.



**Fig. 8.** Effects of Cl<sub>Ca</sub> channel blockers on trans-endothelial permeability. To reveal the involvement of Cl<sub>Ca</sub> channels in the trans-endothelial permeability, the TEER of the confluent monolayer of t-BBEC117 cells was measured. Summarized data showing effects of the Cl<sub>Ca</sub> channel blockers, NFA and T16A<sub>inh</sub>-A01 (T16A), on the TEER ( $n = 6$ ). Data are shown as means  $\pm$  S.D. \*\* $P < 0.01$  vs. control (Tukey’s test).

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