Ion Transport Function of SLC4A11 in Corneal Endothelium

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PURPOSE. Mutations in SLC4A11, a member of the SLC4 superfamily of bicarbonate transporters, give rise to corneal endothelial cell dystrophies. SLC4A11 is a putative Na⁺ borate and Na⁺:OH[−] transporter. Therefore we ask whether SLC4A11 in corneal endothelium transports borate (B(OH)₄[−]), bicarbonate (HCO₃[−]), or hydroxyl (OH[−]) anions coupled to Na⁺.

METHODS. SLC4A11 expression in cultured primary bovine corneal endothelial cells (BCECs) was determined by semiquantitative PCR, SDS-PAGE/Western blotting, and immunofluorescence staining. Ion transport function was examined by measuring intracellular pH (pH_i) or Na⁺ ([Na⁺]_i) in response to Ringer solutions with/without B(OH)₄[−] or HCO₃[−] after overexpressing or small interfering RNA (siRNA) silencing of SLC4A11.

RESULTS. SLC4A11 is localized to the basolateral membrane in BCEC. B(OH)₄[−] (2.5–10 mM) in bicarbonate-free Ringer induced a rapid small acidification (0.01 pH unit) followed by alkalization (0.05–0.1 pH unit), consistent with diffusion of boric acid into the cell followed by B(OH)₄[−]. However, the rate of B(OH)₄[−]-induced pH_i change was unaffected by overexpression of SLC4A11. B(OH)₄[−] did not induce significant changes in resting [Na⁺]_i or the amplitude and rate of acidification caused by Na⁺ removal. siRNA-mediated knockdown of SLC4A11 (~70%) did not alter pH_i responses to CO₂/HCO₃[−]-rich Ringer, Na⁺-free induced acidification, or the rate of Na⁺ influx in the presence of bicarbonate. However, in the absence of bicarbonate, siSLC4A11 knockdown significantly decreased the rate (43%) and amplitude (48%) of acidification due to Na⁺ removal and recovery (53%) upon add-back. Additionally, the rate of acid recovery following NH₄⁺ prepulse was decreased significantly (27%) by SLC4A11 silencing.

CONCLUSIONS. In corneal endothelium, SLC4A11 displays robust Na⁺-coupled OH[−] transport, but does not transport B(OH)₄[−] or HCO₃[−].

Keywords: SLC4A11, intracellular pH, intracellular Na⁺, bicarbonate/borate transport

The corneal endothelium is a confluent monolayer of thin (4 μm) wide (diameter: 20–30 μm) cells on the posterior surface of the cornea that function to regulate the hydration and optical transparency of the cornea through active ion-transport processes.¹ Ion-transport dysfunction through trauma, inflammation, toxicity, or endothelial dystrophy leads to corneal edema, loss of transparency, and poor vision. For example, Fuchs' endothelial corneal dystrophy (FECED), congenital hereditary endothelial dystrophy (CHED), and Harboyan Syndrome (HS) are characterized by bilateral loss of vision, decreased endothelial cell density, and abnormal Descemet's membrane. Because of the nonregenerative nature of the corneal endothelium and lack of specific pharmacologic remedy, corneal transplantation is the only current successful therapeutic strategy for restoring corneal transparency.^{2,3}

Mutations in *SLC4A11*,^{4–10} *COL8A2*,^{11–14} *TCF8*,^{15,16} or *LOXHD1*¹⁷ genes are associated with endothelial dystrophies. These genes encode a novel anion-transporter, a Descemet's membrane component secreted by the endothelium, a transcription factor associated with embryonic epithelial develop-

ment, and a stereociliary protein, respectively. Although different hypotheses have been suggested, the pathophysiology of these dystrophies has not been fully delineated. *SLC4A11* is a ubiquitously expressing gene that encodes a 100-kDa protein with 14 transmembrane domains,^{18,19} assembling as dimers within the plasma membrane.²⁰ In the eye, it is expressed in the corneal epithelium and endothelium.²¹ Because of its membership in the Solute Carrier 4 (SLC4) superfamily, SLC4A11 was assumed to be a bicarbonate transporter.¹⁹ The only functional investigation, however, suggests that SLC4A11 does not transport bicarbonate, but is a Na⁺:2B(OH)₄[−] (electrogenic sodium borate) cotransporter, and may behave as a Na⁺:OH[−] permeable channel.²² HCO₃[−] transport is known to significantly affect the fluid pump activity of the corneal endothelium.^{23–26} The absence of HCO₃[−], reducing the expression of NBCe1 (Na⁺:HCO₃[−] cotransporter-1) or inhibition of carbonic anhydrase, which catalyzes: CO₂ + H₂O ↔ HCO₃[−] + H⁺, reduces fluid flux across the corneal endothelium.^{23–26} Since SLC4A11 belongs to the bicarbonate transport family, lack of function, or reduced expression²⁷ of a HCO₃[−]

transporter in corneal endothelium could compromise the endothelial pump. Although the biological significance of putative B(OH)_4^- transport in animal cells is unknown, the potential presence in the eye is intriguing. Therefore, using overexpression and small interfering RNA (siRNA) knockdown approaches in cultured primary bovine corneal endothelial cells (BCECs), we examined the role of SLC4A11 as a potential HCO_3^- or B(OH)_4^- transporter. In addition, we tested whether SLC4A11 is a $\text{Na}^+:\text{OH}^-$ cotransporter in BCECs. Determination of the function of SLC4A11 in the corneal endothelium will provide important information for understanding the pathology of *SLC4A11* mutations in endothelial dystrophies.

MATERIALS AND METHODS

BCEC Primary Cultures and Other Cell Type

All experiments, except where indicated, were carried out using BCECs obtained as described previously.²⁸ Briefly, corneas were isolated from bovine eyes procured from a local slaughterhouse and placed in concave molds with posterior surface facing upward. Endothelial cells were detached from the surface after incubation with 0.25% trypsin at 37°C for 15 minutes and gentle scraping. The cells were dispersed in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum and 1% penicillin (100 U/mL)/fungizone (0.25 µg/mL) mixture in T-25 flasks at 37°C with 5% CO_2 until confluent, 5 to 7 days. Cells (3.5×10^5) were subcultured into six-well plates or six 25-mm coverslips for experiments, allowed to come to 100% confluence, and used within 24 to 48 hours. Rabbit eyes (Pel-Freez Biologicals, Roger, AR) were used to obtain corneas from which the endothelial layers from the posterior side were peeled using pointed forceps to obtain native rabbit corneal endothelium. Human corneal endothelial cells (HCECs) were cultured as described previously.²⁹

Semiquantitative PCR

Total RNA extracted from confluent BCEC cultures (TRIzol method; Life Technologies Corp., Carlsbad, CA) was reverse transcribed and probed for mRNA expression of SLC4A11 with specific primers (SuperScript III One-Step RT-PCR system Platinum Taq DNA polymerase kit; Invitrogen, Carlsbad, CA). Primer sequences were designed based on bovine and rabbit *SLC4A11* mRNA sequences (Bovine, SLC4A11-1: forward [FW] ACT GCC TAC CAC TGG GAC CT, reverse [RV] CTC GTA AAT GTG CCC GTT CT; SLC4A11-2: FW CAT CAT CGG GAA AAA CAA GG, RV ATG GCT CCA TTT GTG TTC TCAT; β -actin FW ATC AAG GAG AAG CTC TGC TACG, RV TTG AAG GTA GTT TCG TGA ATGC); (Rabbit, SLC4A11-1: FW AGA GTG CCC CAA AGG AAG AT, RV ATG ATG AGC GGA AAG ACC AT; SLC4A11-2: FW CCA TGA AGT CCC TAC AGA AGC, RV ACC AGG ATG ACA AAG CGA AC). The PCR product obtained after 40 cycles at 55°C annealing temperature was visualized by running samples on 2% agarose gel stained with gel red. β -Actin expression was used as a reference.

SDS-PAGE and Western Blotting

BCECs grown on six-well plates were rinsed with chilled PBS and total protein extracted using RIPA lysis buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The extract was sonicated and centrifuged (13,000g for 20 minutes) and the supernatant was analyzed for protein content (Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford, IL). Protein (20–30 µg) in 5× sample buffer was resolved on 9% SDS gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Blots were then

blocked with 5% nonfat milk and probed with a custom SLC4A11 antibody (raised against the C-terminal region: IIEAKYLDVMDAEH; Covance, Richmond, CA), 1:1000 dilution, overnight followed by antirabbit secondary antibody conjugated to horseradish peroxidase for 1 hour and developed (Pierce Supersignal West Pico Chemiluminescent substrate; Thermo Scientific). Blots were subjected to densitometric analysis using UN-SCAN-IT 6.1. β -Actin was also probed each time to ensure equal protein loading. Purified SLC4A11 custom antibody was tested by Western blot. The band obtained using the custom antibody was at the predicted size and the predicted band was absent when the antigen peptide was included with the sample.

Immunofluorescence Staining

BCECs grown on coverslips were fixed and prepared for staining as described previously.³⁰ Cells were incubated overnight with antirabbit SLC4A11 (1:50) and antimouse ZO-1 (Zonula Occludens 1:30; Invitrogen) followed by washing and secondary antibody treatment for 1 hour (antirabbit Alexa 488 and antimouse Alexa 635; Molecular Probes, Eugene, OR). The coverslips were mounted on glass slides using commercial mounting media (Prolong; Molecular Probes) containing DAPI.

Physiologic Measurement of Intracellular pH and Na^+ Concentration

Measurement protocol and system design were similar to those of previous studies.^{31,32} Briefly, BCECs grown to 100% confluence on 25-mm coverslips were loaded with BCECF-AM (10 µM) or SBFI-AM (10 µM) (Molecular Probes), pH, and Na^+ -sensitive dyes, respectively. The coverslips were mounted in a perfusion chamber on an inverted microscope and perfused with Ringer's solutions (37°C). Bicarbonate-free (BF) Ringer's solution contained (in mM) 150 Na^+ , 4 K^+ , 0.6 Mg^{2+} , 1.4 Ca^{2+} , 118 Cl^- , 1 HPO_4^- , 10 HEPES, 28.5 gluconate⁻, and 5 glucose. Bicarbonate-rich (BR) Ringer's solution was BF Ringer's with equimolar substitution of sodium gluconate with NaHCO_3^- . B(OH)_4^- containing Ringer's solution was prepared by dissolving boric acid and adjusting pH with NaOH. Na^+ -free Ringer's solution contained NMDG-Cl or tetramethyl ammonium chloride (TMA)-Cl (for borate experiments) as a Na^+ substitute. All Ringer's solutions were equilibrated with air bubbled through NaOH (BF) or 5% CO_2 (BR) and pH was adjusted to 7.50 with NaOH or Tris base at 37°C. Osmolarities of all solutions were adjusted to 295 to 300 mOsm by adding sucrose. 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA; Sigma-Aldrich, St. Louis, MO) was added to the Ringer's solution on the day of the experiment.

The cells were excited at 440 and 495 nm for BCECF and 340 and 380 nm for SBFI fluorescence. The ratio of emitted fluorescence (520–550 nm for BCECF and above 450 nm for SBFI) was obtained at 1 Hz. BCECF ratios were calibrated against pH_i by the high- K^+ -nigericin technique as described previously.^{31,32} Relative changes in $[\text{Na}]_i$ were expressed as the fluorescence ratio 340/380 or change in fluorescence ratio ($\Delta F/F$).

Overexpression of SLC4A11

Cells grown on six-well plates or 25-mm coverslips were transfected with human influenza hemagglutinin (HA)-tagged hSLC4A11 containing phCMV2 vector or phCMV2 empty vector⁵ using Lipofectamine-2000 (Invitrogen) as per manufacturer's protocol. Expression of the induced protein was examined 48 hours posttransfection by Western blot using

FIGURE 1. Expression of SLC4A11 in corneal endothelium. (A) Bovine PCR. (B) Rabbit PCR. Different sets of primers were used to check and confirm the mRNA expression of SLC4A11. (C) Western blot shows protein expression in human, rabbit, and bovine endothelium. Fresh tissue was obtained from dissected corneas by peeling the Descemet's membrane along with the corneal endothelium.

Mouse monoclonal anti-HA antibody, 1:5000 dilution (AB-3212; Santa Cruz Biotechnology, Santa Cruz, CA).

Membrane Protein Expression of SLC4A11

BCECs grown in T-75 flasks were transfected with HA-tagged hSLC4A11 containing pCMV2 vector or empty vector as described above. Cells were processed for extraction of membrane proteins 48 hours posttransfection using surface biotinylation according to the manufacturer's directions (89881; Pierce Biotechnology, Rockford, IL). Briefly, cells were treated with 0.1 mg/mL Sulfo-NHS-SS-Biotin in PBS at 4°C for 30 minutes, collected using a cell scraper, lysed in the presence of protease inhibitors, centrifuged, supernatant applied to a commercial column (NeutrAvidin Agarose Resin; Thermo Scientific) for 1 hour, and centrifuged to collect the unbound flow through. The column was washed and bound protein eluted with sample buffer containing DTT (bound fraction of the lysate). Membrane expression was tested by Western blot using both anti-HA-tag and anti-SLC4A11 antibodies in both membrane bound and unbound fractions of the lysate as well as total lysate. Equal amounts of total, bound, and unbound protein fractions were analyzed by SDS-PAGE.

siRNA Knockdown of SLC4A11 in BCECs

SLC4A11 knockdown was achieved using an siRNA mixture (BTR1 siRNA [cow], sc-270244; Santa Cruz Biotechnology). The mix is a pool of three different siRNA duplexes: sc-270244A: Sense: CAC AGA GGA AUU CAA Att, Antisense: UUU GAA UUC CUC CUC UGU Gtt; sc-270244B: Sense: CCA UGC UCU UCU UCA UAG Att, Antisense: UCU AUG AAG AAG AGC AUG Gtt; sc-270244C: Sense: CCA CUG CUC UAC AUG AAG Att, Antisense: UCU UCA UGU AGA GCA GUG Gtt. BCECs,

grown in six-well plates or on 25-mm coverslips at 50% confluence, were washed once (Opti-MEM wash; Invitrogen) to remove serum and antibiotics. Lipofectamine-2000 was used to transfer either scrambled siRNA (Control; Invitrogen) or 10 to 50 nM siSLC4A11. Following transfection (5–6 hours at 37°C), the first washing agent (Opti-MEM; Invitrogen) was replaced with DMEM. Cell lysates were tested for maximum knockdown at different times (24–96 hours) posttransfection.

Measurement of Borate Concentration and Effect on Cell Viability

Borate concentration in media was estimated by a colorimetric assay using azomethine-H.³³ Samples were concentrated 2× by vacuum centrifugation to ensure measurable boron concentrations as low as 0.1 μM. Boron was depleted in the media with and without serum using a boron-specific ionic exchange resin (Amberlite IRA-743; Sigma-Aldrich). The UV-sterilized resin (Amberlite, 0.6 g per 50 mL of medium; Sigma-Aldrich) was added to the medium and agitated on a rotator at 80 rpm for 24 hours at 4°C. The treatment was repeated with a fresh batch of exchange resin for the next 24 hours. Medium was collected and filtered before use. The [borate] of DMEM + serum was 25 μM, which was reduced to <10 μM by resin treatment. The effect of borate on cell growth and viability was measured by MTT assay. BCECs or HEK293 cells were seeded on 24-well plates and incubated with borate-depleted medium alone or supplemented with different concentrations of borate for 24 to 48 hours. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to a final concentration of 0.45 mg/mL and incubated 1 hour at 37°C. Insoluble formazan (converted from MTT by mitochondrial reductases) was solubilized with 500 μL DMSO. Absorbance was read at 540 nm in a microplate reader.

FIGURE 2. Immunofluorescence basolateral localization of SLC4A11. (A–C) Cultured BCEC, SLC4A11 (green), ZO-1 (red), and nuclei (blue); (D–F) fresh rabbit corneal endothelium, SLC4A11 (green), and nuclei (blue). (D) Inset, $\times 20$.

Statistical Analysis

Statistical significance was determined using Student's *t*-test. A value of $P < 0.05$ was considered significant. Histogram data are presented as mean values $\pm$ SE.

RESULTS

Expression of SLC4A11 mRNA was determined in both cultured BCECs and native rabbit corneal endothelium, the two commonly used models for investigating physiologic function of the corneal endothelium. RT-PCR using multiple sets of genome specific primers for *SLC4A11* yielded single bands with the expected product size (Figs. 1A, 1B). β -Actin was used as a control for all PCR protocols. Figure 1C is a Western blot showing a SLC4A11-specific band at approximately 100 kD, consistent with previous reports,^{19,21} obtained using cultured (BCECs and HCECs) and native (bovine and rabbit) corneal endothelium protein extracts. SLC4A11 immunostaining in BCECs (Fig. 2A) and fresh rabbit corneal endothelium (Fig. 2B) showed predominant lateral membrane localization. Rabbit cells more clearly showed the lateral localization of SLC4A11, consistent with a previous report.²² Although peripheral staining was evident in cultured BCECs, the staining was not confined to the membrane in some areas, probably due to less specific antibody binding in the cultured cells.

Using SLC4A11-transfected HEK293 cells Park and colleagues²² suggest that SLC4A11 cotransports $\text{Na}^+:\text{B}(\text{OH})_4^-$ or Na^+ coupled to OH^- in the absence of borate. To test $\text{Na}^+:\text{B}(\text{OH})_4^-$ as a substrate for SLC4A11 in corneal endothelium, BCECs loaded with the pH-sensitive dye BCECF were perfused with 2.5 and 10 mM $\text{B}(\text{OH})_4^-$ in BF Ringer's solution. Figure 3A shows that $\text{B}(\text{OH})_4^-$ induced a small but brief acidification (~ 0.01 pH units), followed by a small alkalization ($+0.06$ and $+0.11$ pH units with 2.5, $n = 12$; and 10 mM $\text{B}[\text{OH}]_4^-$ [$n = 7$], respectively). The inset in Figure 3A shows reversal after removal of $\text{B}(\text{OH})_4^-$. Figure 3B summarizes the pH_i changes due to $\text{B}(\text{OH})_4^-$. To determine if this borate-dependent alkalization represents SLC4A11-mediated $\text{Na}^+:\text{B}(\text{OH})_4^-$ transport we overexpressed SLC4A11 in BCEC (Fig. 3C). Membrane expression of the induced protein was ascertained by Western blot of the isolated membrane fraction by surface biotinylation (Fig. 3D). HA-tagged protein was found in the bound fraction confirming membrane localization of the overexpressed SLC4A11. The unbound fraction had a very faint band for SLC4A11, indicative of relatively low cytosolic retention of the overexpressed protein. Figure 3E shows that BCECs overexpressing SLC4A11 did not show any difference in the rate (and amplitude) of $\text{B}(\text{OH})_4^-$ -induced alkalization compared with untransfected cells in response to 10 mM $\text{B}(\text{OH})_4^-$.

The primary evidence for $\text{Na}^+:\text{B}(\text{OH})_4^-$ cotransport was the acceleration of Na^+ -free induced acidification in the presence of $\text{B}(\text{OH})_4^-$.²² In that study the Na^+/H^+ exchanger (NHE1) was blocked by the amiloride analog EIPA, to magnify the effect.

FIGURE 3. Effect of borate on pH_i in BCEC overexpressing SLC4A11. (A) Addition of 2.5 or 10 mM borate induced slight acidification followed by alkalization. *Inset:* Reversal on borate removal. (B) Average amplitude of acidification and alkalization (2.5 and 10 mM, $n = 12$ and 7, respectively). (C) SLC4A11 expression detected with HA-tag antibody at different plasmid to lipofectamine 2000 concentration ratios (4:6 3:9, 4:10, 3:6, 2:5 μL), 48-hour posttransfection. (D) Membrane expression of the transfected SLC4A11 by surface biotinylation (total lysate [TL], unbound fraction [UB], and bound fraction [BD]). (E) Average rate ($n = 5$) of alkalization in SLC4A11 overexpressing cells relative to control.

FIGURE 4. Lack of borate-dependent Na^+ flux. (A) Cell acidification caused by Na^+ removal in the presence and the absence of borate. All solutions had 0.25 μM EIPA. (B) Average rate. (C) Average amplitude of acidification in the presence and the absence of borate ($n = 9$).

FIGURE 5. Effect of borate on intracellular Na^+ concentration. **(A)** No change in SBFI fluorescence ratio with 5 or 10 mM borate in control mock-transfected cells ($n = 7$); *inset*: change in SBFI fluorescence due to bicarbonate-rich (BR) perfusion. **(B)** No change in SBFI fluorescence ratio with 5 or 10 mM borate in SLC4A11 overexpressing cells ($n = 7$). Ouabain (100 μM), a sodium potassium ATPase inhibitor, was used as a positive control.

FIGURE 6. SLC4A11 knockdown using siRNA in BCEC. **(A)** Western blot of siSLC4A11-treated cells at different concentrations of siRNA. **(B)** Different time points at 30 nM siRNA. The percentage knockdown was compared with the protein levels in scrambled siRNA-treated cells, C, control. Approximately 70% knockdown was estimated compared with scramble siRNA in BCEC at 72 hours posttransfection.

FIGURE 7. Effect of SLC4A11 knockdown on HCO_3^- induced changes in pH_i . **(A)** Representative traces of pH_i for control (scrambled siRNA, $n = 8$). **(B)** siSLC4A11 ($n = 14$) treated BCEC. **(C, D)** The amplitudes and the initial rates of alkalinization, respectively, for siSLC4A11 compared with control.

FIGURE 8. Effect of SLC4A11 knockdown on HCO_3^- induced changes in intracellular Na^+ . (A) Representative traces depicting change in SBF ratio (340/380) for control (scrambled siRNA, $n = 8$). (B) siSLC4A11 ($n = 8$) treated BCEC in response to BR. (C) Summary of initial rates of fluorescence ratio.

The optimum effective dose of EIPA in BCEC was estimated by determining inhibition of the initial rate of pH_i recovery from acid load induced by an NH_4^+ pulse. We found that 0.25 μM EIPA produced an approximately 70% to 80% decrease in initial rate of recovery (data not shown). Higher concentrations

showed cell toxicity. Therefore, the following experiment was performed in the presence of 0.25 μM EIPA. Figure 4A shows BCEC acidification following removal of Na^+ in the absence and then in the presence of 5 mM B(OH)_4^- . Figures 4B and 4C summarize the data and show that the rate and amplitude of

FIGURE 9. SLC4A11 knockdown has no effect on Na^+ -dependent HCO_3^- flux. (A) Representative traces of pH_i for Control (scrambled siRNA, $n = 6$). (B) siSLC4A11 ($n = 8$) treated BCEC when Na^+ was removed in the presence of bicarbonate. (C, D) Summary of amplitude and rate of pH_i changes.

FIGURE 10. SLC4A11 knockdown slows Na⁺ free induced changes in pH_i in the absence of HCO₃⁻. (A) Representative trace of pH_i for control (scrambled siRNA, *n* = 7). (B) siSLC4A11 (*n* = 9) treated BCEC. (C–E) Summary bar graphs of rate and amplitude of acidification, and rate of recovery, respectively, in control and siSLC4A11-treated cells (**P* < 0.05).

acidification following removal of Na⁺ did not change significantly in the presence of 5 mM B(OH)₄⁻ compared with control.

We further examined potential Na⁺-coupled B(OH)₄⁻ transport by measuring [Na⁺_i] in response to either 5 or 10 mM B(OH)₄⁻ exposure in control or SLC4A11 overexpressing BCEC. For comparison, we also demonstrated the robust change in [Na⁺_i] known to occur upon exposure to CO₂/HCO₃⁻ due to the 1Na⁺:2HCO₃⁻ cotransporter (NBCe1)³² (Fig. 5A, inset). Figure 5A shows no change in SBFI fluorescence upon addition of B(OH)₄⁻ in control BCEC, indicating no significant Na-linked B(OH)₄⁻ transport by cells endogenously expressing SLC4A11. Figure 5B shows that overexpression of SLC4A11 also did not alter the response to B(OH)₄⁻. The previously reported results (Figs. 3–5) are consistent with the absence of Na⁺-dependent B(OH)₄⁻ transport by SLC4A11. Moreover, since putative B(OH)₄⁻ transport by SLC4A11 appeared to have effects on cell proliferation and survival,²² we examined the effect of borate on BCEC cell survival. Borate (10–300 μM; physiologic concentrations of borate are generally

<100 μM³⁴) had no significant effect on endothelial cell proliferation or survival (see Supplementary Fig. S1).

SLC4A11, a new addition to the SLC4 superfamily of bicarbonate transporters has not been thoroughly tested for possible HCO₃⁻ transport. Three different experiments were conducted to test possible Na⁺-coupled HCO₃⁻ transport by SLC4A11 in BCECs transfected with *siSLC4A11*. Figure 6 shows that transfection with *siSLC4A11* in BCECs caused an approximately 70% decrease in SLC4A11 expression between 48- and 72-hour posttransfection. First, the effect of SLC4A11 knockdown on CO₂/HCO₃⁻-induced changes in pH_i was determined. Figure 7A shows that in control cells a typical CO₂/HCO₃⁻ response includes slight acidification due to CO₂ influx followed by alkalinization due to Na⁺:HCO₃⁻ influx that is persistent in the presence of CO₂/HCO₃⁻. Contribution of SLC4A11 to the net bicarbonate transport should be indicated by a decrease in the rate and amplitude of alkalinization in *siSLC4A11*-transfected cells. Figure 7B shows a response similar to control in *siSLC4A11*-transfected cells. Figures 7C, 7D summarize the rate and amplitude of alkalinization, respectively, indicating no significant difference due to

FIGURE 11. SLC4A11 knockdown slows recovery from acid load induced by an NH_4^+ pulse under HCO_3^- -free conditions. (A) Representative trace of pH_i for control (scrambled siRNA, $n = 10$). (B) siSLC4A11 ($n = 14$) treated BCEC. NH_4^+ , 20 mM ammonium chloride. (C) Summary bar graph of rate of recovery from acid load (* $P = 0.0075$).

SLC4A11 knockdown from control. Second, intracellular Na^+ concentrations were measured with a similar protocol to further assess $\text{Na}^+:\text{HCO}_3^-$ transport. If SLC4A11 is a Na^+ -dependent HCO_3^- transporter, then siSLC4A11 knockdown should reduce the rate of Na^+ influx in response to $\text{CO}_2/\text{HCO}_3^-$. Figures 8A and 8B show representative $[\text{Na}^+]_i$ traces from control and transfected cells, respectively. Figure 8C summarizes the data from different trials and shows that the rate of Na^+ influx was not significantly different between the control and the transfected. Third, the effect of siSLC4A11 knockdown on Na^+ -free induced acidification in the presence of $\text{CO}_2/\text{HCO}_3^-$ was measured. In siSLC4A11-transfected cells the rate and amplitude of acidification and the rate of recovery should be reduced if SLC4A11 has Na^+ -coupled HCO_3^- cotransport activity. Figures 9A and 9B are representative pH_i traces from control and transfected cells. Figures 9C and 9D summarize the results from different trials and indicate that the rate and amplitude of acidification and the rate of recovery in the presence of $\text{CO}_2/\text{HCO}_3^-$ was not significantly different between control and transfected cells. In summary, these results indicate that SLC4A11 does not produce any measurable $\text{Na}^+:\text{HCO}_3^-$ transport in BCECs.

Finally, we examined the potential for SLC4A11 to act as a $\text{Na}^+:\text{OH}^-$ cotransporter, as suggested in a previous study.²² To test this hypothesis, pH_i was measured during a Na^+ -free pulse in the absence of bicarbonate. Figures 10A and 10B show representative pH_i traces from control and siSLC4A11-transfected cells. Figures 10C–E summarize the data and indicate that the rate and amplitude of acidification was reduced by 43% and 48% ($P = 0.02$ and 0.0006 , respectively) and the rate of recovery, following Na^+ add-back, was reduced by 53% ($P = 0.04$) in the transfected cells. We further confirmed this activity by measuring the rates of recovery from an acid load induced by a 1-minute pulse of 20 mM NH_4Cl in the absence of bicarbonate. Cells exposed to NH_4Cl solutions first alkalinize

due to inward diffusion of NH_3 , followed by a slower acidification due to NH_4^+ uptake. Removal of NH_4Cl causes rapid NH_3 efflux, trapping protons, and acidifying the cells. Figures 11A and 11B show representative traces from control and transfected cells and indicate a slowing of acid recovery in siSLC4A11-treated cells. Figure 11C summarizes these results and shows that knockdown of SLC4A11 significantly decreased the rate of recovery (~30%) from an acid load ($P = 0.007$).

DISCUSSION

Putative B(OH)_4^- transport by SLC4A11 was intriguing since B(OH)_4^- appeared to have effects on cell proliferation and survival.²² However, we could find no evidence for B(OH)_4^- transport in BCECs or an effect of borate on cell survival (Supplementary Fig. S1). HCO_3^- transport has an important role in endothelial function. However, from the results of the current study we can say that SLC4A11 does not provide significant HCO_3^- transport, consistent with an earlier report.²² Finally, we confirmed that SLC4A11 does have Na^+ -dependent OH^- cotransport activity.²² This functions similar to the Na^+/H^+ exchange (NHE1), which would regulate pH_i , and could facilitate lactic acid flux across the endothelium.^{35,36}

SLC4A11 is considered a human homolog of the plant B(OH)_4^- transporter (AtBor1) and is suggested to transport B(OH)_4^- coupled to Na^+ based on a significant B(OH)_4^- -dependent increase in the rate of acidification under Na^+ -free conditions in SLC4A11-transfected HEK293 cells.²² That study however, is problematic because the Na^+ substitute used, *N*-methyl-D-glucamine (NMDG⁺), is a borate chelator (forms a 1:1 complex with B(OH)_4^-).^{37–39} NMDG would deplete the available borate in the solutions and the acidification due to Na^+ removal in the presence of B(OH)_4^- cannot be attributed to $\text{Na}^+:\text{B(OH)}_4^-$ transport. Moreover, the chelation of borate

leads to deprotonation of boric acid, thereby acidifying the solutions, which may explain the faster rate of acidification. Our experiments substituted Na^+ with equimolar amounts of TMA (Fig. 4). Under these conditions, we did not find any B(OH)_4^- -dependent Na^+ fluxes. Moreover, overexpression of SLC4A11 did not alter the pH_i response to 2.5 or 10 mM B(OH)_4^- (Fig. 3E). Consistent with these findings, $[\text{Na}^+]_i$ was unaffected by the presence of B(OH)_4^- (Fig. 5) in both control (mock-transfected cells) and SLC4A11 overexpressing cells. From these results we conclude that SLC4A11 is not a $\text{Na}^+:\text{B(OH)}_4^-$ transporter in corneal endothelium.

SLC4A11, having some homology ($\sim 20\%$)⁴⁰ to other SLC4 transporters, was at first presumed to be a bicarbonate transporter (bicarbonate transporter-related protein-1 [BTR1])¹⁹ and having mutations associated with endothelial dystrophies^{5,6} suggests a possible loss of function disease phenotype. However, experiments demonstrating HCO_3^- transport by SLC4A11 have not been reported. The SLC4 superfamily consists of three subfamilies of HCO_3^- transporters that transport HCO_3^- either in a Cl^- or Na^+ -dependent manner. To test if SLC4A11 is a HCO_3^- transporter and contributed to the corneal endothelial $\text{CO}_2/\text{HCO}_3^-$ response (Fig. 7A); we used siRNA to knock down SLC4A11. However, responses to $\text{CO}_2/\text{HCO}_3^-$ in the presence or absence of extracellular Na^+ were not altered (Figs. 7, 9). Additionally, $[\text{Na}^+]_i$ in the presence of $\text{CO}_2/\text{HCO}_3^-$ was not affected by SLC4A11 knockdown (Fig. 8). From these experiments we conclude that SLC4A11 does not possess significant Na^+ -dependent HCO_3^- cotransport activity consistent with results from Park and colleagues,²² who found that SLC4A11 provides $\text{Na}^+:\text{OH}^-$ permeability in the absence of bicarbonate and borate. We tested this activity by determining the rate of pH_i changes induced by extracellular Na^+ depletion and add-back and from an acid load induced by NH_4^+ pulse in the absence of HCO_3^- . We found that the rate and extent of acidification caused by Na^+ depletion were reduced significantly as well as the rate of recovery on Na^+ add-back in *siSLC4A11*-treated cells (Fig. 10). These results strongly suggest SLC4A11 contributes to the Na^+ -free acidification and recovery upon add-back. In addition, the rate of recovery from an NH_4^+ pulse induced acid load was also significantly reduced due to SLC4A11 knockdown (Fig. 11). These findings confirm that SLC4A11 is transporting Na^+ coupled to OH^- contributing to pH_i regulation in corneal endothelium.

SLC4A11 is associated with the pathology of late-onset FECD,^{4,6} early-onset CHED2,^{5,7,8,41,42} and Harboyan Syndrome.^{9,10} The role of SLC4A11 in these endothelial dystrophies is supported by the loss of function due to low levels of protein indicated by retention of SLC4A11 mutants and the wild type (in the case of heterozygous dominant FECD) in the ER^{4,5} along with reduced levels of SLC4A11 in FECD corneal endothelium.²⁷ Loss of functional SLC4A11 could be a causative factor for low endothelial cell density due to apoptosis^{43–45} implicated in these dystrophies. Similar to NHE1 as a pH_i modulator, SLC4A11 may regulate cell survival through antiapoptotic mechanisms.^{46,47} On the other hand, impaired SLC4A11 expression could lead to compromised pH_i regulation affecting HCO_3^- and lactic acid transport and depressing endothelial pump function characteristic of these dystrophies. Consistent with this hypothesis, SLC4A11 knock-out mice present corneal edema and accumulation of NaCl in the stroma.⁴⁸ Further studies are needed to determine if mutational loss of this functional activity or other secondary effects (e.g., stress induced by UPR) contribute to corneal edema and subsequent loss of endothelial cells. However, mutations in genes other than *SLC4A11* (e.g., *COL8A2*), where a mutant knock-in model depicted phenotypes associated with

FECD suggest multiple pathologic mechanisms for the development of these dystrophies.

In summary, SLC4A11 is a basolateral membrane protein in corneal endothelial cells. Functional characterization revealed that SLC4A11 acts as a Na^+ -dependent pH_i modulator transporting OH^- with no significant affinity to B(OH)_4^- or HCO_3^- anions.

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