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Identification and cloning of the Na/HCO_3^- cotransporter (NBC) in human corneal endothelium

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Abstract

Fluid secretion by the corneal endothelium is associated with the net flux of HCO_3^- from basolateral (stromal) to apical (anterior chamber) sides of the tissue. In this study we asked if Na^+/HCO_3^- cotransporter (NBC-1) protein expression and functional activity are present in freshly isolated human corneal endothelium. Immunoblot analysis using a polyclonal antibody to NBC-1 showed a single band at ~130 kDa. Indirect immunofluorescence indicated that NBC-1 is expressed on the basolateral, but not apical side of human corneal endothelium. RT-PCR was used to determine whether the kidney or pancreatic isoform of NBC-1 is expressed. Using the specific primers for pNBC and kNBC isoforms, RT-PCR showed that only pNBC could be detected in human corneal endothelium. The product was cloned and confirmed by sequencing. Full-length NBC-1 was also cloned from human corneal endothelium. This clone (hcNBC) is 100% identical to the longer, more common form of NBC [pNBC; 1079 amino acids (aa); 122 kDa in human heart, pancreas and prostate]. To test for functional activity of NBC-1, freshly isolated endothelium was loaded with the pH sensitive fluorescent dye BCECF and HCO_3^- fluxes were measured. HCO_3^- fluxes were Na^+-dependent, electrogenic and H_2-DIDS sensitive. We conclude that the long isoform of the sodium bicarbonate cotransporter (pNBC-1) is expressed on the basolateral side of fresh human corneal endothelium (hcNBC). The shorter form, kNBC, could not be detected. As in bovine corneal endothelium, hcNBC is instrumental in loading HCO_3^- into endothelial cells from the basolateral membrane.

Keywords

corneal endothelium; NBC; bicarbonate transport; human

1. Introduction

Animal studies have shown that ion-coupled fluid transport by the corneal endothelium maintains corneal hydration and transparency. This process is dependent on the presence of HCO_3^- and is sensitive to carbonic anhydrase inhibitors (CAIs) (Fischbarg and Lim, 1974; Hodson, 1974; Hodson and Miller, 1976; Kuang et al., 1990; Riley et al., 1995). One in vitro study has also demonstrated this HCO_3^- dependency in human corneas (Wigham and

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Hodson, 1981). In humans, endothelial cell density and corresponding membrane Na^+–K^+ATPase density slowly decrease with age (Geroski et al., 1985). Nevertheless, because of a significant functional reserve, corneal hydration and transparency are maintained in the presence of the cell loss and are apparently insensitive to the clinical use of topical CAIs (Egan et al., 1998; Giasson et al., 2000). On the other hand, when endothelial cell loss is accelerated, (e.g. Fuchs' Endothelial Dystrophy, trauma, chronic inflammation), corneal hydration will increase and sensitivity to topical CAIs can be uncovered (Konowal et al., 1999). These findings indicate that human corneal endothelium is likely to possess similar HCO_3^- transport function as shown in animal studies.

In bovine corneal endothelium, the basolateral $Na^+/2HCO_3^-$ cotransporter (NBC-1) loads corneal endothelial cells with HCO_3^- from the stromal side (Sun et al., 2000). The robust HCO_3^- entry drives the formation of CO_2 in the cell catalyzed by carbonic anhydrase II. Three possible mechanisms for apical HCO_3^- efflux from endothelial cells to aqueous humor have been postulated. They are (1) Cl^-/HCO_3^- exchange (Bonanno et al., 1998), (2) CO_2 efflux and conversion to HCO_3^- by a membrane bound carbonic anhydrase (CAIV) (Bonanno et al., 1999), and (3) conductive flux via anion channels (Sun et al., 2000).

Currently, two isoforms of NBC-1, kidney (kNBC) (Burnham et al., 1998; Romero et al., 1998) and pancreas (pNBC) (Abuladze et al., 1998; Thevenod et al., 1999) have been cloned. The NH_2-terminal sequences of the two isoforms are different (pNBC, MEDE; kNBC, MSTE) and pNBC contains additional cAMP-dependent protein kinase A and protein kinase C phosphorylation consensus sites. In the proximal tubule, kNBC has a $1Na^+:3HCO_3^-$ stoichiometry, which favors HCO_3^- efflux from the cell. In the pancreatic duct, pNBC has a $1Na^+:2HCO_3^-$ stoichiometry, which favors HCO_3^- influx. However, the pNBC isoform can have a 1:3 stoichiometry when expressed in proximal tubule cells and kNBC can have a 1:2 stoichiometry depending on the cell type in which it is expressed (Gross et al., 2001a). Switching from 1:3 to 1:2 appears to be favored by phosphorylation at the COOH-terminus of NBC-1 (Gross et al., 2001b; Gross and Kurtz, 2002). The functional consequences, if any, of the differences at the NH_2-terminal end have not been elucidated. Our recent study using bovine corneal endothelium has demonstrated that pNBC is strongly expressed, and kNBC is undetectable (Sun et al., 2000). However, using RT-PCR, products for both pNBC and kNBC were found in cultured human corneal endothelium (Usui et al., 1999), which was consistent with immunofluorescence staining in fresh endothelium (Usui et al., 2001). On the other hand, using pNBC and kNBC specific antibodies, only pNBC was detected in corneal endothelium of the rat (Bok et al., 2001). In the current study, we demonstrate that fresh human corneal endothelium expresses only the pNBC isoform and that functional NBC-1 locates to the basolateral membrane.

2. Materials and methods

2.1. Cloning of human corneal endothelial NBC cDNA

2.1.1. PCR primers—For screening, gene specific primers for human pNBC and kNBC (AF027362, kNBC; and AF069510, pNBC) were used as previously described for bovine corneal endothelium (Sun et al., 2000). In addition, to clone the full length of NBC cDNA in

human corneal endothelium, additional pairs of specific primers for pNBC and kNBC were constructed according to the published cDNA sequence from GenBank. Since pNBC and kNBC vary only at the NH_2-terminus, we amplified between both amino terminal ends (MEDE_f = 5′-ATGGAGGATGAAGCTGTCCTGGACGAGGGG-3′ and MSTE_f = 5′-ATGTCCACTGAAAATGTGGAAGGGA-AGCCC-3′) and the common COOH-terminus (ERHT_r = 5′-TTATCAGCATGATGTGTGGCGTTC-3′). To assure fidelity we performed PCR using TaKaRa-ExTaq Polymerase (Panvera, Milwaukee, WI, USA).

2.1.2. Reverse transcription-polymerase chain reaction (RT-PCR)—Corneal endothelial total RNA was extracted from 10 fresh human corneas, which were provided by the Lions Eye Bank of Indiana, using TRIzol Reagent (Gibco BRL) according to the manufacturer's instruction. Time of RNA extraction after death varied from 2 to 5 days. To generate the first-strand cDNA, extracted total RNA (5 μg) was reverse-transcribed (total incubation mixture, 20 μl) at 42°C for 50 min in first strand buffer (50 mM Tris, 75 mM KCl, 3·0 mM $MgCl_2$, pH 8·4), containing 10 mM dithiothreitol, 0·5 mM of each dNTP, oligo-dT primer (3 μg μl^{-1}, 1·5 μl) (Gibco BRL) and superscript II reverse transcriptase (40 U μl^{-1}, Gibco BRL). First-strand cDNA was used in PCR amplification reactions (total incubation mixture, 25 μl) in a reaction buffer containing 20 mM Tris (pH 8·4), 50 mM KCl, 2·0 mM $MgCl_2$, TaKaRa Ex Taq™ polymerase 2·5 units and 0·2 mM of each dNTP, with the specific primer set described above. Final concentration of primers was 0·1 μM.

PCR amplifications were carried out in a thermocycler using the same conditions as previously described (Sun et al., 2000) for bovine NBC. For cloning the full-length cDNA, the following conditions were used: (1) 1 cycle of 2 min 94°C, 2 min 60°C, 2 min 68°C; (2) 30 cycles of 20 sec at 94°C, 20 sec at 60°C, 3 min at 68°C; then (3) final extension of 10 min 68°C. The PCR products were loaded onto a 1% agarose gel, electrophoresed and stained with 0·5 μg ml^{-1} ethidium bromide.

2.1.3. Subcloning and sequencing—Approximately 1 and 3·2 kb pNBC PCR products were purified by electrophoresis using a 1% low melting point agarose gel. Freshly purified products were mixed for 5 min with the PCR® 4-TOPO® vector (Invitrogen; San Diego, CA, USA). The TOPO® cloning reaction was added into a vial of One Shot® cells for plasmid transformation. The transformed bacteria were plated on agar culture media containing Ampicillin (50 μg ml^{-1}) and incubated at 37°C overnight. Inserts from selected clones were digested with EcoR1 (GIBCO BRL) and sizes were confirmed using 1% agarose minigel electrophoresis. The vectors with inserts of predicted size were isolated using a plasmid miniprep kit (Qiagen; Chatsworth, CA, USA). Sequencing was performed using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction mix (PE Applied Biosystems) according to the manufacturer's instructions. To sequence the full length of NBC cDNA, we used 6 walking primers. Sequencing electrophoresis was run on the ABI Prism 377 DNA sequencer in the Indiana University Molecular Biology Institute. Sequences were assembled and compared using Vector NTI ver5·2 software (InforMax, Inc.; North Bethesda, MD, USA).

2.2. Immunoblotting

Fresh human corneal endothelial cells were scraped from dissected human corneas that had been kept at 4°C for 2–5 days since death. The cell scrapings were placed into ice-cold PBS containing a protease inhibitor cocktail (Complete™, Boehringer Mannheim) and centrifuged at low speed for ~5 min. Cell pellets were re-suspended in 2% SDS sample buffer containing protease inhibitors. The preparations were sonicated (Branson 250) briefly on ice and then centrifuged at 10 000 g for 5–10 min. An aliquot of the supernatant was taken for protein assay using the Lowry method (Bio-Rad). Five percent β-mercaptoethanol and bromphenol blue were added to the remainder of supernatant. The samples (not heated) were applied to an 8% polyacrylamide gel with 4·5% stacking gel (60 μg/lane). After electrophoresis, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane overnight at 4°C. Membranes were incubated in PBS containing 5% nonfat dry milk for 1 hr at room temperature. The blots were then incubated with rabbit anti-NBC-1 polyclonal antibody (Schmitt et al., 1999) (kind gift from B.M. Schmitt and W.F. Boron, Yale University), (1:1000) in PBS containing 5% nonfat dry milk for 1 hr at room temperature with shaking. Next, the blots were washed five times for 5 min each with PBS/Tween-20, incubated with goat anti-rabbit secondary antibody coupled to horseradish peroxidase (Sigma) for 1 hr at room temperature, then washed with PBS/Tween-20 five times for 5 min each and developed by enhanced chemiluminescence (ECL). Films were scanned to produce digital images and were then assembled and labeled using Microsoft Powerpoint software. This antibody cannot distinguish between the k and p isoforms of NBC-1. Specificity of antibody binding to kidney and pancreas NBC-1 has been previously demonstrated (Marino et al., 1999; Schmitt et al., 1999; Thevenod et al., 1999).

2.3. Immunofluorescence

To prepare fresh endothelial cells for immunofluorescence staining, dissected human corneas were washed with PBS and immediately fixed with PLP fixation buffer (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate, 45 mM sodium phosphate, pH 7·4) for 10 min. Corneas were rinsed with PBS and endothelium/Descemet's strips were peeled off using jeweler's forceps and flattened onto Superfrost microscope slides (Fisher Scientific). Strips were fixed again at room temperature for 20 min and washed with PBS. Slides were then kept in PBS for 5 min containing 1% SDS to unmask epitopes and washed 3 times in PBS. Cells were blocked for 1 hr in PBS containing 0·2% bovine serum albumin (BSA), 5% goat serum, 0·01% saponin and 50 mM NH_4Cl. Rabbit polyclonal NBC-1 antibody diluted 1:100 in PBS/goat serum (1:1), was added onto cells and incubated for 1 hr at room temperature. Slides were washed 3 times for 15 min in PBS containing 0·01% saponin. Then secondary antibody conjugated to Oregon Green (Molecular Probes) (1:1000) was applied for 1 hr at room temperature. Slides were washed and mounted with Prolong anti-fade medium according to the manufacturer's (Molecular Probes) instructions. Fluorescence was observed with a standard epifluorescence microscope equipped with a cooled CCD camera.

2.4. $Na^+/2HCO_3^-$ cotransport physiology

2.4.1. Microscope perfusion—For measurement of pH_i using fluorescent dyes, fresh strips of human corneal endothelium were placed in a perfusion chamber that has been described previously in detail (Bonanno and Machen, 1989). The assembled chamber was placed on a water-jacketed (37°C) brass collar held on the stage of an inverted microscope (Nikon Diaphot) and viewed with a long working distance (1·2 mm) water immersion objective (Zeiss, 40 ×). The chamber was connected to hanging syringes containing Ringer solutions in a Plexiglas warming box (37°C) using Phar-Med tubing. The flow of the perfusate (~0·5 ml min^{-1}) was achieved by gravity. An independent eight-way valve was employed to select the desired perfusate for the chamber. The composition of the HCO_3^--rich Ringer's solution used throughout the study was (in mM): 150 Na^+; 4 K^+; 0·6 Mg^{2+}; 1·4 Ca^{2+}; 118·2 Cl^-; 1 HPO_4^-; 10 $HEPES^-$; 28·5 HCO_3^-; 2 $gluconate^-$; 5 glucose, equilibrated with 5% CO_2 and pH adjusted to 7·50 at 37°C. HCO_3^--free Ringer's (pH 7·5) was prepared by equimolar substitution of $NaHCO_3$ with Na-gluconate. Na^+-free HCO_3^--rich Ringer's was prepared by substitution of NaCl with 50 mM KCl, balance *N*-methyl-D-glucamine ($NMDG^+$) chloride and substitution of $NaHCO_3^-$ with $KHCO_3^-$. Osmolarity was adjusted to 300 ± 5 mosM with sucrose.

2.4.2. Measurement of pH_i—Dissected fresh human corneas were placed in HCO_3^--rich Ringer's. Descemet's endothelium was grasped with jeweler's forceps at the limbus and strips were peeled off. Large flat or curled strips (bigger than 3 × 5 mm^2) were loaded with the pH-sensitive fluorescent dye BCECF by incubation in HCO_3^--rich Ringer's solution containing 2 μM BCECF-AM at room temperature for 10–15 min. Dye-loaded strips were then kept in Ringer's for at least 30 min before use. Fluorescence excitation (495 and 440 nm) and data collection were obtained using a ratio fluorescence imaging system controlled by MetaFluor software (Universal Imaging, West Chester, PA, USA). Fluorescence ratios were obtained at 1 sec^{-1} and were calibrated against pH_i by the high K^+-nigericin technique (Bonanno and Giasson, 1992a).

3. Results

To confirm the presence of NBC in human corneal endothelium, we used immunoblotting with rabbit anti-NBC-1 antibody. Fig. 1 shows positive bands for fresh human corneal endothelium at ~130 kDa, which is the expected range for mammalian NBC (Romero et al., 1997; Abuladze et al., 1998; Burnham et al., 1998; Schmitt et al., 1999; Thevenod et al., 1999), and also consistent with our previous results from bovine corneal endothelium (Sun et al., 2000). Fig. 2 shows indirect immunofluorescence distribution of NBC in fresh human corneal endothelium using this same antibody. Prominent basolateral membrane staining is apparent in fresh human corneal endothelium, which is consistent with the staining observed in bovine corneal endothelium (Sun et al., 2000).

To determine which isoform(s) of NBC are expressed in human corneal endothelium, RT-PCR was performed using specific primers for kNBC and pNBC using fresh human corneal endothelium RNA. Fig. 3(A) shows that RT-PCR gave positive bands at ~1 kb for pNBC. However, we did not get positive bands for kNBC from fresh human corneal endothelium.

This result is consistent with our previous report in bovine corneal endothelium (Sun et al., 2000). The specificity of the primers had been confirmed previously using RNA from kidney cortex (Sun et al., 2000). Furthermore, Fig. 3(B) shows that only pNBC full-length cDNA was obtained, kNBC full-length cDNA was not found in human corneal endothelium.

Sequencing confirmed the identity of pNBC in human corneal endothelial cells. The first 1050 bases of pNBC published for other species were used for alignment with our result. Nucleotide sequence alignment showed 100% homology with hpNBC and 93% with bovine corneal endothelial NBC (bcNBC). Fig. 4 shows the amino acid alignment of our product sequence with hpNBC, rpNBC, bcNBC and rkNBC. Amino acid homology was 100% with hpNBC and 99% with bcNBC for the first 350 amino acids. The full-length cDNA sequence is available in GenBank accession #AF310248.

Functional evidence for an active NBC in human corneal endothelium is shown in Fig. 5. Dissected strips of Descemet's/Endothelium were perfused initially in a HCO_3^--free Ringer's. Upon exposure to HCO_3^--rich Ringer's, pH_i increases significantly over background indicating significant HCO_3^- influx. The small initial acidification due to rapid CO_2 entry that is usually observed in bovine endothelium could not be elicited in the human tissue. This may be due to more robust HCO_3^- influx in the human cells. On removal of HCO_3^- Ringer's there is an increase in pH_i due to CO_2 efflux and a slow return to baseline pH_i. When this sequence was repeated in the presence of H_2DIDS, an inhibitor of $Na^+/2HCO_3^-$ cotransport, the HCO_3^- fluxes were slowed by 50% after 10 min (n = 4). Since $Na^+/2HCO_3^-$ cotransport is electrogenic, depolarizing the membrane potential using the K^+ channel blocker Ba^{2+} alkalinizes cells secondary to HCO_3^- influx (Bonanno and Giasson, 1992b). Fig. 6 shows the effects of 5 mM Ba^{2+} on human corneal endothelial pH_i. In the presence of HCO_3^-, application of a 1 min pulse of Ba^{2+} caused an immediate increase in pH_i (initial dpH_i/dt = 0·004 ± 0·0012 pH sec^{-1}, n = 3). HCO_3^- was removed and another 1 min pulse of Ba^{2+} was applied that caused a 78% slower increase in pH_i (initial dpH_i/dt = 0·0009 ± 0·0001 pH sec^{-1}, n = 3). Cells were then perfused again with HCO_3^-. Application of 250 μM H_2DIDS caused pH_i to drop by ~0·3 pH units, consistent with inhibition of active $Na^+/2HCO_3^-$ cotransport. In the presence of H_2DIDS, the pH_i increase caused by Ba^{2+} was slowed by 68% (initial dpH_i/dt = 0·0013 ± 0·0008 pH sec^{-1}, n = 3) relative to control. Lastly, in Fig. 7, dissected strips of Descemet's/Endothelium perfused in HCO_3^--Rich Ringer's were exposed to 10 mmol l^{-1} $(NH_4)_2SO_4$ for three minutes. This induces an alkalinization because of entry of NH_3, followed by a slower acidification resulting from the entry of NH_4^+ and other cellular acidifying forces. When the $(NH_4)_2SO_4$ was removed, cells acidify due to rapid efflux of NH_3 leading to the dissociation of the NH_4^+ that remains in the cell. This was followed by perfusion in Na^+ free Ringer's, which prevents recovery of pH_i. Cells were then exposed to 10 μM EIPA (blocks Na^+/H^+ exchange) and 250 μM H_2DIDS (blocks Na^+:HCO_3^- cotransport). Na^+ was then re-introduced and a partial acid recovery (~40%) ensued. Cells were then washed to remove H_2DIDS and the same protocol was repeated, but in the absence of H_2DIDS. Under these conditions, acid recovery was >90%,

indicating that a significant portion of recovery was H_2DIDS sensitive. Similar results were obtained in three other experiments.

4. Discussion

Functional studies using rabbit (Fischbarg and Lim, 1974; Hodson and Miller, 1976; Riley et al., 1995 and human corneas (Wigham, 1981 #1796) have shown that HCO_3^--dependent transport is essential for the maintenance of corneal hydration. Furthermore, HCO_3^- plays a key role in regulation of endothelial cell intracellular pH (Bonanno and Giasson, 1992b). Among several transporters, such as the Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger, the NA/HCO_3^- cotransporter has been suggested to play a key role in corneal endothelial HCO_3^- transport (Sun et al., 2000). Previously it has been reported that cultured human corneal endothelium expresses functional NBC-1 (Usui et al., 1999). Here we provide molecular and functional evidence for expression of NBC-1 in fresh human corneal endothelium, as well.

4.1. Molecular evidence for expression of NBC-1

RT-PCR using specific primers for pNBC and kNBC indicate that pNBC is expressed in fresh human corneal endothelium, and kNBC is undetectable. In cultured human corneal endothelium however, RT-PCR analysis suggested that both isoforms of NBC-1 are expressed (Usui et al., 1999). The RT-PCR result using the cultured cells showed a very strong pNBC product and a relatively weak kNBC product. Using the same primers from that study (Usui et al., 1999), we have been unable to obtain a kNBC product from either bovine or human corneal endothelium. On the other hand, the kNBC screening primers used in the current study yield a strong kNBC product from bovine kidney that was verified by sequencing, (Sun et al., 2000). Since the sequence homology between bovine and human in the region spanned by these primers is 100%, this finding indicates that kNBC would easily be detected, if present. Moreover, the cloning and sequencing of full-length NBC-1 cDNA from fresh human corneal endothelium confirmed the expression of pNBC and failed to show a kNBC product.

Immunoblot analysis using polyclonal NBC antibody showed strong expression of NBC-1 in fresh human corneal endothelium (see Fig. 1) and immunofluorescence staining showed a basolateral location (see Fig. 2). However, this antibody cannot distinguish between kNBC and pNBC. Two groups (Bok et al., 2001; Usui et al., 2001) have developed k and pNBC specific antibodies. Immunoblotting and immunohistochemistry using rat cornea indicated that only pNBC is expressed (Bok et al., 2001) and that it is localized to the basolateral membrane of corneal endothelium. On the other hand, immunohistochemistry of rat and human cornea by the other group (Usui et al., 2001) suggested that both pNBC and kNBC are expressed in endothelium, however immunoblots from cornea were not reported. Further, there have not been any other reports suggesting that k and pNBC are co-expressed in the same cell type. In sum, the immunofluorescence studies consistently show basolateral staining for NBC-1 in corneal endothelium, however there are conflicting results in regard to the specificity of the isoform specific antibodies.

Expression of kNBC in human corneal endothelium may be either very weak or variable depending on the age of eyes or conditions when the eyes were processed. Expression of pNBC in corneal endothelium however is more consistent. Although it had been thought for some time that kNBC had a 1:3 stoichiometry and pNBC had a 1:2 stoichiometry, recent studies indicate that the functional stoichiometry is not dependent on the isoform, but on the cell type within which NBC-1 is expressed (Heyer et al., 1999; Gross et al., 2001a). There are differences in PKA and PKC regulatory sites, however there has not been a clear demonstration of PKA or PKC sensitivity. While expression of only pNBC in corneal endothelium may not affect the transport stoichiometry, it may have a yet undiscovered role in regulation of NBC function.

4.2. Functional evidence for $Na^+/2HCO_3^-$ cotransport

Here we show for the first time evidence consistent with functional Na^+-dependent, DIDS sensitive, electrogenic $Na^+/2HCO_3^-$ cotransport activity in fresh human corneal endothelium. First, HCO_3^- entry into fresh human corneal endothelial cells was significantly slowed by H_2DIDS. Second, membrane potential depolarization with Ba^{2+} induced an alkalinization that was ~4 times faster in the presence of HCO_3^-. Since buffering capacity (β) is approximately 6 times greater in the presence of HCO_3^- than in its absence (Bonanno and Giasson, 1992a), the actual HCO_3^- flux [(dpH/d*t*) × β] is approximately 24 times greater than the equivalent proton flux that occurred in the absence of HCO_3^-. Third, application of H_2DIDS caused a substantial acidification in human cells similar to that found in fresh and cultured bovine endothelium (Bonanno and Giasson, 1992b). This indicates that Na^+/HCO_3^- cotransport is active in resting cells. Fourth, the barium-induced alkalinization was significantly inhibited by H_2DIDS. Interestingly, there is a significant amount of barium-induced alkalinization that is H_2DIDS insensitive. This may be due to HCO_3^- flux through conductive channels (Bonanno and Srinivas, 1997). Lastly, stimulating $Na^+/2HCO_3^-$ cotransport by acidification showed that recovery from the acid load is Na-dependent and H_2DIDS sensitive. These results are consistent with the well-known properties of electrogenic $Na^+-HCO_3^-$ cotransport (Romero and Boron, 1999) and are similar to results using cultured human corneal endothelium (Usui et al., 1999). Our results are also consistent with the view that the $Na^+-HCO_3^-$ cotransporter mediates HCO_3^- influx in human corneal endothelium as proposed previously for bovine cornea (Sun et al., 2000). Since transendothelial HCO_3^- flux is from basolateral to apical, a basolateral NBC should facilitate HCO_3^- influx. Further, pH_i is significantly higher in HCO_3^- solutions than in the absence of HCO_3^- indicating that there is net HCO_3^- uptake in relation to the production of carbonic acid from CO_2. Lastly, the finding that application of H_2DIDS reduced pH_i, while cells were perfused in standard HCO_3^- Ringer's, indicates that Na^+/HCO_3^- flux is inward.

In summary, fresh human corneal endothelial cells express the Na^+/HCO_3^- cotransporter (NBC-1). RT-PCR analysis and full-length cloning indicate that only the long form of NBC-1 (pNBC) is expressed. HCO_3^- flux is Na-dependent, electrogenic and H_2DIDS

sensitive. Human corneal endothelial $Na^+/2HCO_3^-$ cotransport is located on the basolateral membrane consistent with its role in HCO_3^- uptake.

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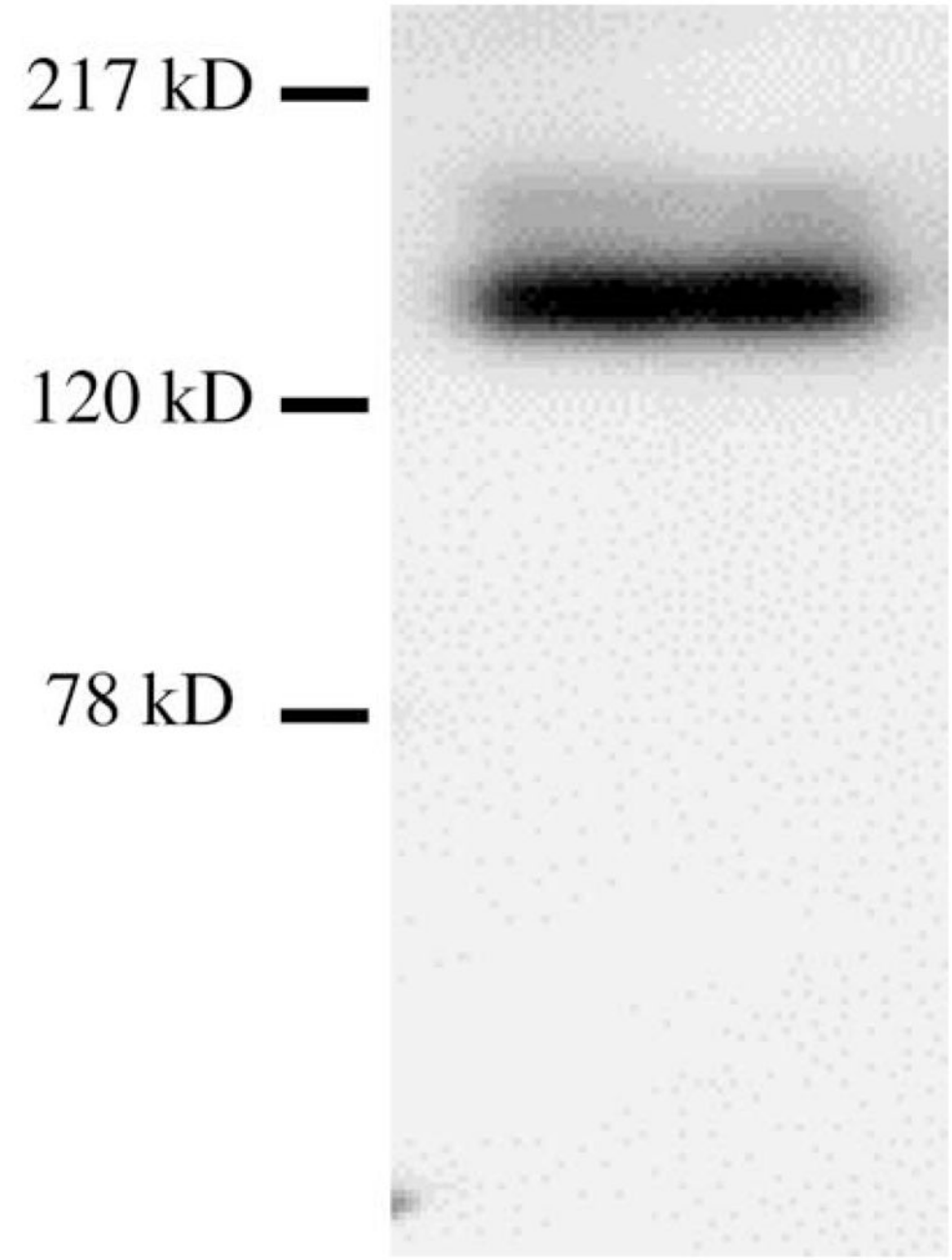

Fig. 1.

Western blot for NBC-1. Total cellular protein was extracted from human corneal endothelial cells. 60 μg of protein was subjected to SDS-PAGE and transferred to PVDF membranes overnight and stained by ECL using rabbit polyclonal anti-NBC-1. The major positive band is at ~130 kDa.

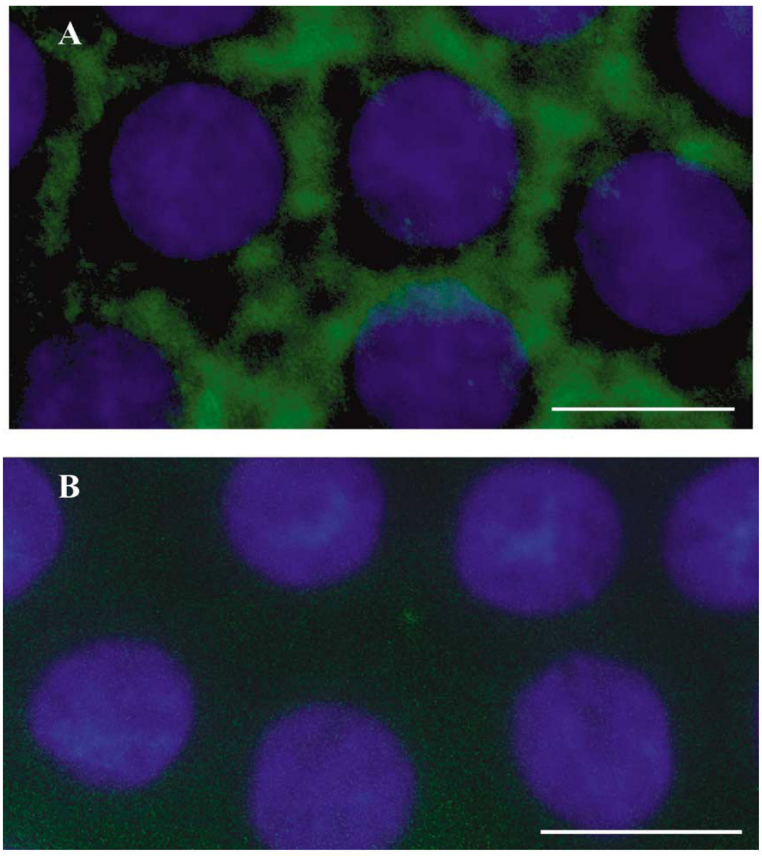

Fig. 2.

Immunofluorescence localization of NBC. Dissected strips of human Descemet's membrane/endothelium were fixed and flattened onto glass slides. Slides were processed for immunofluorescence using the rabbit polyclonal anti-NBC-1 antibody and nuclei stained using DAPI. Bar = 25 μm.

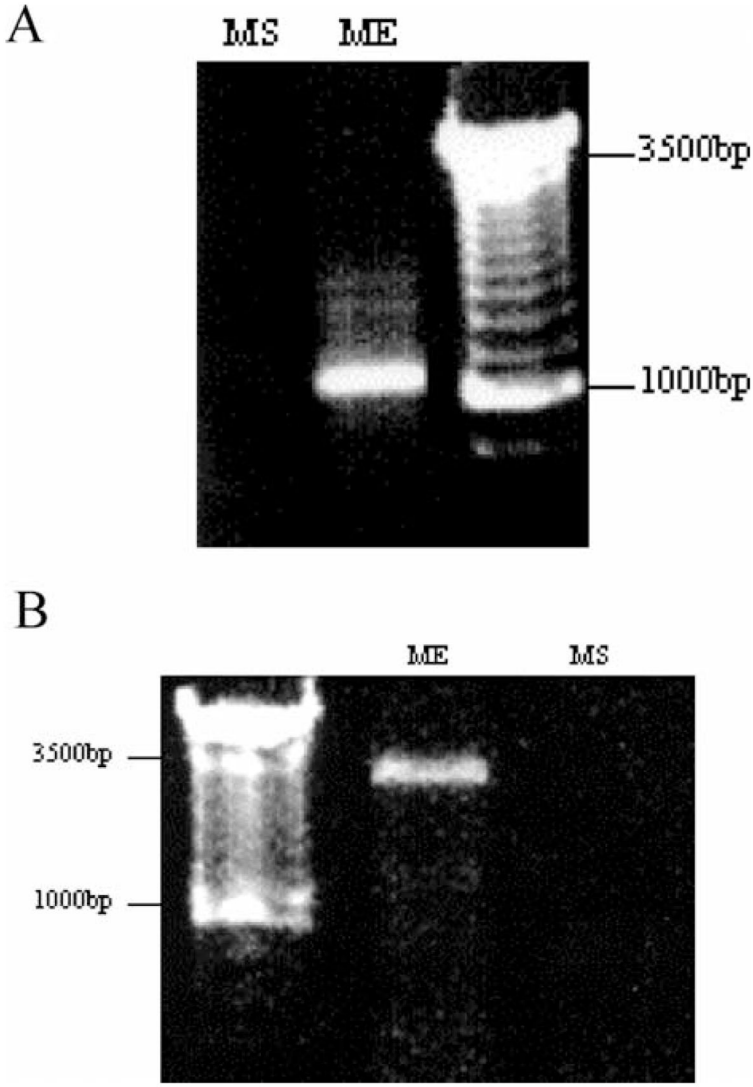

Fig. 3.

(A) RT-PCR screening for NBC-1 isoforms, kNBC and pNBC. The predicted product size for kNBC (MS) and pNBC (ME) was 0·9 and 1 kb, respectively. (B) Full length NBC cDNA. PCR amplifications using primers from amino terminal end of kNBC (MS) or pNBC (ME) to common COOH-terminus. Only pNBC product was obtained.

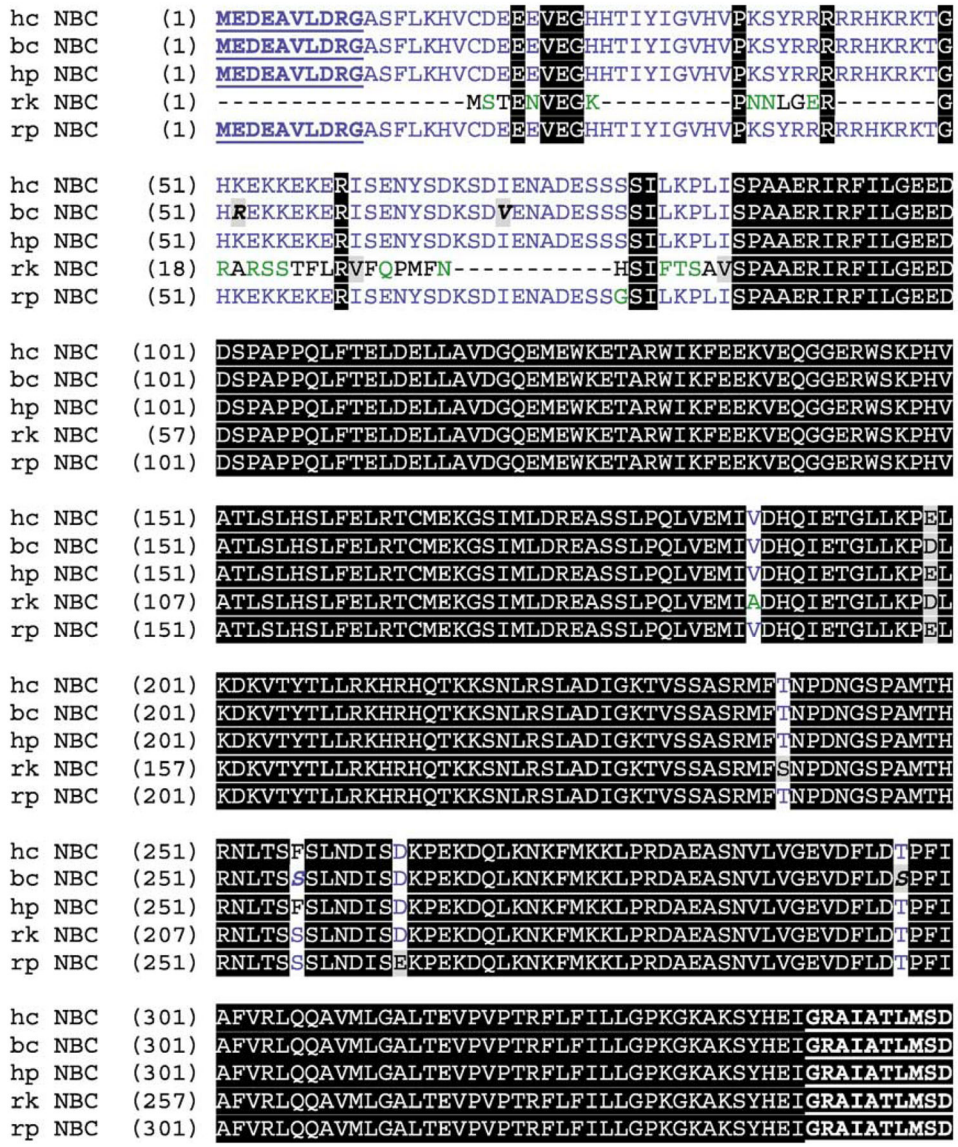

Fig. 4.

Amino acid alignment of human corneal endothelial NBC (hcNBC) with hpNBC, rpNBC and rkNBC for the first 350 amino acids. Underlined/bold amino acids are promer sequences used for RT-PCR. Solid background indicates complete homology. Shaded italics indicate no homology with the other NBCs. The full length cDNA sequence is available from GenBank accession # AF310248.

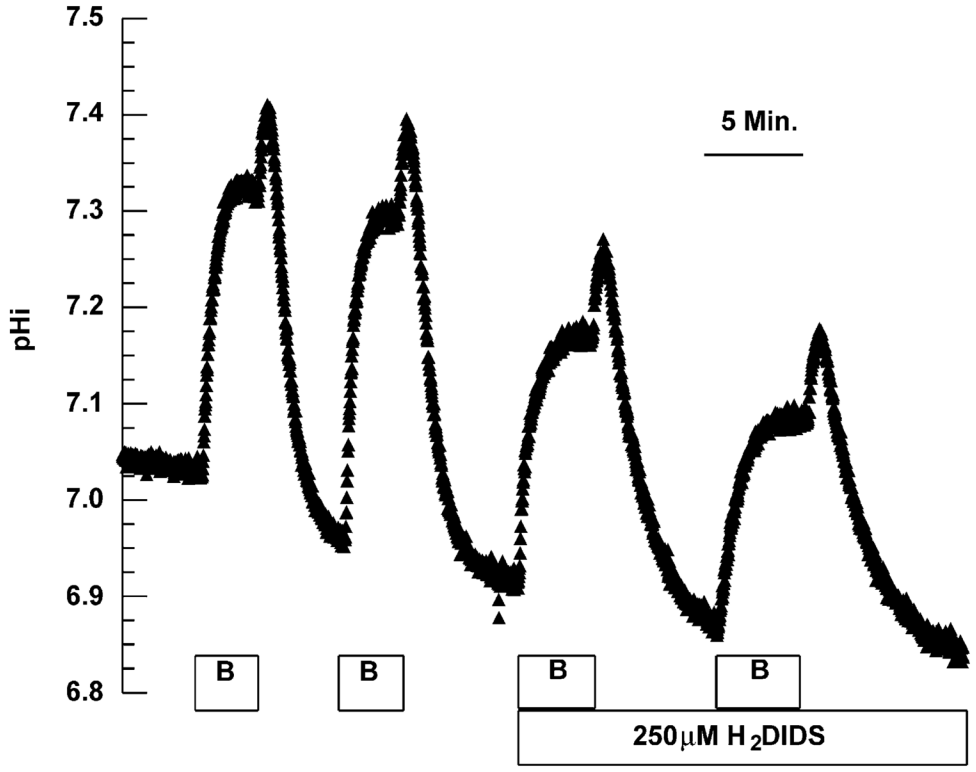

Fig. 5.

H_2DIDS sensitive HCO_3^- flux. Strips of Descemet's/Endothelium was dissected from human corneas, cells loaded with the pH sensitive fluorescent dye BCECF and placed into a microscope perfusion chamber. Cells were perfused in bicarbonate-free ringer. Boxes indicate when cells were exposed to experimental solutions (B, CO_2/HCO_3^- ringer).

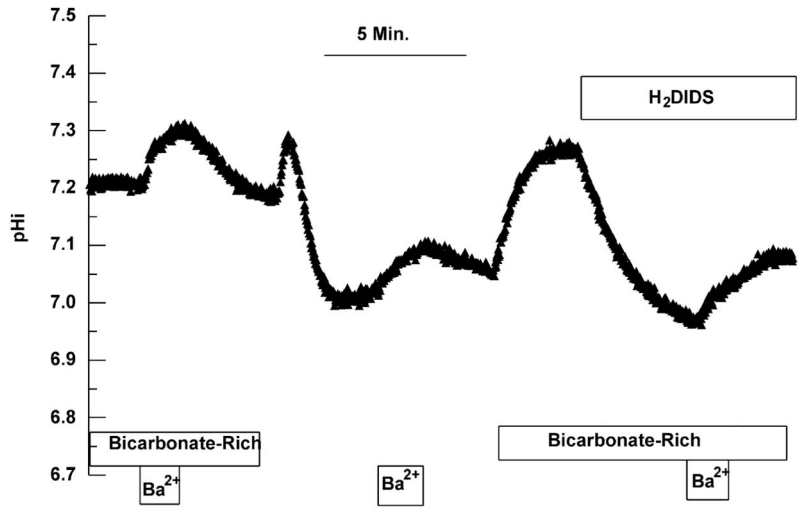

Fig. 6.

Depolarization induced HCO_3^- flux. Cells were depolarized by exposure to 5 mM Ba^{2+} where indicated, in the presence or absence of HCO_3^- and 250 μM H_2DIDS.

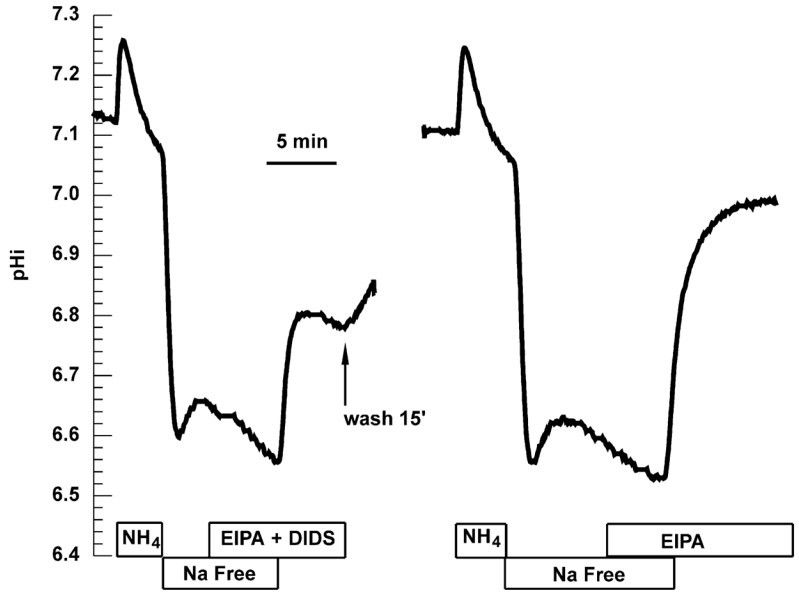

Fig. 7.

Na-dependent, H_2DIDS-sensitive acid recovery. Cells were acidified in HCO_3^--Rich ringer by ammonium sulfate prepulse and perfused in the absence of Na^+ to prevent acid recovery. Cells were then pre-exposed to 10 μM EIPA (to block Na^+/H^+ exchange) and 250 μM H_2DIDS. Then in the continued presence of drugs, Na^+ was re-introduced. This sequence was then repeated, but in the absence of H_2DIDS.