Published in final edited form as:

Am J Physiol Cell Physiol. 2005 March ; 288(3): C739–C746. doi:10.1152/ajpcell.00405.2004.

Role of NBC1 in apical and basolateral HCO_3^- permeabilities and transendothelial HCO_3^- fluxes in bovine corneal endothelium

Jinhua Li, Xing Cai Sun, and Joseph A. Bonanno

Indiana University School of Optometry, Bloomington, Indiana

Abstract

Corneal transparency and hydration control are dependent on HCO_3^- transport properties of the corneal endothelium. Recent work (13) suggested the presence of an apical $1\text{Na}^+ - 3\text{HCO}_3^-$ cotransporter (NBC1) in addition to a basolateral $1\text{Na}^+ - 2\text{HCO}_3^-$ cotransporter. We examined whether the NBC1 cotransporter contributes significantly to basolateral or apical HCO_3^- permeability and whether the cotransporter participates in transendothelial net HCO_3^- flux in cultured bovine corneal endothelium. NBC1 protein expression was reduced using small interfering RNA (siRNA). Immunoblot analysis showed that 5–15 nM siRNA decreased NBC1 expression by 80–95%, 4 days posttransfection. Apical and basolateral HCO_3^- permeabilities were determined by measuring the rate of pH_i change when HCO_3^- was removed from the bath under constant pH or constant CO_2 conditions. Using either protocol, we found that cultures treated with NBC1 siRNA had sixfold lower basolateral HCO_3^- permeability than untreated or siCONTROL siRNA-treated cells. Apical HCO_3^- permeability was unaffected by NBC1 siRNA treatment. Net non-steady-state HCO_3^- flux was $0.707 \pm 0.009 \text{ mM} \cdot \text{min}^{-1} \cdot \text{cm}^2$ in the basolateral-to-apical direction and increased to 1.74 ± 0.15 when cells were stimulated with 2 μM forskolin. Treatment with 5 nM siRNA decreased basolateral-to-apical flux by 67%, whereas apical-to-basolateral flux was unaffected, significantly decreasing net HCO_3^- flux to 0.236 ± 0.002 . NBC1 siRNA treatment or 100 μM ouabain also eliminated steady-state HCO_3^- flux, as measured by apical compartment alkalinization. Collectively, reduced basolateral HCO_3^- permeability, basolateral-to-apical fluxes, and net HCO_3^- flux as a result of reduced expression of NBC1 indicate that NBC1 plays a key role in transendothelial HCO_3^- flux and is functional only at the basolateral membrane.

Keywords

corneal endothelium; sodium bicarbonate cotransporter; small interfering RNA; bicarbonate transport

THE ION AND FLUID TRANSPORT PROPERTIES of the corneal endothelium are responsible for maintaining the hydration and transparency of the cornea. Corneal endothelial fluid transport requires the presence of HCO_3^- (15, 18, 29) and Cl^- (44), is sensitive to carbonic anhydrase inhibitors

(19, 22, 29), and is completely eliminated by ouabain, a $\text{Na}^+\text{-K}^+\text{-ATPase}$ inhibitor. Net stroma to anterior chamber HCO_3^- flux is responsible for the measured short-circuit current and the small, negative transendothelial potential (19), suggesting that HCO_3^- is the primary secreted anion. Although significant progress has been made in identifying and locating plasma membrane transporters in the corneal endothelium, the contribution of the transporters to net HCO_3^- transport is largely unknown. In this study we have examined the role of the sodium bicarbonate cotransporter (NBC1) in transendothelial HCO_3^- transport.

Previous studies have shown that the uptake of HCO_3^- across the basolateral membrane of corneal endothelial cells occurs by a potent Na^+ -dependent, Cl^- -independent, DIDS-sensitive, and electrogenic $\text{Na}^+ - 2\text{HCO}_3^-$ cotransporter (5, 8, 21, 35). The activity of this cotransporter has a significant effect on intracellular pH (pH_i), and it appears to be the major entry point for HCO_3^- flux across the endothelium (5, 8). Recent molecular cloning experiments have identified several Na^+ -dependent bicarbonate transporters (3, 10, 20, 23, 27, 31, 40). Two variants of NBC1 have been found: the kidney proximal tubule form of NBC (kNBC) (11, 30) has a 1:3 stoichiometry, and the pancreas form of NBC (pNBC) (1, 38) has a 1:2 stoichiometry. However, more recent studies have shown that the stoichiometry of either kNBC or pNBC can change depending on the cell type in which it is expressed (17).

Our previous studies have shown that human (35) and bovine corneal endothelial cells (36) express the pNBC isoform only. An earlier report (42), however, suggested that both pNBC and kNBC are expressed in human corneal endothelium. Immunohistochemistry studies in cultured and fresh bovine (36), rat (4), and human endothelium (35, 41) indicate that NBC1 exclusively locate to the basolateral membrane; however, a recent report (13) suggests apical expression as well. Whereas HCO_3^- uptake by a basolateral $\text{Na}^+ - 2\text{HCO}_3^-$ cotransporter is certain, the mechanism for apical efflux is not clear. Evidence has been provided suggesting that HCO_3^- can exit the endothelial cells through anion channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+} -activated Cl^- channels (CaCC) (13, 34, 45). In addition, CO_2 diffusion and conversion to HCO_3^- by an apical membrane-bound extracellular carbonic anhydrase (CAIV) could also provide for net apical efflux (5, 6). If an apical NBC1 exists, then a $1\text{Na}^+:3\text{HCO}_3^-$ stoichiometry could also potentially contribute to the apical efflux pathways (13).

In the present study we have investigated the role of NBC1 in HCO_3^- permeability and transendothelial HCO_3^- fluxes in cultured corneal endothelial cells by using a short interfering RNA (siRNA) knockdown approach. siRNA has significant advantages over pharmacological agents such as DIDS, which can block several anion transporters and channels. We found that siRNA transiently inhibited NBC1 expression, significantly reduced basolateral but not apical HCO_3^- permeability, and reduced non-steady-state basolateral-to-apical HCO_3^- flux and net transendothelial HCO_3^- flux, indicating that an apical NBC1, if present, does not significantly contribute to net HCO_3^- flux.

MATERIALS AND METHODS

Cell culture

Bovine corneal endothelial cells (BCEC) were cultured to confluence onto 25-mm round coverslips, 13-mm Anodisc filters, or T-25 flasks as previously described (7, 24). Briefly, primary cultures from fresh cow eyes were established in T-25 flasks with 3 ml of Dulbecco's modified Eagle's medium (DMEM), 10% bovine calf serum, and antibiotic (100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 µg/ml Fungizone), 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 or Anodiscs and allowed to reach confluence within 5–7 days.

siRNA transfection

Four sense and antisense oligonucleotides corresponding to the following NBC1 cDNAs were designed and blasted using the Ambion siRNA-targeting design tool and were purchased from Invitrogen: AAGTTTGAAGAAAAAGTGGAA (397–417), AAAGAATATGTACTCAGGTGG (1209–1229), AATTGTGCCAAGTGAGTTCAA (2259–2279), and AAAAAGAAGGAGGATGAGAAG (3034–3054). Using these oligonucleotides, four siRNAs for NBC1 were synthesized using the Silencer siRNA construction kit from Ambion. A siCONTROL nontargeting siRNA (no known mammalian homology) was purchased from Dharmacom. Cells were transfected when 70–80% confluent by using Oligofectamine (Invitrogen) according to the manufacturer's protocol in the presence of siRNA. Cell-coated coverslips or Anodiscs in six-well plates were incubated with 1 ml of Opti-MEM I (GIBCO) containing siRNA for 4 h, followed by addition of 2 ml of standard DMEM with serum. T-25 flasks were treated with 2 ml of Opti-MEM I containing siRNA, followed by addition of 4 ml of culture medium. Medium was then changed every 2 days.

Immunoblotting

BCEC were dissolved directly in 2% SDS sample buffer that contained protease inhibitors. The preparations were sonicated (Branson 250) briefly on ice and centrifuged at 10,000 g for 5–10 min. An aliquot of the supernatant was taken for protein concentration measurement using the Bradford assay (Bio-Rad). Samples (30 µg, not heated) were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). Blots were then probed with NBC1 polyclonal antibody (AB-3212, 1:2,000; Chemicon) or α-subunit of Na⁺-K⁺-ATPase antibody (1:1,000; Developmental Hybridoma Bank, Iowa University), and bound antibody was detected using enhanced chemiluminescence (ECL). The membrane was then stripped using Re-blot plus strong antibody stripping solution (Chemicon) to remove NBC1 antibody or Na⁺-K⁺-ATPase antibody, and blots were incubated with β-actin polyclonal antibody (1:10,000; Sigma) and developed using ECL. Films were scanned to produce digital images that were then assembled and labeled using Photoshop software.

Immunofluorescence

Cultured cells grown to confluence on coverslips were washed three to four times with warmed (37°C) PBS and fixed for 30 min in warmed 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 to four times with PBS. Coverslips were then kept for 5 min in PBS that contained 1% SDS to unmask epitopes and washed three times in PBS. Cells were blocked for 1 h in PBS that contained 0.2% bovine serum albumin, 5% goat serum, 0.01% saponin, and 50 mM NH_4Cl . Rabbit polyclonal NBC1 antibody and rat monoclonal ZO-1 antibody (MAB1520; Chemicon) diluted 1:100 together in PBS-goat serum (1:1) were added onto coverslips and incubated for 1 h at room temperature or overnight at 4°C. Coverslips were washed three times for 15 min in PBS that contained 0.01% saponin. Secondary antibodies conjugated to Alexa 488 (NBC1) (1:1,000; Molecular Probes) and Alexa Fluor 594 (ZO-1) (1:1,000; Molecular Probes) were applied for 1 h at room temperature. Coverslips were washed and then stained with 1 $\mu\text{g}/\text{ml}$ Hoechst nuclear dye for 5 min. Coverslips were washed with water and mounted with Prolong antifade medium (Molecular Probes) according to the manufacturer's instructions. Fluorescence was observed with a standard epifluorescence microscope equipped with a cooled charge-coupled device camera.

Microscope perfusion

For measurement of pH_i and HCO_3^- transendothelial flux, cells were cultured to confluence on 13-mm diameter, 0.2- μm Anodisc membranes. Anodiscs were placed in a double-sided perfusion chamber designed for independent perfusion of the apical and basolateral sides (9). The assembled chamber was placed on a water-jacketed (37°C) brass collar held on the stage of an inverted microscope (Nikon Diaphot 200) and viewed with a long-working-distance (2 mm) water-immersion objective ($\times 40$; Nikon). Apical and basolateral compartments were connected to hanging syringes that contained Ringer solution in a Plexiglas warming box (37°C) by using Phar-Med tubing. The flow of the perfusate (~ 0.5 ml/min) was achieved by gravity. Two independent eight-way valves were employed to select the desired perfusate for the apical and basolateral chambers. The composition of the standard HCO_3^- -rich Ringer solution used throughout the study was (in mM) 150 Na^+ , 4 K^+ , 0.6 Mg^{2+} , 1.4 Ca^{2+} , 118 Cl^- , 1 HPO_4^{2-} , 10 HEPES $^-$, 28.5 HCO_3^- , 2 gluconate $^-$, and 5 glucose, equilibrated with 5% CO_2 and pH adjusted to 7.50 at 37°C. HCO_3^- -free Ringer solution (pH 7.50) was prepared by equimolar substitution of NaHCO_3 with sodium gluconate. Low- HCO_3^- Ringer solution (2.85 mM HCO_3^- , pH 6.5) was prepared by replacing 25.65 mM NaHCO_3 with sodium gluconate.

Measurement of HCO_3^- permeability

BCEC cultured onto permeable Anodisc filters were loaded with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by incubation in HCO_3^- -free Ringer solution that contained 1 μM BCECF-AM at room temperature for 30–60 min. Dye-loaded cells were then kept in Ringer solution for at least 30 min before use. Fluorescence excitation (495 and 440 nm) and data collection were obtained using a DeltaRam ratio fluorescence system (Photon Technology International, Monmouth Junction,

NJ) controlled by Felix software. Fluorescence ratios were obtained at 1 s^{-1} and were calibrated against pH_i by using the high-potassium-nigericin technique (39). A calibration curve, which follows a pH titration equation, has been constructed for BCEC (7). HCO_3^- permeabilities of apical and basolateral membranes was determined using the constant- CO_2 or constant-pH protocols as described previously (5). Briefly, in the constant-pH protocol, the HCO_3^- -rich Ringer on the apical or basolateral side is replaced with a CO_2 - and HCO_3^- -free Ringer of the same pH (HEPES buffered). Under this protocol, the initial pH_i change is due to rapid CO_2 efflux (increase in pH_i), followed by HCO_3^- efflux (decrease in pH_i). The maximum slope of the pH_i decrease is taken as an estimate of HCO_3^- permeability. In the constant- CO_2 protocol, the HCO_3^- -rich Ringer (28.5 mM HCO_3^- , 5% CO_2 , pH 7.5) is replaced with a low- HCO_3^- solution (2.85 mM HCO_3^- , 5% CO_2 , pH 6.5). Under this protocol, the initial pH_i change is predominantly due to HCO_3^- efflux, because there is no CO_2 gradient. However, there is a pH gradient that can contribute (~15%) to the pH_i decrease (5). Separate control experiments are performed in the absence of $\text{CO}_2/\text{HCO}_3^-$ to estimate this contribution.

Measurement of transendothelial HCO_3^- flux

BCEC cultured onto permeable Anodisc filters, perfused in a double-sided chamber, were exposed to the standard HCO_3^- -rich solution (5% CO_2 , 28.5 mM HCO_3^- , pH 7.5) on the basolateral and apical sides at 37°C . A low- HCO_3^- solution (5% CO_2 /2.85 mM HCO_3^- , pH 6.5) without HEPES buffer and containing $1 \mu\text{M}$ BCECF free acid was then quickly exchanged on one side of the chamber, and the exit tube on that side was clamped. The pH of the low- HCO_3^- solution was estimated (1 Hz) at $\sim 200 \mu\text{m}$ from the surface of the cells by measuring the fluorescence ratio of BCECF using the microscope fluorometer. The pH of the low- HCO_3^- solution rose from 6.5, and the initial rate of change over the first 20 s after clamping was estimated. After the initial pH change was recorded, HCO_3^- -rich solution was returned, the chamber was flipped over on the microscope stage, and the same measurement procedure was repeated so that unidirectional fluxes in the apical-to-basolateral and basolateral-to-apical directions were obtained for each Anodisc. Separate control experiments also were performed with the same pH gradient across the cells in 1 mM HEPES⁻ but in the complete absence of $\text{CO}_2/\text{HCO}_3^-$. This pH change was used to estimate the initial OH^- flux (J_{OH}) across the Anodisc, which was subtracted from the total flux calculated in the presence of HCO_3^- . Each unidirectional HCO_3^- flux can be calculated as

$$J_{\text{HCO}_3^-} = \left[\frac{dpH}{dt} \times \frac{\beta}{A} \right] - J_{\text{OH}}$$

where J is flux ($\text{mM} \cdot \text{min}^{-1} \cdot \text{cm}^2$), A is the area of the exposed Anodisc (0.23 cm^2), dpH/dt is the initial rate of pH change from 6.5, and β is the buffering capacity, which is $2.3 \times [\text{HCO}_3^-]$, or 6.55 mM/pH. J is the OH^- flux calculated in the same manner, where β is 0.5 mM/pH. The difference between basolateral-to-apical and apical-to-basolateral unidirectional fluxes is the net HCO_3^- flux. This is called non-steady-state flux because it was measured in the presence of a large HCO_3^- gradient.

Measurement of steady-state HCO_3^- flux

BCEC cultured to confluence on 0.2- μm Anopore membrane tissue culture inserts were washed with DMEM containing 2% bovine calf serum. Then, 200 μl of this culture medium containing 1 μM BCECF free acid were placed on the apical side and 300 μl on the basolateral side. After 6 h in a standard 5% CO_2 incubator at 37°C, cultures were placed in a large glove box equilibrated with 5% CO_2 at 37°C. Samples (50 μl) were taken from the apical and basolateral sides with separate glass capillary tubes, and both ends were sealed with wax. The tubes were then taken to the microscope fluorometer, and the fluorescence ratio of BCECF was measured. The pH of each sample was then determined using a standard curve constructed by using solutions of known pH that had been placed within capillary tubes. The difference in pH was calculated as $\text{pH} = \text{apical pH} - \text{basolateral pH}$. A positive pH indicates apical HCO_3^- accumulation.

RESULTS

NBC1 siRNA was used to knock down NBC1 expression in cultured BCEC. To confirm the reduction and select the best knockdown condition, we performed immunoblotting with rabbit anti-NBC1 antibody. BCEC were transfected with four siRNAs, and Western blotting results showed that the siRNA against the target AAAAAGAAGGAGGATGAGAAG was the most efficient (data not shown), so this was selected for use in the remainder of the study. Figure 1, A and B, shows that NBC1 expression in BCEC was slightly decreased 2 or 3 days after cells were treated with siRNA. At the highest concentration, 25 nM, NBC1 expression was reduced 46 and 49%, at 2 and 3 days posttransfection, respectively, compared with control. However, 4 or 5 days after being treated with siRNA, most of the NBC1 expression was suppressed. At 4 days posttransfection with 5 nM siRNA, at least 80% of protein expression was inhibited compared with control, with slightly better knockdown at higher starting siRNA concentration. Figure 1C shows that immunofluorescence for NBC1 was barely detectable at 4 days posttransfection with 15 nM siRNA, while cell and tight junction morphology were normal. NBC1 expression began to recover by the sixth day posttransfection. Although morphological appearance and cell confluence were normal, we did notice an increase in numbers of floating cells in cultures treated at the highest (25 nM) siRNA concentration. We chose to use lower concentrations, 15 nM or less, for the remainder of the study.

To determine that the reduction of NBC1 expression mediated by siRNA was not a nonspecific effect of siRNA transfection, we used a siCONTROL nontargeting siRNA to transfect BCEC and performed immunoblotting with anti-NBC1 antibody. As Fig. 2 shows, there was no significant change in NBC1 expression 4 days after cells were treated with 5 and 15 nM siCONTROL siRNA compared with untreated cells, whereas an obvious decrease in NBC1 expression occurred when cells were treated with 5 nM NBC1 siRNA. These results show that the reduction in NBC1 expression is not simply due to the introduction of foreign siRNA.

More importantly, we must consider that any physiological effects of NBC1 siRNA treatment could be due to the nonspecific knockdown of expression of other transporters that

could participate in “NBC1-like” transport. Given our current knowledge of transport mechanisms in corneal endothelium (6), candidates that might affect HCO_3^- flux include the Na^+/H^+ exchanger, Cl^-/HCO_3^- exchanger, and the $Na^+-K^+-ATPase$. In some systems, Na^+/H^+ exchange activity can indirectly contribute to HCO_3^- uptake. However, in corneal endothelium perfused in the presence of HCO_3^- , inhibition of Na^+/H^+ exchange with amiloride could slow recovery of pH_i from acid loading but would have no effect on steady-state pH_i , indicating that there is no significant Na^+/H^+ activity in resting cells. The Cl^-/HCO_3^- exchanger AE2 is present on the basolateral membrane of fresh dissected endothelium (6), but it is not expressed in cultured endothelium and no Cl^-/HCO_3^- exchange activity can be detected in cultured cells (9). On the other hand, reduced expression of the $Na^+-K^+-ATPase$ would collapse the Na^+ gradients that drive $Na^+ - 2HCO_3^-$ cotransport, and we found in the present study (see below) that ouabain treatment significantly inhibited net HCO_3^- fluxes. Therefore, we tested whether NBC siRNA treatment might affect $Na^+-K^+-ATPase$ expression. Figure 2 shows that $Na^+-K^+-ATPase$ expression level did not change 4 days after BCEC cells were treated with NBC1 siRNA, indicating that physiological effects of NBC1 siRNA treatment are not due to nonspecific knockdown of the $Na^+-K^+-ATPase$.

Previous investigations have shown that HCO_3^- plays a key role in regulation of endothelial cell pH_i and that NBC1 has an exclusive role in basolateral HCO_3^- transport (5, 8, 21, 35). These conclusions are based on experiments showing Na^+ dependent and DIDS-sensitive, electrogenic HCO_3^- transport at the basolateral membrane (36). More recently, data have been presented suggesting that NBC1 is also present on the apical membrane and could have a role in transendothelial HCO_3^- flux (13). We used a constant-pH and a constant- CO_2 protocol (5) to examine the apical and basolateral HCO_3^- permeabilities of cultured corneal endothelial cells. In the constant-pH protocol, the HCO_3^- -rich Ringer is replaced with HCO_3^- -free Ringer. The initial pH_i change is due to rapid CO_2 efflux. Although HCO_3^- efflux is occurring concomitantly, a decrease in pH_i is not observed until the $[CO_2]$ has equilibrated (~ 15 s). The maximum rate of pH_i decrease (averaged over 20 s) is taken as an indirect measure of HCO_3^- efflux. In the constant- CO_2 protocol, the HCO_3^- -rich Ringer (28.5 mM, pH 7.5) is replaced with low- HCO_3^- (2.85 mM, pH 6.5) Ringer. The initial pH_i drop is predominantly from HCO_3^- efflux with a small (15%) contribution from H^+ flux (5). Figure 3 shows that there was a sharp and rapid acidification due to HCO_3^- efflux (-0.004467 ± 0.003187 pH_i/s , $n = 6$) below the baseline after the initial alkalization from CO_2 efflux during the constant-pH protocol on the basolateral side; however, there was a significantly slower acidification (-0.00073 ± 0.0000544 pH_i/s , $n = 6$, $P < 0.001$) in cells that had been treated with 5 nM siRNA, indicating a more than sixfold decrease in basolateral HCO_3^- permeability. When the constant- CO_2 protocol was used to remove the effect of CO_2 fluxes, similar results were obtained. There was a significantly slower drop in pH_i (-0.000812 ± 0.0000918 pH_i/s , $n = 6$, $P < 0.001$) in the siRNA-treated cells compared with the control cells (-0.00506 ± 0.000338 pH_i/s , $n = 6$). Conversely, there was no significant difference in the rate of pH_i decrease on the apical side between the control and siRNA-treated cells, under either the constant-pH or constant- CO_2 protocol. To test that the procedure of introducing foreign siRNA could have nonspecific effects on the measured

HCO_3^- permeabilities, we transfected the siCONTROL siRNA into BCEC and measured pH_i using the constant- CO_2 protocol. Figure 4 shows that there was no significant difference in HCO_3^- permeabilities between control and siCONTROL siRNA-treated cells on either the basolateral or apical side. These results indicate that NBC1 plays a major role in the regulation of basolateral HCO_3^- permeability but not apical HCO_3^- permeability in corneal endothelial cells.

We next focused on the effect of NBC1 on the transendothelial HCO_3^- flux in corneal endothelial cells. Figure 5, A and B, shows that basolateral-to-apical exceeded apical-to-basolateral unidirectional HCO_3^- fluxes by approximately sixfold in control cells, consistent with a net stroma-to-anterior chamber HCO_3^- flux. Increasing cell cAMP concentration with forskolin increases the permeability of the apical CFTR to HCO_3^- by twofold without having a significant effect on basolateral permeability (34). Figure 5 shows that 2 μM forskolin increased basolateral-to-apical but not apical-to-basolateral unidirectional HCO_3^- fluxes and increased net flux by more than twofold, consistent with the effect on apical permeability. In the NBC1 siRNA treated cells, basolateral-to-apical flux (0.010405 ± 0.000618 pH/s) was significantly slowed compared with the untreated cells (0.0294 ± 0.00319 pH/s), but there was no difference in apical-to-basolateral unidirectional fluxes. When cells were treated with 100 μM ouabain, basolateral-to-apical flux (0.00863 ± 0.000874 pH/s) also was significantly slow compared with the untreated cells. Figure 5C summarizes the results from six separate experiments and shows that the net initial HCO_3^- flux was 0.7066 ± 0.009144 $\text{mM} \cdot \text{min}^{-1} \cdot \text{cm}^2$, and it was increased to 1.74 ± 0.15 $\text{mM} \cdot \text{min}^{-1} \cdot \text{cm}^2$ when cells were stimulated with 2 μM forskolin ($P < 0.001$). In siRNA-treated cells, net initial HCO_3^- flux was reduced threefold to 0.236 ± 0.0024 $\text{mM} \cdot \text{min}^{-1} \cdot \text{cm}^2$ ($P < 0.005$). A similar result was observed in ouabain-treated cells, where net initial HCO_3^- flux was reduced to 0.134 ± 0.0336 $\text{mM} \cdot \text{min}^{-1} \cdot \text{cm}^2$ ($P < 0.005$).

To obtain a measure of the contribution of NBC1 to steady-state transendothelial HCO_3^- flux, BCEC were cultured on 0.2- μm Anopore membrane tissue culture inserts and transfected with siRNA. Four days posttransfection, the cultures were washed with DMEM containing 2% bovine calf serum and 1 μM BCECF free acid; 200 μl of culture medium were placed on the apical side and 300 μl on the basolateral side. Six hours later, samples of apical and basolateral medium were collected and the BCECF fluorescence ratio was determined. Consistent with net basolateral-to-apical HCO_3^- flux, control untreated filters showed a net relative alkalization ($\text{pH} = \text{apical pH} - \text{basolateral pH}$) of the apical medium ($+0.077 \pm 0.018$ pH unit). As a further control, cultures were treated with ouabain, which was expected to inhibit all Na^+ -dependent active transport processes and reduce the net pH change to zero. However, we found that 100 μM ouabain reversed the pH gradient to -0.073 ± 0.017 pH unit. NBC1 siRNA treatment caused a small alkalization of the basolateral medium and a larger acidification of the apical medium, producing a net pH change of -0.098 ± 0.032 , very similar to ouabain treatment. Figure 6 summarizes these data and indicates that NBC1 plays a major role in transendothelial HCO_3^- transport.

DISCUSSION

We have previously reported that a basolateral $Na^+ - 2HCO_3^-$ cotransporter (NBC1) provides for robust HCO_3^- influx in bovine (36) and human (35) corneal endothelial cells. This activity is Na^+ dependent, DIDS sensitive, and electrogenic. To examine the role of this transporter in membrane HCO_3^- permeabilities and transendothelial HCO_3^- flux, we inhibited NBC1 expression by using a specific siRNA approach. Our findings are consistent with a major role of a basolateral NBC1 in transendothelial HCO_3^- flux but do not support the function of an apical NBC1. This is in contrast to immunofluorescence data recently reported that suggested the presence of an apical NBC1; however, no functional tests for apical $Na^+ - 3HCO_3^-$ exchange were performed (13).

The RNA interference (RNAi) technique has been extended to a wide range of commonly used mammalian cells. RNAi is a highly specific form of posttranscriptional gene silencing using 21- to 23-nt siRNA molecules and has been shown to be an effective and specific method for examining functional roles of specific proteins (2, 14, 26). RNAi is very attractive for reducing NBC1 activity, because the primary pharmacological inhibiting agent DIDS is nonspecific and can block other anion transporters and channels. Moreover, the information obtained from selective application of DIDS is difficult to interpret. For example, slowing HCO_3^- efflux through a DIDS-sensitive apical transporter or channel should increase pH_i . However, we have observed that apical application of DIDS produces a delayed reduction in pH_i of corneal endothelial cells. This result suggests that little, if any, apical DIDS-sensitive HCO_3^- flux is present and that the drug was diffusing to the lateral membrane and inhibiting the basolateral $Na^+ - 2HCO_3^-$ cotransporter. In the present study, we have shown that transfection of siRNA designed to target NBC1 markedly attenuated the expression of NBC1 in BCEC. In general, it is best to use the lowest effective concentration of siRNA, because high doses can have nonspecific effects (32). We found that high concentrations of siRNA (> 20 nM) showed some toxicity to the cells 4 days posttransfection, so we chose 5–15 nM siRNA as the optimal concentration throughout this study. Some studies have indicated that there is no significant nonspecific interference and degradation of endogenous mRNAs in mammalian cells subject to siRNA (12, 32); however, small mismatches with non-target genes can risk inhibiting expression of these genes (33). In addition to the posttranscriptional, sequence-specific effect mediated by the siRNA, the introduction of sequence nonspecific siRNA can sometimes have unpredictable effects on gene expression (14). To exclude these nonspecific effects, we used a siCONTROL nontargeting siRNA to determine whether the activation of the RNAi-induced silencing complex (RISC) could nonspecifically affect NBC1 expression or function. Figure 2 shows that siCONTROL siRNA did not have any effect on NBC1 expression, whereas NBC1 siRNA decreased NBC1 expression but did not affect $Na^+ K^+$ -ATPase expression. We chose to examine $Na^+ K^+$ -ATPase expression because inhibition of this protein could produce functional changes similar to NBC1 inhibition. Furthermore, transfection with siCONTROL had no functional effect on HCO_3^- permeabilities (Fig. 4). These results suggest that siRNA targeted to NBC1 is specific and effective in reducing expression in BCEC.

Basolateral, but not apical, HCO_3^- permeability was significantly affected by NBC1 siRNA treatment. This was determined by measuring the rate of pH_i change after removal of HCO_3^- from apical or basolateral perfusion chambers. Removal of HCO_3^- and CO_2 from the perfusing Ringer solution maintains solution pH (constant pH protocol); however, the pH_i changes induced are confounded by initial CO_2 efflux, which must be cleared before the rate of pH_i decrease can be measured. The constant- CO_2 protocol avoids confounding by CO_2 fluxes but adds instead the reduced pH of the perfusate. Previously, we (37) showed that the pH_i changes measured due to the bath pH changes contributed only ~15% to the initial rate of pH_i change. Nevertheless, both protocols showed similar sixfold drops in basolateral HCO_3^- permeability and no effect on apical permeability. The absence of an effect on apical HCO_3^- permeability is consistent with previous studies showing no change in apical HCO_3^- permeability under low- Na^+ conditions (5) and an influx of Na^+ upon removal of apical HCO_3^- (36). A siCONTROL siRNA was also transfected into endothelial cells, but this had no effect on HCO_3^- permeability in both basolateral and apical sides, indicating that NBC1 siRNA affects the basolateral permeability specifically.

The role of NBC1 in transendothelial HCO_3^- flux was measured under non-steady-state and steady-state conditions. Under non-steady-state conditions we introduced a large HCO_3^- gradient across the cell monolayer and measured initial unidirectional HCO_3^- fluxes, which were adjusted for OH^- fluxes. In control cultures, we found that basolateral-to-apical flux was significantly larger than apical-to-basolateral flux. Forskolin, which produces a rapid increase in cell cAMP concentration, has been shown to stimulate endothelial fluid transport (28) and activate an apical CFTR channel, leading to increased HCO_3^- permeability across the apical, but not basolateral, membrane (34, 46). We used forskolin as a positive control for the non-steady-state flux experiments. We found (Fig. 5) that 2 μM forskolin increased basolateral-to-apical but not apical-to-basolateral fluxes. The absence of a change in apical-to-basolateral fluxes in the presence of forskolin may be explained by a lack of significant change in driving force for HCO_3^- from the outside to the inside of cells across an apical membrane channel. When HCO_3^- is totally removed, the membrane potential depolarizes by only 5 mV (21). In apical-to-basolateral fluxes, basolateral HCO_3^- concentration is reduced from 28 to 2.85 mM. The initial intracellular HCO_3^- concentration is ~20 mM (8), so there is a significant electrochemical gradient for HCO_3^- exit across the basolateral membrane, but initially there is no change in chemical gradient across the apical membrane and only a small change in electrical gradient that would still limit HCO_3^- influx across an apical channel. On the other hand, NBC1 siRNA inhibited basolateral-to-apical but not apical-to-basolateral fluxes, consistent with NBC1 contributing to a net HCO_3^- flux from the stroma to the anterior chamber (19, 25, 43).

Steady-state HCO_3^- flux was determined by measuring the relative pH change in apical and basolateral compartments in cultures bathed with identical HCO_3^- -rich starting culture media. After 6 h of incubation, the apical side was relatively alkaline in control cultures. Similar types of measurements have been made in cultured endothelium using a pH-stat technique (13). Ouabain, which is expected to inhibit the accumulation of HCO_3^- , actually produced a relative apical acidification. The reason for the apical acidification is not clear.

One possibility is acidification by an apical lactate- H^+ cotransporter (16), which would be unaffected by ouabain's effect on Na^+ gradients. siRNA treatment, which also was expected to inhibit apical compartment HCO_3^- accumulation, also produced a relative apical acidification consistent with the ouabain treatment and inhibition of net basolateral-to-apical HCO_3^- flux.

In summary, our results show conclusively that basolateral NBC1 ($1Na^+ - 2HCO_3^-$) has a significant role in transendothelial HCO_3^- flux. The basolateral $Na^+ - 2HCO_3^-$ cotransporter loads endothelial cells with HCO_3^- . HCO_3^- flux from the cell across the apical membrane can be significantly stimulated by increasing cAMP concentration, consistent with an increase in apical HCO_3^- permeability through CFTR. However, we found no evidence for NBC1 on the apical membrane.

Acknowledgments

We thank Miao Cui for excellent technical assistance.

GRANTS This research was supported by National Eye Institute Grant EY-08834.

REFERENCES

1. Abuladze N, Lee I, Newman D, Hwang J, Boorer K, Pushkin A, Kurtz I. Molecular cloning, chromosomal localization, tissue distribution, and functional expression of the human pancreatic sodium bicarbonate cotransporter. *J Biol Chem*. 1998; 273:17689–17695. [PubMed: 9651366]
2. Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ. Desensitization, internalization, and signaling functions of β -arrestins demonstrated by RNA interference. *Proc Natl Acad Sci USA*. 2003; 100:1740–1744. [PubMed: 12582207]
3. Amlal H, Burnham CE, Soleimani M. Characterization of Na^+/HCO_3^- cotransporter isoform NBC-3. *Am J Physiol Renal Physiol*. 1999; 276:F903–F913.
4. Bok D, Schibler MJ, Pushkin A, Sassani P, Abuladze N, Naser Z, Kurtz I. Immunolocalization of electrogenic sodium-bicarbonate cotransporters pNBC1 and kNBC1 in the rat eye. *Am J Physiol Renal Physiol*. 2001; 281:F920–F935. [PubMed: 11592950]
5. Bonanno J, Guan Y, Jelamskii S, Kang X. Apical and basolateral CO_2 - HCO_3^- permeability in cultured bovine corneal endothelial cells. *Am J Physiol Cell Physiol*. 1999; 277:C545–C553.
6. Bonanno JA. Identity and regulation of ion transport mechanisms in the corneal endothelium. *Prog Retin Eye Res*. 2003; 22:69–94. [PubMed: 12597924]
7. Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium. I. Na^+/H^+ exchange in the absence and presence of HCO_3^- . *Invest Ophthalmol Vis Sci*. 1992; 33:3058–3067. [PubMed: 1328110]
8. Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium. II. Na^+/HCO_3^- cotransport and Cl^-/HCO_3^- exchange. *Invest Ophthalmol Vis Sci*. 1992; 33:3068–3079. [PubMed: 1399410]
9. Bonanno JA, Guan Y, Xiao J, Srinivas SP. Reevaluation of Cl^-/HCO_3^- exchange in cultured bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1998; 39:2713–2722. [PubMed: 9856782]
10. Burnham CE, Amlal H, Wang Z, Shull GE, Soleimani M. Cloning and functional expression of a human kidney Na^+/HCO_3^- cotransporter. *J Biol Chem*. 1997; 272:19111–19114. [PubMed: 9235899]
11. Burnham CE, Flagella M, Wang Z, Amlal H, Schull GE, Soleimani M. Cloning, renal distribution, and regulation of the rat Na^+/HCO_3^- cotransporter. *Am J Physiol Renal Physiol*. 1998; 274:F1119–F1126.

12. Chi JT, Chang HY, Wang NN, Chang DS, Dunphy N, Brown PO. Genomewide view of gene silencing by small interfering RNAs. *Proc Natl Acad Sci USA*. 2003; 100:6343–6346. [PubMed: 12730368]
13. Diecke FP, Wen Q, Sanchez JM, Kuang K, Fischbarg J. Immunocytochemical localization of Na⁺–HCO₃[–] cotransporters and carbonic anhydrase dependence of fluid transport in corneal endothelial cells. *Am J Physiol Cell Physiol*. 2004; 286:C1434–C1442. [PubMed: 14960417]
14. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. 2001; 411:494–498. [PubMed: 11373684]
15. Fischbarg J, Lim J. Role of cations, anions, and carbonic anhydrase in fluid transport across rabbit corneal endothelium. *J Physiol*. 1974; 241:647–675. [PubMed: 4215880]
16. Giasson C, Bonanno JA. Facilitated transport of lactate by rabbit corneal endothelium. *Exp Eye Res*. 1994; 59:73–81. [PubMed: 7835399]
17. Gross E, Hawkins K, Abuladze N, Pushkin A, Cotton CU, Hopfer U, Kurtz I. The stoichiometry of the electrogenic sodium bicarbonate cotransporter NBC1 is cell-type dependent. *J Physiol*. 2001; 531:597–603. [PubMed: 11251043]
18. Hodson S. The regulation of corneal hydration by a salt pump requiring the presence of sodium and bicarbonate ions. *J Physiol*. 1974; 236:271–302. [PubMed: 16992435]
19. Hodson S, Miller F. The bicarbonate ion pump in the endothelium which regulates the hydration of rabbit cornea. *J Physiol*. 1976; 263:563–577. [PubMed: 828203]
20. Ishibashi K, Sasaki S, Marumo F. Molecular cloning of a new sodium bicarbonate cotransporter cDNA from human retina. *Biochem Biophys Res Commun*. 1998; 246:535–538. [PubMed: 9610397]
21. Jentsch T, Keller S, Koch M, Wiederholt M. Evidence for coupled transport of bicarbonate and sodium in cultured bovine corneal endothelial cells. *J Membr Biol*. 1984; 81:189–204. [PubMed: 6502693]
22. Kuang K, Xu M, Koniarek J, Fischbarg J. Effects of ambient bicarbonate, phosphate and carbonic anhydrase inhibitors on fluid transport across rabbit endothelium. *Exp Eye Res*. 1990; 50:487–493. [PubMed: 2373152]
23. Kurtz I, Petrasek D, Tatishchev S. Molecular mechanisms of electrogenic sodium bicarbonate cotransport: structural and equilibrium thermodynamic considerations. *J Membr Biol*. 2004; 197:77–90. [PubMed: 15014910]
24. MacCallum D, Lillie J, Scaletta L, Occhino J, Frederick W, Ledbetter S. Bovine corneal endothelium in vitro. *Exp Cell Res*. 1982; 139:1–13. [PubMed: 7084312]
25. Mayes KR, Hodson S. Local osmotic coupling to the active transendothelial bicarbonate flux in the rabbit cornea. *Biochim Biophys Acta*. 1978; 514:286–293. [PubMed: 737174]
26. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet*. 2002; 3:737–747. [PubMed: 12360232]
27. Pushkin A, Abuladze N, Newman D, Lee I, Xu G, Kurtz I. Two C-terminal variants of NBC4, a new member of the sodium bicarbonate cotransporter family: cloning, characterization, and localization. *IUBMB Life*. 2000; 50:13–19. [PubMed: 11087115]
28. Riley M, Winkler B, Starnes C, Peters M. Adenosine promotes regulation of corneal hydration through cyclic adenosine monophosphate. *Invest Ophthalmol Vis Sci*. 1996; 37:1–10. [PubMed: 8550312]
29. Riley MV, Winkler BS, Czajkowski CA, Peters MI. The roles of bicarbonate and CO₂ in transendothelial fluid movement and control of corneal thickness. *Invest Ophthalmol Vis Sci*. 1995; 36:103–112. [PubMed: 7822137]
30. Romero MF, Fong P, Berger UV, Hediger MA, Boron WF. Cloning and functional expression of rNBC, an electrogenic Na⁺–HCO₃[–] cotransporter from rat kidney. *Am J Physiol Renal Physiol*. 1998; 274:F425–F432.
31. Romero MF, Hediger MA, Boulpaep EL, Boron WF. Expression cloning and characterization of a renal electrogenic Na⁺/HCO₃[–] cotransporter. *Nature*. 1997; 387:409–413. [PubMed: 9163427]

32. Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW. Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci USA*. 2003; 100:6347–6352. [PubMed: 12746500]
33. Snove O Jr, Holen T. Many commonly used siRNAs risk off-target activity. *Biochem Biophys Res Commun*. 2004; 319:256–263. [PubMed: 15158470]
34. Sun XC, Bonanno JA. Expression, localization, and functional evaluation of CFTR in bovine corneal endothelial cells. *Am J Physiol Cell Physiol*. 2002; 282:C673–C683. [PubMed: 11880256]
35. Sun XC, Bonanno JA. Identification and cloning of the NaHCO_3^- cotransporter (NBC) in human corneal endothelium. *Exp Eye Res*. 2003; 77:287–295. [PubMed: 12907161]
36. Sun XC, Bonanno JA, Jelamskii S, Xie Q. Expression and localization of NaHCO_3^- cotransporter in bovine corneal endothelium. *Am J Physiol Cell Physiol*. 2000; 279:C1648–C1655. [PubMed: 11029313]
37. Sun XC, Zhai CB, Cui M, Chen Y, Levin LR, Buck J, Bonanno JA. HCO_3^- —dependent soluble adenylyl cyclase activates cystic fibrosis transmembrane conductance regulator in corneal endothelium. *Am J Physiol Cell Physiol*. 2003; 284:C1114–C1122. [PubMed: 12519749]
38. Thevenod F, Roussa E, Schmitt BM, Romero MF. Cloning and immunolocalization of a rat pancreatic Na^+ bicarbonate cotransporter. *Biochem Biophys Res Commun*. 1999; 264:291–298. [PubMed: 10527880]
39. Thomas J, Buchsbaum R, Zimniak A, Racker E. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry*. 1979; 18:2210–2218. [PubMed: 36128]
40. Tsuganezawa H, Kobayashi K, Iyori M, Araki T, Koizumi A, Watanabe S, Kaneko A, Fukao T, Monkawa T, Yoshida T, Kim DK, Kanai Y, Endou H, Hayashi M, Saruta T. A new member of the HCO_3^- transporter superfamily is an apical anion exchanger of β -intercalated cells in the kidney. *J Biol Chem*. 2001; 276:8180–8189. [PubMed: 11102437]
41. Usui T, Hara M, Satoh H, Moriyama N, Kagaya H, Amano S, Oshika T, Ishii Y, Ibaraki N, Hara C, Kunimi M, Noiri E, Tsukamoto K, Inatomi J, Kawakami H, Endou H, Igarashi T, Goto A, Fujita T, Araie M, Seki G. Molecular basis of ocular abnormalities associated with proximal renal tubular acidosis. *J Clin Invest*. 2001; 108:107–115. [PubMed: 11435462]
42. Usui T, Seki G, Amano S, Oshika T, Miyata K, Kunimi M, Taniguchi S, Uwatoko S, Fujita T, Araie M. Functional and molecular evidence for $\text{Na}^+/\text{HCO}_3^-$ cotransporter in human corneal endothelial cells. *Pflügers Arch*. 1999; 438:458–462. [PubMed: 10519138]
43. Wigham C, Hodson S. Bicarbonate and the trans-endothelial short-circuit current of human cornea. *Curr Eye Res*. 1981; 1:285–290. [PubMed: 7307537]
44. Winkler B, Riley M, Peters M, Williams F. Chloride is required for fluid transport by the rabbit corneal endothelium. *Am J Physiol Cell Physiol*. 1992; 262:C1167–C1174.
45. Xie Q, Zhang Y, Zhai C, Bonanno JA. Calcium influx factor from cytochrome P-450 metabolism and secretion-like coupling mechanisms for capacitative calcium entry in corneal endothelial cells. *J Biol Chem*. 2002; 277:16559–16566. [PubMed: 11867616]
46. Zhang Y, Xie Q, Sun XC, Bonanno JA. Enhancement of HCO_3^- permeability across the apical membrane of bovine corneal endothelium by multiple signaling pathways. *Invest Ophthalmol Vis Sci*. 2002; 43:1146–1153. [PubMed: 11923259]

Fig. 1.

Effect of $1Na^+ - 3HCO_3^-$ cotransporter (NBC1) small interfering RNA (siRNA) on NBC1 expression. **A:** Western blot analysis of NBC1 in bovine corneal endothelial cells (BCEC). Total cellular protein was extracted from BCEC treated or untreated with different concentrations of NBC1 siRNA and examined at different time points. Protein (30 μ g) was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes overnight and stained with enhanced chemiluminescence (ECL) using rabbit polyclonal anti-NBC1. The major band position of NBC1 is at ~130 kDa. **B:** densitometric analyses of protein expression from experiments as shown in A. Data were quantified relative to control band intensity, respectively ($n = 3$). **C:** decrease in NBC1 protein 4 days after 15 nM siRNA treatment examined using immunofluorescence. Green, NBC1; red, ZO-1; blue, nuclei.

Fig. 2.

Western blot analysis of NBC1 and Na⁺-K⁺-ATPase. Total cellular proteins were extracted from BCEC treated or untreated (control, Con) with different concentrations of NBC1 siRNA or siCONTROL siRNA (si-Con), and Western blot analysis was performed using NBC1 antibody, α-subunit of Na⁺-K⁺-ATPase antibody, or β-actin antibody as detailed in

MATERIALS AND METHODS.

Fig. 3.

Effect of NBC1 siRNA treatment on basolateral and apical HCO_3^- fluxes. **A:** cells untreated or treated with 5 nM NBC siRNA were perfused in $\text{CO}_2/\text{HCO}_3^-$ -rich Ringer on both sides. Basolateral $\text{CO}_2/\text{HCO}_3^-$ was totally removed under the constant-pH protocol (1: BF, bicarbonate free), and then, after reequilibration, basolateral HCO_3^- concentration was reduced from 28.5 to 2.85 mM under the constant- CO_2 protocol (2: LB, low bicarbonate). This sequence was then repeated on the apical side using the same protocols (3 and 4). 1'-4' indicate the same procedures using siRNA-treated cells. **B:** maximum change in intracellular pH (pHi) over the initial 20 s ($-\text{dpHi}/\text{dt}$). Values are means $\pm$ SE ($n = 6$). ** $P < 0.001$ vs. control.

Fig. 4.

Effect of siCONTROL siRNA on basolateral and apical HCO_3^- fluxes. **A:** cells untreated or treated with 5 nM siCONTROL siRNA were perfused in CO_2/HCO_3^- -rich Ringer on both sides. The basolateral and then apical HCO_3^- concentration was reduced from 28.5 to 2.85 mM under the constant- CO_2 protocol. **B:** maximum change in pH_i over the initial 20 s. Values are means $\pm$ SE.

Fig. 5.

Effect of NBC1 siRNA on non-steady-state transendothelial net HCO_3^- flux. **A:** cells treated or untreated with 5 nM NBC1 siRNA were initially perfused in $\text{CO}_2/\text{HCO}_3^-$ -rich Ringer on the basolateral and apical sides. LB solution without HEPES containing 1 μM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) free acid was then quickly exchanged on the apical side followed by clamping the exit tube on that side. After the initial rise in pH was recorded, the apical solution was replaced with $\text{CO}_2/\text{HCO}_3^-$ -rich ringer. The chamber was then flipped over, and the same experiment was done for the basolateral side. For forskolin or ouabain experiments, LB solution containing 2 μM forskolin or 100 μM ouabain was perfused across the cells for 2 min before clamping on apical or basolateral side as described above. B to A, basolateral to apical flux; A to B, apical to basolateral. **B:** maximum slope (pH/s) over the first 20 s. Values are means $\pm$ SE (n = 6). *P = 0.005 vs. control. **P = 0.001 vs. control. **C:** transendothelial net HCO_3^- flux. Values are means $\pm$ SE (n = 6). *P = 0.005 vs. control. **P = 0.001 vs. control.

Fig. 6.

Effect of NBC1 siRNA on steady-state net HCO_3^- flux. Cells cultured on Anopore membrane tissue culture inserts were treated or untreated with 15 nM siRNA. Four days later when cells were confluent, the inserts were washed and 200 μ l of DMEM containing 1 μ M BCECF free acid were placed in the apical compartment, 300 μ l were placed on the basolateral side, and cells were incubated for 6 h. Similarly, non-siRNA-treated inserts were prepared in the same manner but were incubated with 100 μ M ouabain for 24 h. Samples from apical and basolateral compartments were collected and pH was measured as described in MATERIALS AND METHODS. $pH = \text{apical pH} - \text{basolateral pH}$. Values are means $\pm$ SE ($n = 6$). ** $P < 0.001$ vs. control.