Published in final edited form as:

Exp Eye Res. 2006 November ; 83(5): 1215–1224. doi:10.1016/j.exer.2006.06.011.

Molecular expression and functional involvement of the bovine calcium-activated chloride channel 1 (bCLCA1) in apical HCO_3^- permeability of bovine corneal endothelium

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Abstract

Corneal endothelium secretes HCO_3^- from basolateral (stroma) to apical (anterior chamber) compartments. Apical HCO_3^- permeability can be enhanced by increasing $[\text{Ca}^{2+}]_i$. We hypothesized that the bovine calcium-activated chloride channel 1 (bCLCA1), shown previously by PCR screening to be expressed in corneal endothelium, is involved in Ca^{2+} activated apical HCO_3^- permeability. bCLCA1 expression in cultured bovine corneal endothelial cells (CBCEC) was examined by *in situ* hybridization analysis, immunoblotting, immunofluorescence and confocal microscopy. Rabbit polyclonal antibodies were generated using a 14 aa polypeptide (417–430) from the predicted sequence of bCLCA1. The small interference RNA (siRNA) knock down technique was used to evaluate the functional involvement of bCLCA1 in apical HCO_3^- permeability. *In situ* hybridization confirmed prominent bCLCA1-specific mRNA expression in CBCEC. bCLCA1 antiserum detected the heterologously expressed bCLCA1 in HEK293 cells and a 90 kDa band in CBCEC, which was absent when using the pre-immune serum or antigen absorption of serum. Immunofluorescence staining with anti-bCLCA1 antibody and confocal microscopy indicates an apical membrane location in CBCEC. In CBCEC transfected with bCLCA1 specific siRNA, bCLCA1 expression was reduced by 80%, while transfection with siControl scrambled sequence had no effect. Increasing $[\text{Ca}_i^{2+}]$ by application of $\text{ATP}\gamma\text{S}$ or cyclopiazonic acid (CPA) increased apical HCO_3^- permeability in siControl transfected CBCEC, while having no effect on apical HCO_3^- permeability in bCLCA1 specific siRNA transfected cells. Baseline HCO_3^- permeability, however, was not different between controls and siRNA treated cells. We conclude that the calcium-activated chloride channel (bCLCA1) is expressed in bovine corneal endothelial cells and can contribute to Ca^{2+} dependent apical HCO_3^- permeability, but not resting permeability, across the corneal endothelium.

Keywords

cornea endothelium; calcium; bCLCA1; HCO_3^- transport

1. Introduction

The corneal endothelium is a thin monolayer of cells covering the posterior surface of the cornea. The primary function of the corneal endothelium is to maintain corneal transparency through active transport of ions and fluid (Maurice, 1972; Maurice and Giardini, 1951). The glycosaminoglycans in the cornea stroma exert a net swelling pressure that gives rise to fluid

imbibition by the stroma. This fluid influx is compensated by the endothelium with an ion-coupled fluid transport mechanism directed from stroma to anterior chamber. Fluid transport by the corneal endothelium is dependent on the presence of HCO_3^- (Riley et al., 1995).

Previous studies have shown that basolateral HCO_3^- permeability is significantly higher than apical (Bonanno et al., 1999). Therefore, the rate-limiting step in transendothelial HCO_3^- flux is at the apical membrane. Recent studies have shown that apical HCO_3^- permeability can be enhanced by increasing $[\text{Ca}^{2+}]_i$ via either activation of P2 purinergic receptors or inhibition of the sarcoendoplasmic reticulum calcium-ATPase (SERCA) (Zhang et al., 2002). However, the individual components involved in this process have not been fully described.

Recently, a new family of membrane proteins sensitive to Ca^{2+} and involved in anion transport and cell adhesion has been identified (Fuller et al., 1994; Fuller and Benos, 2000a,b). By GenBank nomenclature, this family is known as CLCA (chloride channel, calcium activated). As so far investigated, this family is widely expressed in all mammalian species. Two bovine, four human, six mouse and two porcine homologues have been found and deposited on the NCBI Gen-Bank database (Fuller and Benos, 2000a). Each CLCA protein exhibits a distinct tissue distribution pattern. Most CLCA proteins are synthesized as an approximately 125 kDa precursor transmembrane glycoprotein which is rapidly cleaved into 90 kDa and 35 kDa subunits (Pauli et al., 2000).

Bovine calcium-activated chloride channel-1 (bCLCA1) was first isolated by the Benos' laboratory (Ran and Benos, 1991). They purified a 38-kDa protein from bovine trachea that gave rise to 25–30 pS anion channels with an $\text{I}^- > \text{Cl}^-$ selectivity when reconstituted into lipid vesicles (Ran and Benos, 1991, 1992; Ran et al., 1992). The immunoaffinity-purified protein could be phosphorylated by CaMKII in vitro, and channel activity could be increased by Ca^{2+} and CaMKII (Fuller et al., 1994). Based on this 38-kDa protein, Cunningham et al. (1995) cloned a cDNA thought to encode this putative channel protein. The open reading frame predicted a protein of 903 aa (named bCLCA1). When expressed in *Xenopus* oocytes, largely time-independent and outwardly rectified currents were reported and these currents were observed even without raising $[\text{Ca}^{2+}]_i$. In contrast, currents in transfected COS-7 cells were linear and were only observed upon raising $[\text{Ca}^{2+}]_i$. Hydropathy plot of the amino acid sequence of bCLCA1 predicted four major transmembrane domains, while motif analysis showed several consensus sites for N-linked glycosylation, consistent with a role as a membrane protein. In addition, there were several consensus sites for phosphorylation by CaMKII, PKC and tyrosine kinase on bCLCA1 expressed in respiratory epithelia of bovine trachea and bronchi (Cunningham et al., 1995).

CLCAs increase anion permeability non-specifically and can enhance HCO_3^- permeability (Hartzell et al., 2005; Loewen and Forsyth, 2005). HCO_3^- permeability in the apical membrane of BCECs is enhanced by increasing $[\text{Ca}^{2+}]_i$ and can be reduced by the CaMKII inhibitor KN-62 and the PKC inhibitor BIM1 (Zhang et al., 2002), suggesting that a CLCA is involved. Since HCO_3^- flux across the cornea endothelium is an important component of the endothelial fluid pump, we asked whether bCLCA1 is expressed in the apical membrane of bovine corneal endothelium, and whether bCLCA1 plays a role in the Ca^{2+} dependent enhancement of HCO_3^- permeability in the apical membrane of these cells.

2. Materials and methods

2.1. Cell culture

BCECs were cultured to confluence onto 25 mm round coverslips, 13 mm anodiscs, or T-25 flasks as previously described (Bonanno and Giasson, 1992; MacCallum et al., 1982). Briefly, primary cultures from fresh cow eyes were established in T-25 flasks with 3 ml of

Dulbecco's modified Eagle's medium, 10% bovine calf serum, and antibiotics (penicillin 100 u/ml), gassed with 5% CO₂–95% air at 37 °C and fed every 2–3 days. Primary cultures were subcultured to three T-25 flasks and grown to confluence in 5–7 days. The resulting second passage cultures were then further subcultured onto coverslips, anodiscs, and petridishes to reach confluence within 5–7 days.

2.2. In situ hybridization

A 457 bp biotin-labeled antisense probe was synthesized according to the manufacturer's recommendation (Biotin RNA labeling mix from Roche Applied Science, Mannheim, Germany) from pcDNA3.1-bCLCA1 (a kind gift from Dr Catherine M. Fuller), which was linearized by the restriction enzyme, PpuMI. In situ hybridization was performed on confluent cultured cells grown on coverslips. Cells were washed with PBS at 37 °C and then fixed at room temperature for 30 min in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl. The fixed cells were dehydrated by 70%, 90%, 100% ethanol successively, washed in 100% xylene to remove residual lipids, and rehydrated by 100%, 90%, 70% ethanol successively. After washing with PBS, the cells were permeabilized by macromolecular reagents with 0.1% (w/v) pepsin in 0.1 N HCl at 37 °C, washed with PBS for 5 min and then post-fixed with 1% formaldehyde for 10 min and washed again with PBS. Hybridization with biotin-labeled RNA probe was at 37 °C for 16 h in hybridization solution, which contains 60% deionized formamide, 300 mM NaCl, 30 mM sodium citrate, 10 mM EDTA, 25 mM Na H₂PO₄ (pH 7.4), 5% dextran sulfate, and 250 ng/μL sheared salmon sperm DNA. After hybridization, the cells were washed in a solution of 60% formamide, 300 mM NaCl, and 30 mM sodium citrate three times at room temperature and once at 37 °C and washed with PBS for 5 min at room temperature. Finally the probe-labeled samples were detected by streptavidin conjugated with alkaline phosphatase and ELF 97 phosphatase substrate according to the manufacturer's instructions (ELF97 mRNA In situ Hybridization Kits, Molecular Probes, Eugene, OR).

2.3. Generation of bCLCA1 antibody

The anti-bCLCA1 antibody is a custom rabbit polyclonal antibody that was created (Sigma-Genosys, The Woodlands, TX) against a 14 aa peptide selected from the bCLCA1 protein sequence (417–430 aa), which is highly hydrophilic and predicted to have good immunogenicity.

The sera were purified with protein A/G (Sigma-Aldrich, St. Louis, MO) as described by Xie et al. (2005). The fusion protein GST-bCLCA417-430 was created from the corresponding cDNA sequence from the segment 417–430 aa of bCLCA1, which was PCR amplified and subcloned to the C-terminus of glutathione *S*-transferase (GST) in the pGEX-6P-3 vector (Amersham) by T4 ligase (New England Biolabs). GST-bCLCA417-430 was induced in bacteria by IPTG and purified by affinity chromatography using glutathione sepharose fast flow agarose beads (Amersham, Piscataway, NJ). The purified fusion protein was conjugated to the CNBr-sepharose beads (Amersham). The A/G purified IgGs were further affinity purified using the GST-bCLCA417-430 linked sepharose beads. Mouse monoclonal antibody against β-actin was from Sigma. The secondary antibodies were peroxidase-conjugated goat anti-rabbit or mouse IgG (Sigma, St. Louis, MO) and the blots were detected with an enhanced chemiluminescence's kit (ECL) as described by the manufacturer (Pierce, Rockford, IL).

2.4. Immunoblotting

CBCEC were scraped from Petri dishes and placed into ice-cold PBS that contained a protease inhibitor cocktail (Complete, Boehringer Mannheim) and centrifuged at 1000 × *g* for 10 min. The cell pellet was resuspended in microsome preparation buffer (0.1 M Tris-

Ac, 1 mM EDTA, 0.15 M KCl, pH 7.4), homogenized by sonication, and centrifuged at $12,000 \times g$, 4 °C for 20 min. The supernatant was subjected to ultracentrifugation at $100,000 \times g$, 4 °C for 1 h. The membrane proteins, in the resultant pellets, were solubilized with sample buffer without dye (2% SDS, 0.5 M Tris-HCl 6.8, 20% glycerol). Protein concentration was quantitated by Bio-Rad protein assay reagents (Hercules, CA) and separated by 8% SDS-PAGE. Each lane was loaded with 10 µg of protein. The samples were then transferred to nitrocellulose membranes, which were blocked with 5% non-fat dry milk in PBST for 1 h at room temperature, incubated with primary antibodies (the anti-bCLCA1 antibodies, non-purified or purified, 1:2000 or 1:200, respectively; pre-immune IgG, 1:2000; β -actin, 1:10,000) in 5% milk for one more hour, washed with PBST for 30 min, and then incubated with secondary antibodies at 1:40,000 dilution in PBST containing 5% milk.

2.5. Immunofluorescence and confocal microscopy

CBCEC, grown to confluence on coverslips, were washed three times with warm (37 °C) PBS and fixed for 30 min in warm PLP fixation solution (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate and 45 mM sodium phosphate, pH 7.4) on a rocker. After fixation, the cells were washed three or four times with PBS. Cells were then kept in PBS containing 1% SDS for 5 min to unmask epitopes and washed three times in PBS. Cells were blocked for 1 h in PBS containing 0.2% bovine serum albumin, 5% goat serum, 0.01% saponin and 50 mM NH_4Cl . The rabbit anti-bCLCA1 antibody (1:50) or the pre-immune serum (1:50) and the rat anti-ZO-1 (zonula occludens-1) antibody (1:100) (Zymed) were incubated with the cells for 1 h. After washing with PBS containing 0.01% saponin three times for 15 min, the cells were incubated for 45–60 min with secondary antibody against rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, 1:500), secondary antibody against rat IgG conjugated with Alexa Fluor 594 (Molecular Probes, 1:1000). The cells were further washed three times with PBS containing 0.01% saponin for 15 min and twice with PBS for 10 min. Cell nuclei were counterstained with 100 ng/ml 4',6'-diamidino-2-phenylindole hydrochloride (DAPI). After washing twice with deionized water for 5 min, the coverslips were mounted with Prolong antifade medium according to the manufacturer's instructions (Molecular Probes). The staining was observed with an epifluorescence microscope equipped with a cooled charge-coupled device camera. Fluorescence of selected cells was documented with a Bio-Rad laser scanning confocal microscope to determine protein localization.

For frozen sections of fresh tissue, cow corneas were dissected into 2×5 mm blocks and washed with cold PBS, and transferred to base molds filled with OCT. The base molds were frozen in liquid nitrogen for 30 s, and then stored at -80 °C. Five micrometer sections were cut, transferred to superfrost slides, dried and fixed in cold acetone (-20 °C) for 2 min. Sections were stained for bCLCA1 immunofluorescence and nuclei stained with DAPI.

2.6. Transfection

To test if the anti-bCLCA1 antibody can recognize the bCLCA1 protein, the pcDNA3-bCLCA1 expression construct was transfected into HEK293 cells. HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (Invitrogen, Carlsbad, CA). Transfection was performed using the lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). One day before transfection, HEK293 cells were subcultured in antibiotic-free medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). PcDNA3-bCLCA1 and lipofectamine 2000 were mixed for 30 min before transfection. Cells were transfected for 1 h in serum-free Opti-MEM I medium (Invitrogen, Carlsbad, CA) and then transferred to fresh antibiotic-free medium with 10% fetal calf serum.

2.7. Synthesis and transfection of small interfering RNA

Three 21-nucleotide small interfering RNA (siRNA) sequences specifically targeting bCLCA1 were synthesized by the Silencer™ siRNA Construction Kit according to the manufacturer's instructions (Ambion, Austin, TX). Briefly, an oligonucleotide potentially targeting bCLCA1 was selected using the siRNA finder program at <http://www.ambion.com/>. The three small interference RNAs were synthesized according to the sequence 583–603 (RNAi-583), 989–1009 (RNAi-989), 1498–1518 (RNAi-1498) of bCLCA1 ORF. RNAi-583 sense (5'-aatgtgattgtcaatgccagcctgtctc-3') and antisense oligonucleotides (5'-aactggcatttgacaatcacattcctgtctc-3'), RNAi-989 sense (5'-aagcagcagaattattcttgacctgtctc-3') and antisense oligonucleotides (5'-aatcaagaataattctgctgtctc-3'), and RNAi-1498 sense (5'-aagaaatgggtaaatggtacgcctgtctc-3') and antisense oligonucleotides (5'-aacgtaccattaccatttctctgtctc-3'), all with T7 binding sequences, were synthesized by Integrated DNA Technologies (Coralville, IA). Sense and antisense single strand RNAs were synthesized by using T7 polymerase. The single strand RNAs were incubated together overnight at 37 °C to form double stranded RNA. The synthesized double stranded RNAi was purified by an RNA binding column provided in the kit. A negative control siRNA, siControl Non-Targeting siRNA#1, was synthesized and used according to the manufacturer's instructions (Dharmacon RNA Technologies, Dallas, TX). Cultured BCECs were grown to 30–40% confluence before transfection. Cultured BCECs were transfected overnight with 20 nM of each siRNA, which were mixed with oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) and the OPTI-MEM I reduced serum medium (Gibco, Grand Island, NY). The next day, fresh growth medium without antibiotics was added. At the fourth day after transfection, the cells were collected for further analysis.

2.8. RT-PCR

RT-PCR was performed using 0.4 µg of total RNA from cultured BCEC. The RT step was performed in a total volume of 20 µl by using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The PCR reactions were performed in a total volume of 25 µl by using the high-fidelity Taq DNA polymerase according to the manufacturer's instructions (Roche, Indianapolis, IN). Thirty-five PCR cycles (95 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s) were performed in addition to a beginning denaturation at 95 °C for 2 min and a final extension at 72 °C for 10 min. PCR primers used for amplifying bCLCA1 corresponded to sequence 904–923 and 1030–1049 of bCLCA1 open reading frame, which were synthesized by Integrated DNA Technologies (Coralville, IA). The expected product is 144 bp. The primers for GAPDH were described previously. The RT-PCR products were subject to 2% agarose gel electrophoresis and visualized by ethidium bromide staining and ultraviolet illumination. The band was cut and purified by a gel extraction kit (Qiagen, Valencia, CA). The purified PCR product was subcloned into the pcDNA3.1-V5-His-TOPO vector (Invitrogen). The PCR product was sequenced using the T7 primers (IUPUI DNA Sequencing Facility, Indianapolis, IN). Real-time PCR was performed under the same conditions as the standard PCR with the addition of 1 µM SYBR Green (Molecular Probes) using a real-time PCR thermocycler (Corbett Roto-Gene). After real-time PCR, all PCR products were checked by agarose gel electrophoresis.

2.9. Solutions and chemicals

The composition of the HCO₃⁻-rich (BR) Ringer's solution was (in millimolar) 150 Na⁺, 4 K⁺, 0.6 Mg²⁺, 1.4 Ca²⁺, 118 Cl⁻, 1 HPO₄²⁻, 10 HEPES, 28.5 HCO₃⁻, 2 gluconate⁻, and 5 glucose. Ringer's solution was equilibrated with 5% CO₂, and pH was adjusted to 7.5 at 37 °C. The Low- HCO₃⁻ (LB) Ringer's solution (2.85 mM HCO₃⁻; pH 6.5) was prepared by replacing 25.65 mM NaHCO₃ with sodium gluconate. The Cl⁻- free Ringer's solution was

prepared by equimolar replacement of NaCl with sodium gluconate. Osmolarity was adjusted to 290 ± 5 mOsm with sucrose. 2',7'-Bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR). Cell culture supplies were from Gibco BRL (Grand Island, NY). All other chemicals were from Sigma (St. Louis, MO).

2.10. Perfusion

For independent perfusion of the apical and basolateral sides of cell-coated filters (Anodisc; Fisher Scientific), a double-sided perfusion chamber was used (Bonanno et al., 1998). The assembled chamber was placed on a water-jacketed (37 °C) brass collar held on the stage of an inverted microscope (Diaphot; Nikon, Melville, NY). The apical side was viewed with a long-working-distance objective ($\times 40$, 1.2 mm working distance, 0.75 NA; Carl Zeiss, Oberkochen, Germany). The apical and basolateral compartments were connected to separate sections of tubing (Phar-Med; Fisher Scientific), which, in turn, were connected to syringes containing Ringer's in a Plexiglas warming box (37 °C). Both HCO_3^- -rich and low- HCO_3^- Ringer's were continually bubbled with 5% CO_2 . The flow of the perfusate (~ 0.5 mL/min) was achieved by gravity. Two independent eight-way valves were used to select the desired perfusate for the apical and basolateral chambers.

2.11. Measurements of intracellular pH

Intracellular pH (pH_i) was measured with the pH-sensitive fluorescent dye BCECF as previously described (Zhang et al., 2002). Fluorescence ratios (F_{495}/F_{440}) obtained at 1 Hz were calibrated against pH_i by the high- K^+ -nigreicin technique.

2.12. Statistics

Initial rates of pH_i change (dpH_i/dt) upon switching from HCO_3^- -rich to low- HCO_3^- solutions on the apical side were calculated from 20 s of data centered at the maximal slope and served as a relative measure of HCO_3^- permeability across the apical membrane. The pH_i changes were expressed as the mean $\pm$ SE. Paired *t*-test was used for statistical analysis and $p < 0.05$ was considered significant.

3. Results

3.1. Detection of bCLCA1 mRNA expression in CBCEC by in situ hybridization

Fig. 1A shows that the in situ hybridization with the RNA probe resulted in intense staining of the cytosol of the CBCECs. Conversely, Fig. 1B shows that cells exposed to alkaline phosphatase conjugated streptavidin and alkaline phosphatase substrate without RNA probe (negative control), were not stained. The above result was consistent with our previous RT-PCR finding that bCLCA1 mRNA is expressed in bovine corneal endothelium (Zhang et al., 2002).

3.2. Characterization of bCLCA1 antibody

Antibodies to bCLCA1 are not generally available. Therefore, in order to examine the protein expression and distribution of bCLCA1 in BCEC, a rabbit polyclonal antibody was generated with a peptide antigen from 417 to 430 amino acid sequence of bCLCA1 and then purified by protein A/G and fusion protein antigen GST-bCLCA1 (see Section 2). Fig. 2A shows a 31/32 kDa (26 kDa for GST + 5/6 kDa for 417–430 aa of bCLCA1) fusion protein GST-bCLCA1 induced in bacteria. bCLCA1 specific serum detected the fusion protein (GST-bCLCA1), but the pre-immune serum did not (Fig. 2B). To further test if the antibody is specific to bCLCA1, we heterologously expressed bCLCA1 in HEK293 cells by

transfection with pcDNA3-bCLCA1. Fig. 2C shows that the purified antibody detected a band of molecular weight around 90/95 kDa, which was in the expected range for bCLCA1. In HEK293 cells without bCLCA1 transfection, no band was detected and pre-immune serum also did not detect any band. The above result indicates that the anti-bCLCA1 antibody is specific to the bCLCA1 protein.

The expression, processing and primary structure of CLCA proteins have recently been studied (Pauli et al., 2000). The initial translation product of the open reading frame is about 100 kDa, which is glycosylated into a 125 kDa glycoprotein, and then immediately proteolytically cleaved into 90 kDa and 35 kDa subunits. The proteolytic cleavage site is assumed to be around 709 aa (Pauli et al., 2000). The peptide sequence used to induce our anti-bCLCA1 antibody is upstream to the cleavage site. Therefore, the antibody should detect the 125 kDa, 100 kDa and 90 kDa peptides, but not the 35 kDa, carboxyl-terminal subunit. Indeed, Fig. 2D shows that the anti-bCLCA1 antibody recognized three bands at approximately 125 kDa, 100 kDa and 90 kDa in cultured BCEC. All bands could be absorbed by the fusion protein antigen GST-bCLCA1. The pre-immune IgG did not detect any bands (data not shown). Therefore, these findings show that bCLCA1 is expressed in cultured BCECs. The heterologously expressed bCLCA1 protein in HEK293 cells is larger than those in native tissues. This might be due to different post-translational modifications or different starting translation sites between the in-vivo and in-vitro expression system.

3.3. Distribution of bCLCA1 protein in BCEC

Immunofluorescence was used to further examine the expression of bCLCA1 in CBCECs. In Fig. 3A, CBCECs show intense staining only when they are incubated with the anti-bCLCA1 antibody (lower panel), but not in cells without incubation with primary antibody (upper panel). Co-staining with the tight junction protein ZO-1, showed some co-localization, suggesting that bCLCA1 is present at the apical plasma membrane.

bCLCA1 is expressed on the apical membrane of pancreas acinar cells (Thevenod et al., 2003). Confocal microscopy was used to analyze the immunostaining images of CBCECs. Fig. 3B,C shows sequential confocal montages from the basal to apical sides (total cell thickness 5 μ m). Cells were counter-stained with DAPI (nuclei) and ZO-1, a zonula occludens protein. ZO-1 acts as a lateral membrane marker at the transition between apical and basolateral membrane domains. The X-Z dimensional image with ZO-1 and nuclear staining shows that ZO-1 staining (red) is relatively apical compared to nuclear staining (blue). In the same cells with the bCLCA1 (green) and nuclear staining, bCLCA1 also located relatively apical to the nucleus (Fig. 3B,C, lower panel). These images indicate that bCLCA1 is located on the apical side. This is consistent with previous physiological data that apical HCO_3^- permeability can be enhanced by increasing $[\text{Ca}^{2+}]_i$ (Zhang et al., 2002). Fig. 3D shows that sections of fresh corneas are positive for bCLCA1 along the apical membrane, indicating that bCLCA1 expression is not an artifact of cell culture.

3.4. Small interference RNA suppresses the expression of bCLCA1 in cultured BCEC

The RNAi technique is a new approach to knock down the expression of a specific gene in native cells (Scherr et al., 2003). We have previously shown that expression of the basolateral $\text{1Na}^+/\text{2HCO}_3^-$ cotransporter (NBC1b) in BCEC could be significantly reduced by this approach (Li et al., 2005). Three small interference RNAs were synthesized according to the sequence 583–603 (RNAi-583), 989–1009 (RNAi-989), 1498–1518 (RNAi-1498) of bCLCA1 ORF. Fig. 4A shows that bCLCA1 protein expression can be suppressed by the three RNAi constructs compared to the loading control, β -actin. The percentages of the protein knocked down by RNAi-583, RNAi-989 and RNAi-1498 were 52.9%, 80.4% and 78.2% relative to vehicle control. The siControl transfected cells had no effect on bCLCA1

expression. Fig. 4B shows real-time PCR results from transfected cells. The mRNA expression of the housekeeping gene GAPDH did not vary among samples from endothelium alone, endothelium treated with vehicle, siControl, or RNAi-989 transfected cells indicating equal loading of cDNA to every PCR reaction. bCLCA1 mRNA expression was also the same in endothelium alone, oligofectamine or siControl treated cells, however, bCLCA1 expression was significantly reduced by RNAi-989 transfection. Fig. 4C shows the expected real-time PCR product (144 bp). Sequencing showed 100% homology to the published bCLCA1 sequence in NCBI GenBank. These results demonstrate that the three RNAi constructs can specifically suppress the expression of bCLCA1 in cultured BCEC and among the three RNAi constructs; RNAi-989 had the strongest knock-down effect on bCLCA1 expression. Therefore, RNAi-989 was utilized to evaluate the function of bCLCA1 in cultured BCEC.

3.5. Effects of bCLCA1-siRNA on apical HCO_3^- permeability in cultured BCEC

In order to measure relative changes in apical HCO_3^- permeability, we utilized a constant- CO_2 protocol, which was described previously (Bonanno et al., 1999; Zhang et al., 2002). Briefly, CBCEC grown on permeable anodiscs were perfused in a double-sided chamber and exposed to a bicarbonate-rich (BR), Cl^- -free Ringer's solution (5% CO_2 , 28.5 mM HCO_3^- , pH 7.5) on both apical and basolateral sides at 37 °C. Apical perfusion was then quickly switched to low bicarbonate (LB) Cl^- -free solution (5% CO_2 , 2.85 mM HCO_3^- , pH 6.5). Because the $[\text{CO}_2]$ is constant and the same on both sides, pH_i was affected mostly by HCO_3^- efflux across the apical membrane (Bonanno et al., 1999). Cl^- -free Ringer's was used to increase the sensitivity of the assay because Cl^- can compete with HCO_3^- for anion channels and Cl^- -free Ringer's removes any contribution from the weak apical anion exchanger (Bonanno et al., 1999; Zhang et al., 2002). Considering that the amplitude of pH_i changes during LB pulses may be influenced by basolateral transporters such as NBC (Bonanno et al., 1999; Sun and Bonanno, 2003), we used only the initial rate of pH_i change (dpH_i/dt) instead of the amplitude as the criteria for comparison of HCO_3^- permeability across the apical membrane in the presence or absence of applied agents. Therefore, the apical HCO_3^- permeability will be pre-dominantly contributed by anion channels.

To address the question of whether bCLCA1, apparently expressed at the apical membrane, plays a role in Ca^{2+} activated increase in endothelial cell apical membrane HCO_3^- permeability, we used siRNA knockdown of bCLCA1. Purinergic agonists ATP or ATP γ S can induce Ca^{2+} entry and enhanced apical HCO_3^- permeability in corneal endothelial cells (Zhang et al., 2002; Xie et al., 2005). Since ATP γ S cannot be hydrolyzed to form adenosine, which may enhance apical HCO_3^- permeability independent of Ca^{2+} (Zhang et al., 2002), we examined the effect of siRNA-bCLCA1 on ATP γ S enhanced apical HCO_3^- permeability. Control and ATP γ S (100 μM) enhanced apical HCO_3^- permeability were measured in CBCEC transfected with RNAi-989 or siControl. Fig. 5A,B shows that in CBCEC transfected with siControl, ATP γ S application produced a $78 \pm 21\%$ ($n = 7$, $p < 0.01$) increase in dpH_i/dt (pH units/s) relative to the control LB pulse. Fig. 5C,D shows that in CBCEC transfected with RNAi-989 there was no significant difference in dpH_i/dt during LB perfusion between control or ATP γ S application ($n = 6$, $p > 0.05$). These results indicate that suppression of bCLCA1 expression abrogates the Ca^{2+} mediated enhancement of apical HCO_3^- permeability in CBCEC, while negative control siRNA has no detectable effect on calcium stimulated HCO_3^- permeability at the apical membrane of CBCEC. Interestingly, there was no effect of siRNA knockdown on baseline (control) apical HCO_3^- permeability, suggesting that this channel does not contribute to apical HCO_3^- permeability of unstimulated cells.

To further confirm whether bCLCA1 is involved in the calcium mediated enhancement of apical HCO_3^- permeability, an alternative approach to increase $[\text{Ca}^{2+}]_i$ was used. Cyclopiazonic acid (CPA) is a sarcoendoplasmic reticulum Ca^{2+} -AT-Pase (SERCA) inhibitor, which depletes the calcium store raising cytosolic $[\text{Ca}^{2+}]$ and causes further calcium entry through store-operated calcium channels (SOCs) in corneal endothelial cells (Putney and McKay, 1999). In cultured BCEC, CPA can increase $[\text{Ca}^{2+}]_i$ and enhance apical HCO_3^- permeability (Xie et al., 2005; Zhang et al., 2002). Fig. 6A,B shows that in cells transfected with siControl, CPA increased the apical HCO_3^- permeability (dpH_i/dt) by $67 \pm 27\%$ ($n = 6$, $p < 0.01$, Table 1). Fig. 6C,D shows that in CBCEC transfected with RNAi-989, CPA had no effect on initial dpH_i/dt during LB perfusion ($n = 6$, $p > 0.05$). Therefore, the effect of CPA on increasing apical HCO_3^- permeability in culture BCEC was specifically reduced by RNAi-989 mediated bCLCA1 knockdown. Taken together these results indicate that bCLCA1 is involved in the regulation of calcium activated HCO_3^- transport on the apical side of CBCEC. Similar to the ATPyS series of experiments, there was no decrease in baseline HCO_3^- permeability in siRNA treated cells compared to untreated cells.

4. Discussion

Previously, we had demonstrated that increased $[\text{Ca}^{2+}]_i$ increased apical HCO_3^- permeability in corneal endothelial cells via calmodulin and PKC mediated mechanisms (Zhang et al., 2002). This suggested that a calcium activated anion channel could be present. In this study, we confirmed the expression of bCLCA1 in cultured BCEC by in situ hybridization, PCR, western blot, and confocal immunofluorescence microscopy. Knockdown of bCLCA1 by siRNA transfection eliminated the effect of increased $[\text{Ca}^{2+}]_i$ on apical HCO_3^- permeability. These results demonstrate that bCLCA1, which is expressed at the apical membrane, plays an important role in Ca^{2+} stimulated changes in apical permeability. Further studies would be needed to determine if bCLCA1 is the permeating channel or acts as an accessory protein. bCLCA1 has been heterologously expressed in lipid bilayers, oocytes or other cell lines and whole cell patch-clamp measurements often used very high (2 mM) concentration of Ca^{2+} in the pipette to elicit CLCA-associated currents (Jentsch et al., 2002). This is far above any physiologically meaningful concentration of $[\text{Ca}^{2+}]_i$. Thus, previous studies on bCLCA1 cannot exclude that this protein activated endogenous anion channels rather than being a channel itself (Jentsch et al., 2002). In fact, recent studies have shown that some members of the CLCA family, hCLCA1 and mCLCA3, are not ion channels, but rather accessory membrane proteins (Loewen et al., 2003) or are secreted (Gibson et al., 2005).

Because antibodies to bCLCA1 are not readily available, we purified polyclonal antibodies directed against bCLCA1 protein sequence (417–430 aa) to aid in detection of bCLCA1. Detection of heterologously expressed bCLCA1, abrogation of detection with antigen incubation, absence of detection with pre-immune serum, and detection of the predicted post-translational fragment indicate that the antibody has the required specificity. Using this antibody and confocal microscopy we determined that cultured BCEC express bCLCA1 at an apparent apical membrane location. The bCLCA1-RNAi-989 specifically suppressed the expression of bCLCA1 at both the mRNA and protein levels, while control scrambled sequences had no effect (Fig. 4). The functional consequence of this knockdown was that calcium mediated enhancement of apical HCO_3^- permeability via ATPyS or CPA were inhibited (Figs. 5 and 6), indicating that bCLCA1 has a role in the calcium activated HCO_3^- transport in apical membrane of corneal endothelium.

Corneal endothelial fluid transport is dependent on HCO_3^- (Fischbarg and Lim, 1974; Hodson and Miller, 1976; Riley et al., 1995). Recent studies (Diecke et al., 2004; Li et al.,

2005) have confirmed that HCO_3^- is secreted from the basolateral to apical side. This secretion is currently hypothesized to be the source of the ion-coupled osmotic fluid pumping. Uptake of HCO_3^- at the basolateral membrane is by $1\text{Na}^+/2\text{HCO}_3^-$ co-transport via NBC1b (Li et al., 2005; Sun and Bonanno, 2003; Sun et al., 2000). Apical HCO_3^- permeability however, is 3–4 times smaller than basolateral (Bonanno et al., 1999). Apical HCO_3^- permeability has been hypothesized to be mediated by anion exchange, anion channels, and possibly carbonic anhydrase activity (Bonanno, 2003). Previous patch clamp analyses failed to detect appreciable anion currents under physiological conditions (Rae and Watsky, 1996). However, subsequent PCR screenings have detected several channel types including CFTR and CLC type channels (Davies et al., 2004; Shepard and Rae, 1998; Sun et al., 2001). The results here show that bCLCA1 has a role in stimulated transport of HCO_3^- . However, inspection of Figs. 5 and 6 show that knockdown of bCLCA1 did not reduce unstimulated apical HCO_3^- permeability suggesting that bCLCA1 is not involved in baseline secretion of HCO_3^- across corneal endothelium. We focused on the regulation of apical HCO_3^- permeability by calcium in the Cl^- free condition to remove contributions from anion exchange; it would be interesting to investigate if chloride affects calcium-activated apical HCO_3^- permeability in future studies. The conditions that would invoke stimulation of transport *in vivo* are not known. Intracellular calcium is significantly elevated by P2 purinergic activation. ATP is the most likely ligand for P2 receptors and since release of ATP by endothelium is accelerated by cell swelling (Srinivas et al., 2001), it is likely that trauma or similar perturbations would stimulate transport in response to a break in barrier integrity. Moreover, ATP released by endothelium is converted to adenosine, which activates $\text{A}_{2\text{B}}$ receptors leading to increased [cAMP] (Tan-Allen et al., 2005). Activation of PKA increases apical CFTR permeability (Sun and Bonanno, 2002) and dephosphorylates MLC leading to increased barrier resistance (Srinivas et al., 2004).

In summary, bCLCA1 is expressed in corneal endothelial cells. It is likely that bCLCA1 is activated in a coordinated fashion that increases apical anion permeability and restores barrier integrity in an attempt to maintain corneal transparency.

Acknowledgments

We thank Dr Catherine M. Fuller (University of Alabama at Birmingham) for the kind gift of the plasmid pcDNA3-bCLCA1. We also thank Miao Cui for her excellent technical assistance. This project was funded by NIH grant EY008834 to JAB.

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Fig. 1. Detection of bCLCA1 mRNA expression in cultured BCEC by in situ hybridization with biotin-labeled RNA probe. (A) Staining of cultured BCEC by in situ hybridization with 457 bp RNA probe. (B) Negative control, cultured BCEC hybridized without probe.

Fig. 2. Characterization of the anti-bCLCA1 antibody and detection of bCLCA1 in transfected HEK293 cells, fresh and cultured BCEC. (A) Coomassie staining of induced GST protein (26 kDa) and induced fusion protein GST-bCLCA1 (expected size, 31/32 kDa). (B) Immunoblotting using the anti-bCLCA1 antibody (left lane) and pre-immune serum (right lane) to detect the fusion protein antigen. (C) Immunoblotting using the anti-bCLCA1 antibody (left two lanes) or pre-immune serum (right two lanes) to detect bCLCA1 heterologously expressed in HEK293 cells and negative control, mock HEK293 cells. (D) Immunoblotting using the anti-bCLCA1 antibody to specifically detect the bCLCA1 expression in cultured BCEC (lane 1) and heterologously expressed HEK293 cells (lane 2).

Fig. 3.

Immunostaining and laser scanning confocal microscopy of bCLCA1 in cultured BCEC. (A) Immunofluorescence of cultured BCEC by the rabbit anti-bCLCA1 antibody plus anti-rabbit IgG-conjugated Alexa 488 (lower left panel) and rat anti-ZO-1 Ab plus anti-rat IgG-conjugated Alexa 594 (middle panels). The right panels are the overlay of the left and middle panel. The upper left panel, negative control, excluded addition of anti-bCLCA1 Ab. (B) The upper panel is the confocal montage of cultured BCEC stained with anti-bCLCA1 (Alexa Fluor 488-linked second antibody). Nuclei were counterstained by DAPI. The images are sequential from the most basolateral section (upper left) to the most apical (lower right), the z -axis separation between images is 0.5 μm . The lower panel is an X-Z section that shows the bCLCA1 staining apical to the nuclear staining. (C) ZO-1 staining. The upper panel is a confocal montage of cultured BCEC stained with anti-ZO-1 (Alexa Fluor 594-linked second antibody). Nuclei were counterstained by DAPI. The images are sequential from the most basolateral section (upper left) to the most apical (lower right), the z -axis separation between images is 0.5 μm . The lower panel is an X-Z section showing the ZO-1 relatively apical to nuclear staining. (D) Arrow indicates apical side bCLCA1 staining (green) in fresh frozen section from cow cornea. Nuclei stained with DAPI.

Fig. 4.

siRNA knockdown of bCLCA1 expression. (A) Immunoblotting analysis of the effects of three siRNA constructs on the expression of bCLCA1 at the protein level, compared with the vehicle control (oligofectamine) and negative control siRNA, respectively. β -Actin was an equal-loading control. The histogram shows the protein expression percentage relative to the vehicle control. (B) Real-time PCR analysis of the effects of bCLCA1-RNAi-989 (989, arrow) on the mRNA expression of bCLCA1, compared with endothelium alone (e), the vehicle control (o), and negative control siRNA (cs), respectively. The housekeeping gene, GAPDH, was used as an equal loading control. (C) Gel analysis of the effects of bCLCA1-RNAi-989 on the expression of bCLCA1 at the mRNA level compared with negative control siRNA. GAPDH was a loading control.

Fig. 5.

Effect of bCLCA1-RNAi-989 on the ATP γ S-induced apical HCO $_3^-$ permeability in CBCEC. Cultured BCEC grown on permeable membranes were loaded with the pH-sensitive fluorescent dye, BCECF. Apical and basolateral compartments were perfused with HCO $_3^-$ -rich, Cl $^-$ -free Ringer's. Black boxes: LB (low HCO $_3^-$) pulse applied to the apical side. (A) Effects of negative control siRNA on ATP γ S (100 μ M)-induced apical HCO $_3^-$ permeability. (B) Summary of the effects of negative control siRNA on the initial rates of pH $_i$ changes (dpH $_i$ /dt) between the control and ATP γ S LB pulses. (C) Effects of bCLCA1-RNAi-989 on ATP γ S (100 μ M)-induced apical HCO $_3^-$ permeability. (D) Summary of the effects of bCLCA1-RNAi-989 on the initial rates of pH $_i$ changes (dpH $_i$ /dt) between the control and ATP γ S LB pulses.

Fig. 6.

Effect of bCLCA1-RNAi-989 on the CPA-induced apical HCO_3^- permeability in CBCEC. (A) Effects of negative control siRNA on CPA (20 μM)-induced apical HCO_3^- permeability increase. (B) Summary of the effects of negative control siRNA on the initial rates of pH_i changes (dpH_i/dt) between the control and CPA LB pulses. (C) Effects of bCLCA1-RNAi-989 on CPA (20 μM)-induced apical HCO_3^- permeability. (D) Summary of the effects of bCLCA1-RNAi-989 on the initial rates of pH_i changes (dpH_i/dt) between the control and CPA LB pulses.