Enhancement of $\text{HCO}_3^-$ Permeability across the Apical Membrane of Bovine Corneal Endothelium by Multiple Signaling Pathways

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**PURPOSE.** In this study, the involvement of signaling pathways in the regulation of $\text{HCO}_3^-$ permeability across the apical membrane of the bovine corneal endothelium was examined.

**METHODS.** Cultured bovine corneal endothelial cells (CBCECs) were grown to confluence on permeable membranes. Apical and basolateral sides were perfused with a $\text{HCO}_3^-$-rich Cl⁻-free Ringer’s solution (28.5 mM; pH 7.5). Relative changes in apical $\text{HCO}_3^-$ permeability were assayed by pulsing the apical perfusion bath with a low-$\text{HCO}_3^-$ Cl⁻-free Ringer’s solution (2.85 mM; pH 6.5), in the presence or absence of agonists or inhibitors, and comparing the rates of change in intracellular pH (pHi), as measured with a pH-sensitive dye. Ca²⁺-activated signaling was measured with the Ca²⁺-sensitive dye Fura-2. Qualitative changes in membrane potential ($E_m$) were measured with a voltage-sensitive dye. RT-PCR using calcium-activated chloride channel (CLCA)–specific primers was used to examine the expression of CLCA in the corneal endothelium.

**RESULTS.** The adenoceptor agonist adenosine (20 μM) enhanced $\text{HCO}_3^-$ permeability by a factor of 2. Forskolin (40 μM) exerted a 6.3-fold increase of $\text{HCO}_3^-$ permeability, which was inhibited by the Cl⁻ channel blockers, glibenclamide (50 μM) and niflumic acid (100 μM). Adenosine triphosphate (ATP) and ATPγS, P2 receptor agonists that increased intracellular Ca²⁺ in corneal endothelium, enhanced $\text{HCO}_3^-$ permeability by 87% and 79%, respectively. ATPγS induced depolarization of the $E_m$, consistent with anion channel activation, rather than activation of Ca²⁺-dependent K⁺ channels, which could secondarily increase extrusion of anions by $E_m$ hyperpolarization. Cyclopiazonic acid (CPA), an endoplasmic reticulum (ER) Ca²⁺-pump inhibitor that increased [Ca²⁺], also enhanced $\text{HCO}_3^-$ permeability by 95%. Both the calmodulin kinase II (CaMKII) inhibitor KN-62 and the PKC inhibitor bisindolylmaleimide I (BIM), decreased $\text{HCO}_3^-$ permeability induced by ATPγS. The PKC activator PMA also increased $\text{HCO}_3^-$ permeability by a factor of 1.8. RT-PCR using CLCA-specific primers showed the expression of CLCA1 in both fresh and cultured BGEs.

**CONCLUSIONS.** Activation of adenoceptors and purinoceptors enhances $\text{HCO}_3^-$ permeability across the apical membrane of the cultured corneal endothelium. Multiple signaling pathways (PKA, PKC, and Ca²⁺/CaMKII) contribute to the $\text{HCO}_3^-$ transport in cultured corneal endothelium. Both cAMP and Ca²⁺-activated Cl⁻ channels (possibly CLCA) may be involved in $\text{HCO}_3^-$ transport. (Invest Ophthalmol Vis Sci. 2002;43:1146–1153)

The corneal endothelium is a thin monolayer of cells covering the posterior surface of the cornea. Its primary function is to maintain corneal transparency through active transport of ions and fluid. The glycosaminoglycans of the corneal stroma exert a net swelling pressure that offers a constant potential fluid inhibition by the stroma. This fluid influx is counterbalanced by the endothelium with an ion-coupled fluid transport mechanism directed from stroma to aqueous humor. Numerous studies have shown that endothelial fluid transport is dependent on the presence of $\text{HCO}_3^-$.

Studies in the past two decades have revealed at least four mechanisms that support $\text{HCO}_3^-$ transport: (1) a potent Na⁺-dependent, 4,4'-disothiocyano stilbene-2,2'-disulfonic acid (DIDS)-sensitive, electrogenic Na⁺-HCO3⁻ cotransporter (NBC) on the basolateral membrane; (2) a Cl⁻-$\text{HCO}_3^-$ exchanger; (3) anion/Cl⁻ channels on the apical membrane; and (4) cytosolic and membrane-bound carbonic anhydrases (CAs). All these mechanisms have been demonstrated to exist in both fresh and cultured bovine corneal endothelial cells, although the Cl⁻-$\text{HCO}_3^-$ exchange activity is weak in cultured corneal endothelium. It is well established that $\text{HCO}_3^-$ enters the cell through the basolateral NBC. We have previously shown that basolateral $\text{HCO}_3^-$ permeability is significantly higher than apical. Thus, the rate-limiting step in the transendothelial $\text{HCO}_3^-$ transport is at the apical membrane. We speculate that, most probably, apical $\text{HCO}_3^-$ permeability is controlled by Cl⁻ channels and/or CAs.

We have recently shown that the cytisic fibrosis transmembrane conductance regulator (CFTR) is expressed in fresh and cultured BGEs, which is activated by cAMP, has been shown to have substantial permeability to $\text{HCO}_3^-$, which may be involved in some of the pathogenic aspects of CF. Further, recent studies have identified some CF-causing mutants that demonstrate normal Cl⁻ channel activity but impaired $\text{HCO}_3^-$ transport. Thus, it is possible that CFTR has a significant role in $\text{HCO}_3^-$ transport in corneal endothelium. It is well known that adenosine, which increases cAMP through the A₂ receptor, can increase the ion and fluid transport across the corneal endothelium. Our previous studies have also shown that Cl⁻ and $\text{HCO}_3^-$ permeabilities are enhanced by cAMP in the cultured corneal endothelium. Thus, we hypothesize that adenosine can also enhance $\text{HCO}_3^-$ permeability across the apical membrane of BGEs by activating the cAMP-PKA signaling pathway.

Agonists of P₂ purinergic receptors have been shown to mobilize Ca²⁺ in corneal endothelial cells. $\text{HCO}_3^-$ has also been demonstrated to permeate a Ca²⁺-dependent anion channel in gallbladder. Therefore, we also investigated whether $\text{HCO}_3^-$ permeability across the apical membrane can be activated by a Ca²⁺-signaling mechanism. One possible candidate for $\text{HCO}_3^-$ permeability is the calcium-activated chloride chan-
nel (CLCA)13–15 RT-PCR was used to determine the expression of CLCA at the mRNA level. Furthermore, activation of P2x receptors can also generate diacylglycerol (DAG), which will activate PKC. Last, because constitutive phosphorylation by cellular PKC is a prerequisite for the activation of CFTR by PKA,16,17 we tested to determine whether PKC is involved in HCO3− permeability across the apical membrane.

**Materials and Methods**

**Cell Culture**

Bovine corneal endothelial cells (BCECs) were cultured as described.6–18 Briefly, primary cultures from fresh bovine eyes were established in T-25 flasks in 3 mL DMEM, 10% bovine calf serum with antibiotic-antimycotic agents (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B), gassed with 5% CO2, and fed every 2 to 3 days. These cells were subcultured to three T-25 flasks and grown to confluence in 5 to 7 days. The resultant second-passage cultures were subcultured onto 13-mm permeable filters, reaching confluence within 7 days. Cells were transferred to 0.5% serum-DMEM for at least 24 hours before the experiments began.

**Solutions and Chemicals**

The composition of the HCO3−-rich (bicarbonate-rich; BR) Ringer’s solution was (in millimolar) 150 Na+, 4 K+, 0.6 Mg2+, 1.4 Ca2+, 118 Cl−, 1 HPO4−2−, 10 HEPES, 28.5 HCO3−, 2 glucose−, and 5 glucose. Ringer’s solutions were equilibrated with 5% CO2 and pH was adjusted to 7.50 at 37°C. Low-HCO3− (low bicarbonate; LB) Ringer’s solution contained 5 mOsm with sucrose. 2−,7−Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), bis-oxonol (DiBac4), and Fluo-3 were obtained at 1 second were calibrated against pH i by the high-K+ jacketed (37°C) brass collar held on the stage of an inverted microscope (Diaphot; Nikon, Melville, NY). The apical and basolateral compartments were connected to separate sections of tubing (PharMED; Fisher Scientific), which, in turn, were connected to syringes containing Ringer’s in a Flexiglas warming box (37°C). Both HCO3−-rich and low-HCO3− Ringer’s solutions were continually bubbled with 5% CO2. 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.

**Measurements of Intracellular pH**

Intracellular pH (pHi) was measured with the pH-sensitive fluorescent dye BCECF as previously described.19 Fluorescence ratios (F495/F440) obtained at 1 second were calibrated against pH i by the high-K+−nigericin technique. Initial rates of intracellular pH over time (dpHi/dt; i.e., maximum slope) were measured for 20 seconds after initial responses or pH decreases.

**Measurements of Intracellular Ca2+**

Intracellular Ca2+ ([Ca2+]i) was measured with Fura-2, a calcium-sensitive fluorescent dye. The cells were loaded by incubation in Ringer’s solution containing 5 µM Fura-2-AM for 30 minutes at room temperature, followed by a wash for 45 minutes. Ca2+ measurements were performed at room temperature with a fluorescence imaging system (MetaFluor; Universal Imaging Co., West Chester, PA). Fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm.

**Measurements of Membrane Potential**

Bis-oxonol, a voltage-sensitive fluorescent dye, was used to measure relative changes in membrane potential (EM). Depolarization partitions the dye into the plasma membrane, making it more fluorescent. Hyperpolarization releases the dye from the membrane, resulting in a decrease of fluorescence. For continuous EM measurements, bis-oxonol was included in the perfusing Ringer’s solutions at a concentration of 200 nM. The bis-oxonol fluorescence was excited at 495 ± 10 nm and the emission collected through a barrier filter centered at 520 ± 20 nm.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was extracted from cultured and fresh bovine corneal endothelial using extraction reagent (TRIzol; Gibco BRL), according to the manufacturer’s instructions. Total RNA extracted from fresh bovine corneal epithelium served as a positive control. To generate the first-strand cDNA, extracted total RNA (0.5-5 µg) was reverse-transcribed (total incubation mixture, 20 µL) at 42°C for 50 minutes in first-strand buffer (50 mM Tris, 75 mM KCl, and 3.0 mM MgCl2 [pH 8.3]) that contained 10 mM dithiothreitol, 0.5 mM of each dNTP, oligo dT (3 µg/µL, 1.5 µL; Gibco BRL), and reverse transcriptase (40 U/µL, SuperScript II RT; Gibco BRL). First-strand cDNA was used in PCR amplification reactions (total incubation mixture, 25 µL) in a reaction buffer that contained 20 mM Tris (pH 8.4), 50 mM KCl, 2.0 mM MgCl2, 2.5 units polymerase (Ex Taq; TaKaRa Shuzo, Kyoto, Japan), and 0.2 mM of each dNTP, with the specific primer set. Bovine CLCA1- and CLCA2-specific primers (TC-1 and -3 and Lu-1 and -3, respectively) were synthesized according to Elble et al.20 The final concentration of primers was 0.1 µM. Ultrapure water (Nanopure Filtration Systems; Barnstead International, Dubuque, IA) water substituted for first-strand cDNA served as the negative control.

PCR amplifications were performed in a thermocycler under the following conditions: denaturation at 94°C for 3 minutes for one cycle, 30 cycles of denaturation at 94°C for 1 minute each, annealing at 61°C for 1 minute, extension at 72°C for 2 minutes, and a final extension for one cycle at 72°C for 15 minutes. The PCR products were loaded onto 1% agarose gel, electrophoresed, and stained with 0.5 µg/mL ethidium bromide.

**Subcloning and Sequencing**

The PCR product was purified with a gel extraction kit (Valencia, Chatsworth, CA). Freshly purified products were mixed for 5 minutes with a vector (pcDNA3, L/V5-His TOPO; Invitrogen, San Diego, CA). The cloning reaction was added into a vial of cells (One Shot; Invitrogen) for plasmid transformation. The transformed bacteria were plated on agar culture media that contained ampicillin (50 µg/mL) and incubated at 37°C overnight. The vectors with predicted inserts were isolated using a plasmid miniprep kit (Qiagen, Valencia, CA). Sequencing was performed using a dye terminator cycle-sequencing, ready-reaction mix (Prism BigDye kit; PE- Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Sequencing electrophoresis was run on a DNA sequencer (Prism 377; PE-Applied Biosystems) at the Indiana University Molecular Biology Institute. Sequences were assembled and compared on computer (Vector NTI version 5.2 software; InforMax, Bethesda, MD).
Statistics

Initial slopes of the descending pH responses for the first 20 seconds during LB pulses were calculated and served as a relative measure of HCO$_3^-$ permeability across the apical membrane. Quantitation of the pH changes is expressed as the mean ± SE. Paired t-test was used for statistical analysis and $P < 0.05$ was considered significant.

Results

Regulation of HCO$_3^-$ Permeability across the Apical Membrane by the cAMP-PKA Pathway

In this study, we intended to characterize the signaling pathways in HCO$_3^-$ transport across the apical side of cultured bovine corneal endothelial cells (CBCECs). We have shown that adenosine and forskolin enhance chloride and HCO$_3^-$ permeability in CBCECs cultured on coverslips. Because chloride ions can compete with HCO$_3^-$ to permeate anion channels, we used chloride-free Ringer’s solution in all experiments to measure HCO$_3^-$ permeability. Furthermore, the use of Cl$^-$-free (gluconate-substituted) solutions eliminates any possible involvement of anion exchanger activity in our HCO$_3^-$ permeability measurements. To measure relative changes in apical HCO$_3^-$ permeability, we used a constant CO$_2$ protocol. Constant [CO$_2$] on both sides removes any effect on pH due to rapidly diffusing CO$_2$. Permeable filter-transported cultures perfused in a double-sided chamber were exposed to a BR Cl$^-$-free solution (5% CO$_2$, 28.5 mM HCO$_3^-$ [pH 7.5]) on the basolateral and apical sides at 37°C. Apical perfusion was then quickly changed to an LB Cl$^-$-free solution (5% CO$_2$, 2.85 mM HCO$_3^-$ [pH 6.5]).

Because the CO$_2$ concentration is the same on both sides, pH$_i$ was affected by HCO$_3^-$ efflux and H$^+$ influx across the apical membrane. It has been shown that only 18% of the initial dpH$_i$/dt is due to H$^+$ fluxes. Considering that the amplitude of pH changes during LB pulses may be influenced by basolateral transporters such as NBC, we used only the initial rate of pH$_i$ change 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. When BR was replaced by LB on the apical side, pH$_i$ decreased significantly (Fig. 1A).

After decreasing, pH$_i$ stabilized or recovered slightly to a level that was still much lower than the baseline pH$_i$ before readdition of BR. Readdition of BR caused a small decline in pH$_i$ before it increased to baseline. These pH$_i$ changes during LB pulses are consistent with our previous study. Two sequential LB pulses in the absence of test drugs appeared to have the same initial dpH$_i$/dt ($P = 0.335$, $n = 5$, not shown). In this study, DMSO was often used to solubilize test drugs, and so we tested the effects of DMSO on HCO$_3^-$ permeability. After a control pulse, the second pulse was performed in the presence of 0.5% (vol/vol) DMSO in perfusion solutions. The initial dpH$_i$/dt for the two LB pulses were not significantly different (data not shown, $P = 0.614$, $n = 7$), indicating that DMSO did not affect HCO$_3^-$ permeability.

When 20 μM adenosine was applied in the BR solution, baseline pH$_i$ decreased 0.035 ± 0.008 pH units ($n = 7$; Fig. 1A). Furthermore, in the presence of adenosine, the LB pulse caused an increased rate of pH$_i$ change by a factor of 1.98 ± 0.20, compared with the control LB pulse ($n = 7$, $P < 0.005$, Fig. 1B). These results suggest that apical HCO$_3^-$ permeability can be enhanced by a cAMP-mediated process.

We further examined whether forskolin, a potent activator of adenylyl cyclase, could increase apical HCO$_3^-$ permeability. As shown in Figure 1C, after application of 40 μM forskolin, an immediate sharp decrease in pH$_i$ (0.097 ± 0.013, $n = 6$) was observed, which gradually reached a new steady state level below baseline (0.097 ± 0.013, $n = 6$). After pH$_i$ became stable, a second LB pulse was made in the continued presence of 40 μM forskolin. The rate of pH$_i$ decline was 6.3 ± 2.3 times faster than in the control ($P < 0.005$, $n = 6$, Fig. 1D), indicating that increasing cAMP leads to a significant increase in apical HCO$_3^-$ permeability.

To test whether HCO$_3^-$ is transported through cAMP-activated chloride channels, we used the chloride channel blockers niflumic acid and glibenclamide in the presence of forskolin stimulation. After application of 100 μM niflumic acid or 50 μM glibenclamide, pH$_i$ increased (Figs. 2A, 2C; 0.094 ± 0.008, $n = 4$ and 0.067 ± 0.016, $n = 5$, respectively). Niflumic acid and glibenclamide significantly inhibited forskolin-activated dpH$_i$/dt by 94.8% ± 1.4% and 51% ± 10% ($P < 0.005$, $n = 4$; $P < 0.01$, $n = 5$, respectively; Figs. 2B, 2D). After removing...
ni flumic acid or glibenclamide, the effects of the inhibition were totally reversed by 10 to 15 minutes of washout (Figs. 2A, 2C). The inhibitory effects of chloride channel blockers are consistent with the presence of HCO$_3^-$/H$_2$CO$_3$-permeable anion channels in BCECs.

**Regulation of HCO$_3^-$ Permeability by PKA, PKC, and Ca$^{2+}$**

We attempted to examine whether Ca$^{2+}$ signaling is involved in HCO$_3^-$ permeability on the apical membrane in the corneal endothelium by stimulating purinergic receptors, because this has been shown to increase [Ca$^{2+}$], in cultured CBCECs. ATP (100 μM) induced [Ca$^{2+}$] responses (not shown) similar to those reported previously with BCECs cultured on cover-slips. The response includes a peak and a plateau elevation, which are thought to be primarily composed of Ca$^{2+}$ mobilization and capacitative calcium entry, respectively. ATP (100 μM) also decreased baseline pH gradually (0.04 ± 0.019, $n = 6$; Fig. 3A). The second LB pulse in the presence of ATP induced an 87% ± 27% increase in dpH/dt (Figs. 3A, 3D). ATP

![Figure 2](image2.png)

**Figure 2.** Glibenclamide and ni flumic acid inhibited the FSK-induced enhancement of HCO$_3^-$ permeability across the apical side of CBCECs. (A) Effect of ni flumic acid (100 μM) on forskolin-stimulated HCO$_3^-$ permeability. (B) Comparison of the initial dpH/dt between LB pulses in the presence of forskolin alone and forskolin plus ni flumic acid (NA). Black boxes: LB pulses applied to the apical side. (C) The effects of glibenclamide (GB; 50 μM) on forskolin-enhanced HCO$_3^-$ permeability. (D) Comparison of the initial dpH/dt between LB pulses in the presence of forskolin alone and forskolin plus glibenclamide.

![Figure 3](image3.png)

**Figure 3.** ATP, ATPγS, and CPA enhanced HCO$_3^-$ permeability across the apical side of CBCECs. (A) The effects of ATP (100 μM; $n = 6$), ATPγS (100 μM; $n = 7$), and CPA (20 μM; $n = 7$) on apical HCO$_3^-$ permeability. Black boxes: LB pulses applied to the apical side. (D) Summary of the effects of ATP, ATPγS, and CPA on initial dpH/dt during LB pulses.
The second LB pulse in the presence of ATP\textsubscript{γS} (200 \textmu M) decreased pH\textsubscript{i} immediately by 0.047 \pm 0.009 (n = 7). The second LB pulse in the presence of ATP\textsubscript{γS} showed a 79\% \pm 12\% increase in dpH\textsubscript{i}/dt (Figs. 3B, 3D), indicating that elevated [Ca\textsuperscript{2+}]\textsubscript{i} can lead to increased apical HCO\textsubscript{3}\textsuperscript{-} permeability. To further confirm the involvement of Ca\textsuperscript{2+} signaling in regulation of HCO\textsubscript{3}\textsuperscript{-} permeability, we used an alternative strategy to increase [Ca\textsuperscript{2+}]\textsubscript{i}. Cyclopiazonic acid (CPA) is a sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor, which depletes the calcium store and causes further Ca\textsuperscript{2+} entry through store-operated calcium channels (SOCs) causing capacitative calcium entry (CCE).\textsuperscript{15} CPA (20 \textmu M) caused a two-phase Ca\textsuperscript{2+} increase, which is composed of a peak and an elevated sustained plateau (not shown). This response is also consistent with previous reports.\textsuperscript{11} Application of 20 \textmu M CPA decreased pH\textsubscript{i} followed by a slow settling of pH\textsubscript{i} back to the baseline. The second LB pulse in the presence of CPA showed an increase in dpH\textsubscript{i}/dt 1.95 \pm 0.24 times greater than in the control (Figs. 3C, 3D).

Another possibility for the Ca\textsuperscript{2+}-induced apparent increase in HCO\textsubscript{3}\textsuperscript{-} permeability is an increased driving force for HCO\textsubscript{3}\textsuperscript{-} efflux through E\textsubscript{m} hyperpolarization caused by Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. If this were the case, we would expect a hyperpolarization during the [Ca\textsuperscript{2+}]\textsubscript{i} elevation by ATP\textsubscript{γS}. A depolarization would be an indication of increased anion (HCO\textsubscript{3}\textsuperscript{-}) channel permeability. Therefore, we measured the E\textsubscript{m} with the voltage-sensitive dye bis-oxonol. Under Cl\textsuperscript{-}-rich conditions (Fig. 4), ATP\textsubscript{γS} depolarized the E\textsubscript{m} for approximately 10 minutes, and then the E\textsubscript{m} repolarized to baseline (n = 4). Under Cl\textsuperscript{-}-free conditions, we observed similar, but smaller, changes in E\textsubscript{m} (not shown). These results support a more direct role of Ca\textsuperscript{2+} in anion channel permeability, rather than as an increased driving force for HCO\textsubscript{3}\textsuperscript{-} efflux through E\textsubscript{m} hyperpolarization. Increased [Ca\textsuperscript{2+}]\textsubscript{i} has been shown to activate chloride channels through calmodulin kinases (CaMK).\textsuperscript{15} In this study, we examined whether CaMKs are involved in the regulation of HCO\textsubscript{3}\textsuperscript{-} permeability by using the CaMKII-specific inhibitor KN-62.\textsuperscript{21} It has been reported that CaMKII is ubiquitously present in mammalian tissues.\textsuperscript{22} Figure 5A shows a control response to LB and a response in the presence of both ATP\textsubscript{γS} and KN-62. The initial dpH\textsubscript{i}/dt in the presence of both ATP\textsubscript{γS} (100 \textmu M) and KN-62 (2 \textmu M) was much slower than in the presence of ATP\textsubscript{γS} (100 \textmu M) alone (Fig. 3B). Figure 5B summarizes these results, showing that KN-62 in the presence of ATP\textsubscript{γS} inhibited the initial dpH\textsubscript{i}/dt by 49% \pm 9% compared with the control. We also found that LB-induced apical HCO\textsubscript{3}\textsuperscript{-} efflux was inhibited 60\% \pm 6\% by KN-62 alone (P < 0.01, n = 3, data not shown), which indicates that CaMK phosphorylation may be a potentiating factor, even in resting cells.

The Expression of CLCA in Corneal Endothelial Cells

Recently, two CLCA genes have been described.\textsuperscript{13,14} To examine the expression of CLCA genes in the corneal endothelial cells, we used bovine (b)CLCA1 and bCLCA2 (lung-endothelial cell adhesion molecule (Lu-ECAM)-1)-specific primers in RT-PCR for fresh and cultured BCECs. It has been reported that CLCA1 is expressed in the corneal epithelium,\textsuperscript{23} which we used as a positive control. Our RT-PCR results showed expression of bCLCA1 in both fresh and cultured BCECs (Fig. 6), whereas bCLCA2 mRNA was not detected (data not shown). Sequencing of the PCR product (231 bp) showed 99\% homology to published bCLCA1. The detection of mRNA expression of bCLCA1 in the fresh and cultured corneal endothelial cells is consistent with our data showing Ca\textsuperscript{2+}-dependent HCO\textsubscript{3}\textsuperscript{-} permeability.

Possible Regulation of HCO\textsubscript{3}\textsuperscript{-} Permeability across the Apical Membrane by PKC

Activation of phospholipase C can activate PKC through DAG or Ca\textsuperscript{2+}.\textsuperscript{24} Furthermore, PKC can potentiate the activity of CFTR.\textsuperscript{16,17} Because of the permeability of CFTR to HCO\textsubscript{3}\textsuperscript{-}, we...
hypothesized that PKC activation induced by purinoceptor agonists may be one of the signaling pathways leading to HCO$_3^-$ permeability. To examine the involvement of PKC in HCO$_3^-$ permeability regulation, we tested the effects of phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, and its ineffective analogue, 4a-phorbol 12-myristate 13-acetate. LB pulses in the presence of PMA (1 μM) increased dPH/dt by 79% ± 29% (P < 0.01, n = 9; Fig. 7A, 7B), whereas the PMA analogue (1 μM) appeared to have no significant effect (P > 0.05, n = 6). We also applied ATPγS together with bisindolylmaleimide I (BIMI), a specific PKC inhibitor. Combined application of ATPγS and BIMI (1 μM) significantly inhibited dPH/dt by 71% ± 7% (n = 4; Fig. 8A) compared with control, whereas ATPγS alone increased dPH/dt by 79% ± 12% (Figs. 3B, 3D, 8B). We also found that LB-induced apical HCO$_3^-$ efflux was inhibited 50% by BIMI alone (P < 0.05, n = 6, not shown). Moreover, in the presence of BIMI, forskolin did not enhance HCO$_3^-$ permeability (not shown). These results are consistent with the prerequisite role of PKC in CFTR activation and indicate that PKC is one of the signaling pathways for HCO$_3^-$ permeability regulation.

**DISCUSSION**

In this study, we showed for the first time that activation of purinergic receptors can enhance apical HCO$_3^-$ permeability in corneal endothelial cells, possibly through Ca$^{2+}$-CaMKII- and PKC-signaling pathways, and that CLCA1 is expressed in the corneal endothelium. Together with the stimulatory effect of cAMP-PKA on HCO$_3^-$ permeability, multiple pathways including Ca$^{2+}$-CaMKII and PKC can converge to cause the upregulation of HCO$_3^-$ permeability.

We used a two-LB pulse, constant CO$_2$ protocol to examine the effects of intervention drugs on HCO$_3^-$ permeability across the apical membrane of the corneal endothelium. During the initial part of apical LB perfusion, HCO$_3^-$ leaves the cell and H$^+$ enters from the bath across the apical membrane. Because more than 80% of the initial dPH/dt is due to HCO$_3^-$ flux, we can use this rate as a relative measure of HCO$_3^-$ flux. Further, because the experiments are paired and the driving force for HCO$_3^-$ efflux is the same during control and test pulses, the differences in initial dPH/dt reflect differences in HCO$_3^-$ permeability.

**FIGURE 6.** RT-PCR analysis of the expression of bCLCA1 in corneal endothelial cells. The expected size is 231 bp. Lane 1: marker; lane 2: fresh epithelium; lane 3: fresh endothelium; lane 4: cultured endothelium; lane 5: negative control.

**FIGURE 7.** Enhancement of HCO$_3^-$ permeability across the apical side of CBCECs by PMA. (A) The effects of PMA (1 μM) on apical HCO$_3^-$ permeability. (B) Summary of the effects of PMA and the PMA analogue (ANA) on initial dPH/dt during LB pulses. Black boxes: LB pulses applied to the apical side.
Both the adrenoceptor agonist adenosine and AC activator forskolin enhanced HCO$_3^-$ permeability across the corneal endothelium. Adenosine has been used as a stimulator of ion and fluid transport across the corneal endothelium for 30 years. Adenosine binds to A$_2$ receptors leading to activation of the cAMP pathway. We have shown that cAMP activates Cl$^-$ permeability across cultured BCECs. Our current results show that forskolin increased HCO$_3^-$ permeability across the apical membrane (Fig. 2). The upregulation of HCO$_3^-$ permeability by the cAMP pathway is not surprising. We recently found that CFTR, a cAMP-activated chloride channel, is expressed in corneal endothelial cells. Preliminary studies from our laboratory indicate that CFTR is localized only to the apical membrane of the corneal endothelium (Sun XC, written communication, August 2001). The apical localization of CFTR is consistent with our observation that adenosine and forskolin can enhance HCO$_3^-$ permeability across the apical membrane. Thus, the immediate decrease of pH$_i$ caused by either adenosine or forskolin may be due to activation of a cAMP-dependent anion channel, causing a quick increase in HCO$_3^-$ efflux from the cell (Fig. 1C). The reduced pH$_i$ and E$_m$ depolarization would both lead to increased basolateral HCO$_3^-$ influx through the electronegative NBC, which may explain the following transient pH$_i$ fluctuations. The Cl$^-$ channel blockers glibenclamide and niflumic acid inhibited the increased HCO$_3^-$ permeability induced by forskolin, indicating the involvement of Cl$^-$ channels in HCO$_3^-$ transport (Fig. 2).

ATP, ATP$_y$S, and CPA also enhanced HCO$_3^-$ permeability. All these agents increased [Ca$^{2+}$]$_i$, ATP$_y$S depolarized E$_m$ (Fig. 4), consistent with increased anion conductance. After the depolarization in the continued presence of ATP, E$_m$ repolarized. This may indicate that increased [Ca$^{2+}$]$_i$ also activates K$^+$ channels, thereby maintaining the driving force for HCO$_3^-$ influx. Ca$^{2+}$-activated chloride channels have been cloned in the bovine tracheal epithelium and lung endothelium. HCO$_3^-$ has also been demonstrated to permeate a Ca$^{2+}$-dependent anion channel in gallbladder. Our RT-PCR results showed the expression of bCLCA1 in the fresh and cultured corneal endothelial cells (Fig. 6). This is consistent with the enhancing effects of ATP, ATP$_y$S, and CPA on HCO$_3^-$ permeability. Further, studies on the expression and localization of CLCA are needed to determine the role of CLCA in corneal endothelial HCO$_3^-$ transport.

Constitutive phosphorylation of CFTR by PKC is thought to be required for subsequent CFTR activation by PKA. Due to the permeability of CFTR to HCO$_3^-$, it is not surprising that the PKC specific inhibitor BIMI inhibited HCO$_3^-$ permeability dramatically, even in the presence of ATP$_y$S (Fig. 8). The enhanced HCO$_3^-$ permeability caused by the PKC activator PMA further confirms the importance of PKC phosphorylation in the regulation of HCO$_3^-$ permeability in BCECs (Fig. 7).

Because the corneal endothelium is essential for the dehydration and transparency of the cornea, the regulation of the transport functions of this monolayer would have significant effects on the hydration of the cornea. Involvement of multiple pathways and receptor systems has the advantage of using multiple factors to enhance the transport function of the corneal endothelium. In addition to the adrenoceptor-cAMP-PKA mechanism, activation of purinergic receptors can enhance HCO$_3^-$ permeability through both Ca$^{2+}$ and PK pathways. Purinoreceptor agonists lead to a two-phase [Ca$^{2+}$]$_i$ elevation, including Ca$^{2+}$ mobilization from the endoplasmic reticulum (ER) through IP$_3$ receptors and Ca$^{2+}$ entry through store-operated calcium channels. Ca$^{2+}$ may activate CLCA through CaMKII. However, we could not exclude the possibility that Ca$^{2+}$ can directly activate CLCA. Furthermore, activation of purinoreceptors can stimulate PKC through Ca$^{2+}$ or DAG, which also increased HCO$_3^-$ permeability, probably through activation of CFTR.

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**References**