Activation of a CFTR-Mediated Chloride Current in a Rabbit Corneal Epithelial Cell Line

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PURPOSE. To determine whether there is gene expression and functional activity of cystic fibrosis transmembrane conductance regulator protein (CFTR) in an SV40-immortalized rabbit corneal epithelial cell line, tRCE.

METHODS. Both whole-cell and cell-attached patch-clamp techniques were used to examine the biophysical characteristics of the CFTR-mediated chloride current. The molecular identity of this conductance was evaluated using RT-PCR analysis.

RESULTS. In whole-cell patch-clamp studies, a CFTR-dependent chloride conductance was further facilitated by the known CFTR activator genistein (20 μM). Kinetic analysis of cell-attached patches containing few channels ascertained that genistein increased the chloride channel activity by increasing channel open probability (via an increased channel open time and a decreased channel closed time). In addition, in the presence of a reduced forskolin concentration (i.e., 100 nM), the chloride conductance generated could be augmented by the nonspecific phosphodiesterase enzyme inhibitor, IBMX (100 μM), implicating the importance of intracellular cAMP in the regulation of this conductance. Furthermore, this conductance exhibited voltage-dependent inhibition in the presence of the CFTR chloride channel blocker glibenclamide (250 μM), but was DIDS insensitive (500 μM). Consistent with the presence of a CFTR-mediated chloride conductance, the expression of CFTR-mRNA was detected using RT-PCR. Sequence analysis of the product revealed 99.4% homology to that described for rabbit CFTR.

CONCLUSIONS. In tRCE cells, there is gene expression and functional CFTR activity. Its presence may have important therapeutic implications in corneal epithelial diseases resulting from reduced forskolin concentration (i.e., 100 nM), the chloride conductance generated could be augmented by the nonspecific phosphodiesterase enzyme inhibitor, IBMX (100 μM), implicating the importance of intracellular cAMP in the regulation of this conductance. Furthermore, this conductance exhibited voltage-dependent inhibition in the presence of the CFTR chloride channel blocker glibenclamide (250 μM), but was DIDS insensitive (500 μM). Consistent with the presence of a CFTR-mediated chloride conductance, the expression of CFTR-mRNA was detected using RT-PCR. Sequence analysis of the product revealed 99.4% homology to that described for rabbit CFTR.

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ence of a CFTR-mediated chloride conductance, we detected using RT-PCR, the expression of CFTR-mRNA. Sequence analysis of the PCR product revealed 99.4% homology to the registered rabbit CFTR sequence. These studies may be of benefit in the identification of targets for drug development. Such agents designed to enhance chloride channel activity may have therapeutic applications aimed to rectify corneal epithelial dysfunction.

**MATERIALS AND METHODS**

**Cell Culture**

SV40-immortalized rabbit corneal epithelial cells, tRCE, were grown on plastic culture dishes and flasks and maintained in Dulbecco’s modified Eagle’s medium (DMEM/F12) containing the following: 10% fetal bovine serum, insulin (5 µg/ml), EGF (5 µg/ml), and gentamicin (10 mg/ml). Cells were perpetuated under standard tissue culture conditions (95% air-5% CO2, 5°C) and used for patch-clamp studies within 4 days of growth.

**Determination of Expression of CFTR mRNA by RT-PCR**

mRNA was isolated using a Dynabeads mRNA DIRECT Micro Kit (Dynal Inc., Lake Success, NY), as per manufacturer’s protocol. mRNA was eluted into 10 mM Tris/HCl buffer and stored at -80°C until used. First strand cDNA was synthesized using Superscript II for RT-PCR (as described by Gibco BRL, Life Technologies, Gaithersburg, MD). We used novel primers for two conserved regions of amino acids between rabbit and human CFTR (24 and 21 bases long, respectively); sense 5′-GAGGGATTTGGGGAATTATTTGAG-3′ and antisense 3′-CTTGCTCGTTGACCTCCACTC-3′ (oligonucleotide primers were made by the University of Missouri-Columbia DNA Core facility). Omission of the RT provided a negative control for DNA contamination. PCR reactions (50 µl volume) were performed over 30 cycles using recombinant Taq DNA polymerase (Promega, Madison, WI). Each cycle consisted of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. PCR products (457 bp) were electrophoresed on a 2% agarose gel and visualized with ethidium bromide staining. NIH3T3 cells stably expressing wild-type CFTR were used as a positive control for the presence of CFTR and treated as per tRCE cells.

To increase the yield of the PCR product for sequencing of the CFTR, we used nesting primers. The nesting primers were sense 5′-CCTCTCTTTGATTTTCTC-3′ and antisense 5′-CAAGCTTTGAT-6GAACCTTG-3′ (nested oligonucleotides were made by Open Technologies Inc., Alameda, CA). Again, omission of the RT provided a negative control for DNA contamination. PCR reactions (50 µl volume) were performed over 30 cycles using recombinant Taq DNA polymerase. Each cycle consisted of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 60 seconds. PCR products (390 bp) were visualized as above. DNA bands were excised from the agarose gel and then processed to yield purified DNA using a Qiagen DNA extraction protocol (Qiagen, Hilden, Germany). Purified DNA was then sequenced (DNA Core, University of Missouri-Columbia) and entered into the NCBI site for match with the published rabbit CFTR sequence (AF189720).

**Electrophysiology**

**Whole-Cell Patch-Clamp Experiments.** Cell suspensions were prepared by brief trypsinization (0.25% trypsin in phosphate-buffered saline). Pipette electrodes were made from Kimax 51 brand thin-walled capillaries (Fischer Scientific, Pittsburgh, PA), with a two-stage vertical puller (Narishige, Tokyo, Japan). Pipette tips were fire polished with a homemade microforge and had resistances of ~3 MΩ in the bath solution. The membrane potential was held with an Axopatch 1D amplifier (Axon Instrument, Foster City, CA) at 0 mV (~12 mV after correction of the junction potential), after break-in with suction. I-V relationships were generated using Igor software (WaveMetrics, Lake Oswego, OR) and XOP (developed by Richard Bookman at the University of Miami, FL). Current traces in response to voltage pulses (±100 mV, in 20-mV increments, 100- ms duration) were filtered at 1 kHz and digitized at 2 kHz directly into the computer hard drive (7100/80, Macintosh Computer; Apple Computer, Cupertino, CA) through an ITC-16 interface (Instrutech Corp., Port Washington, NY). CFTR channel currents were recorded at room temperature (~22°C). The pipette solution contained (in mM): 85 aspartic acid, 5 pyruvic acid, 10 EGTA, 20 tetraethylammonium chloride, 5 Triscreatinophosphate, 10 MgATP, 2 MgCl2, 5.5 glucose, and 10 HEPES (pH 7.4 with 8 N CsOH). The bath solution contained (in mM): 150 NaCl, 2 MgCl2, 1 CaCl2, 5 glucose, 5 HEPES, and 20% sucrose (pH 7.4 with 1N NaOH).

**Single-Channel Patch-Clamp Experiments.** Cell suspensions were prepared as described above. Cells were transferred to a continuously perfused chamber located on the stage of an inverted microscope (Olympus, Tokyo, Japan). Pipette electrodes were made from Corning 7056 borosilicate glass capillaries (Warner Instrument Corp., Hammed, CT) with a two-stage vertical puller. Pipette tips were fire-polished as above (resistances of ~4 MΩ in the bath solution). CFTR chloride channel currents were recorded at room temperature (~22°C) with an EPC-9 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany), filtered at 100 Hz with a built-in three-pole Bessel filter, and stored on videotapes. Data were refiltered at 50 Hz with an eight-pole Bessel filter (Frequency Device, Haverill, MA) and captured onto a hard disc (Quadra 650, Macintosh Computer) at a sampling rate of 100 Hz. Solution changes were affected via parallel silastic tubing descending from separate gravity-feed reservoirs into a common perfusion manifold.

The pipette potential was held at +50 mV with reference to the bath. Downward deflections in the recordings represent channel openings. The pipette solution contained (in mM): 140 N-methyl-D-glucamine chloride (NMDG-Cl), 2 MgCl2, 5 CaCl2, and 10 HEPES (pH 7.4 with NMDG). All cell-attached experiments were performed in a perfusion solution containing (in mM): 145 NaCl, 5 KCl, 2 MgCl2, 1 CaCl2, 5 glucose, 5 HEPES, and 20% sucrose (pH 7.4 with 1N NaOH). Addition of sucrose to the perfusion solution circumvented activation of swelling-induced chloride currents. For experiments using excised inside-out patches, the bath solution contained (in mM): 150 NMDG-Cl, 10 EGTA, 10 HEPES, 8 Tris, and 2 MgCl2 (pH 7.4 with NMDG).

**Data Analysis and Statistics**

Mean steady state current amplitudes were calculated with Igor software (WaveMetrics Inc.), from a 1- to 2-minute segment of the steady state CFTR current. All-point histograms were generated and fit with Gaussian functions (using Igor software), and single-channel amplitudes were obtained by measuring the difference between two adjacent peaks (representing the channel opening and closing). Data are presented as means ± SEM. Statistical analyses (t-tests) were performed using SigmaPlot software (Jandel Scientific, San Rafael, CA); significance was given at P < 0.05.

**Reagents**

 Forskolin was purchased from Calbiochem (La Jolla, CA) and stored as 20 mM stock in dimethyl sulfoxide (DMSO) at -20°C. Genistein was purchased from Alexis Corp. (San Diego, CA) and stored as 100 mM stock in DMSO at -20°C. All other chemicals were purchased from Sigma.

**RESULTS**

**Activation of a cAMP-Dependent Chloride Conductance**

We first examined the conductance generated in excised inside-out patches after the application of protein kinase A (PKA, 40 U/ml) and ATP (2.75 mM), both essential for CFTR opening.
Figure 1A shows a typical recording from such an experiment (over the range ±50 mV). One single-channel open event was observed throughout the entire recording. The average I-V relationship shown in Figure 1B is linear, with a conductance of 8.88 ± 0.04 pS (n = 3), typical for CFTR.

Using the whole-cell patch-clamp technique, we examined the ability of tRCE cells to facilitate an increase in chloride conductance (chloride channel activity) in the presence of the adenylate cyclase activator, forskolin. In previous studies the addition of 10 μM forskolin has been shown to be sufficient to maximally activate the cAMP-PKA–dependent pathway.20,28 In tRCE cells, we observed that the whole-cell chloride conductance measured in the presence of 10 μM forskolin alone was unaffected by the addition of a saturating concentration (200 μM) of the membrane permeant cAMP analog CPT-cAMP, in the continued presence of forskolin (n = 4, data not shown). These data suggest that in tRCE cells, 10 μM forskolin is sufficient to maximally activate the whole-cell chloride current via the cAMP-PKA pathway.

A well-documented potent activator of CFTR chloride channels is the isoflavone, genistein.20–24 Because the effects of genistein on CFTR have been extensively studied and a mechanism of action has been proposed, we therefore examined its effects on the forskolin-stimulated chloride current in tRCE cells. Figure 2A shows a typical I-V relationship examining the effects of forskolin (10 μM) and genistein (20 μM) on the whole-cell chloride current (over the range ±100 mV). In the absence of agonists, basal conductance is minimal. The addition of 10 μM forskolin alone generated a slight increase in the whole-cell conductance, with a reversal potential of −30.11 ± 6.30 (n = 9), which is close to the reversal potential for chloride. This conductance was dramatically increased upon the addition of 20 μM genistein in the continued presence of forskolin. The resulting I-V relationship is nonlinear (outwardly rectifying) because of the imposed asymmetric chloride gradient (24 mM chloride in the pipette and 156 mM chloride in the bath). Figure 2B shows the average data obtained at ±100 mV, in the presence of forskolin (F) and 10 μM forskolin plus 20 μM genistein (F + G). Basal current is minimal (B). (A) Average whole-cell chloride conductance at ±100 mV, elicited by the addition of F and then F + G (n = 12, 12/29 cells tested; *significance at P < 0.05). Basal current is subtracted.

Whole-cell patch-clamp technique allows us to monitor chloride conductance in relatively intact cells; however, the kinetic information generated is minimal. To this end, we examined the effect of the same agonists (forskolin and genistein) in cell-attached patch-clamp configuration and thus determined their effects on single-channel open probability (Po). Data from a cell-attached patch recording are shown in Figure 3A. In this recording, which lasted more than 1 hour, only one single opening step was observed, indicating that there was likely only one channel present in the patch. In the presence of forskolin alone (10 μM), the channel spends most of the time in the closed state (see arrow) and opens for relatively little time; thus the Po is low. On application of genistein (20 μM) in the continued presence of forskolin, the channel spends more time open and less time closed, and the Po therefore increases. Indeed, we quantified the open and closed times from three cell-attached patches, each lasting 1 hour (where one single-channel open event was observed throughout). We found that the open time (or burst duration) in the presence of 10 μM forskolin was 5.27 ± 0.62 seconds, increasing to 14.37 ± 1.53 seconds in the presence of 20 μM genistein (108 and 109 events, respectively). Conversely, the
Forskolin was 15.36 m

increases the CPT-cAMP–stimulated CFTR current. In this cell-attached patch traces (200 seconds each) from a recording lasting >1 hour. In the presence of forskolin alone (10 μM), the channel spends most of the time in the closed state, and Po is low (0.23). Application of genistein (20 μM) in the continued presence of forskolin elicits longer channel opening events, and Po increases (0.77). (B) Genistein increases the CPT-cAMP–stimulated CFTR current. In this cell-attached trace showing two channel opening steps, the addition of genistein (20 μM) increases the Po from 0.22 to 0.50. Arrows, closed state. Downward deflections are channel openings. (C) Summary of data from four cell-attached patches, demonstrating the 2.6-fold increase in Po by genistein on the forskolin-stimulated CFTR chloride current (4/38 cells tested).

closed time (or interburst duration) in the presence of 10 μM forskolin was 15.36 ± 2.78 seconds, which decreased to 7.97 ± 0.99 seconds in the presence of 20 μM genistein (112 and 113 events, respectively). This effect of genistein on open and closed times is similar to that reported previously.

Similar increases in Po were observed when genistein (20 μM) was added to a bath perfusate containing 100 μM CPT-cAMP (Fig. 3B). Using Gaussian fits of all-points amplitude histograms, we demonstrate a 9% decrease in the single-channel amplitude in the presence of genistein (data not shown). This reduction in single-channel amplitude is consistent with data previously published. In four cells (4/38 cells tested), there was an average increase in Po from 0.31 ± 0.08 to 0.82 ± 0.06, on addition of genistein (20 μM) to the forskolin-stimulated (10 μM) chloride current (Fig. 3C). Thus, concurrence with previously published data in other cell types, genistein potentiates the cAMP-dependent CFTR chloride current in tRCE cells, via an increase in the channel Po, which is mediated by an increase in the channel open time and a decrease in the channel closed time.

Effect of PDE Inhibition

We hypothesized that in tRCE cells, there may be notable degradation of intracellular cAMP by PDEs. Thus, modulation of intracellular cAMP through the use of PDE inhibitors would likely influence the activity of this cAMP-dependent chloride conductance. Therefore, we tested the effects of the nonspecific PDE inhibitor IBMX on the chloride current. IBMX has previously been described to improve CFTR channel current in several cell types under specified conditions. We have previously shown in Calu-3 cells (endogenously expressing CFTR) that PDE inhibitors are only effective in the presence of lowered (100 nM) forskolin concentrations (i.e., submaximal concentrations of cAMP). However, they are ineffective when tested in the presence of saturating, maximally effective concentrations of forskolin, i.e., 10 μM. Similarly, we have found in tRCE cells, that 100 μM IBMX elicited no increase in the whole-cell chloride current in the presence of 10 μM forskolin (n = 7, data not shown). However, consistent with previously published data, 100 μM IBMX augmented the chloride current generated by a lowered forskolin concentration (i.e., 100 nM). Figure 4A shows a typical whole-cell trace from such an experiment. IBMX (100 μM) increased the forskolin-stimulated chloride current, which was further potentiated by the addition of genistein (20 μM). On washout of all agonists, the whole-cell current returns to basal current levels. The I-V relation to the right shows the data from the trace at points a–d as marked. Average data from several experiments are shown in Figure 4B, and the data are expressed as net current (basal current subtracted). At +100 mV, the CFTR chloride current significantly increased from 10.07 ± 5.36 to 89.82 ± 31.16 pA/pF (n = 7, P < 0.05) in the presence of forskolin alone and forskolin plus IBMX, respectively. The addition of genistein (20 μM) to the perfusate further augmented this chloride current (see Figs. 4A, 4B). These data suggest that the cAMP generated by the application of forskolin is likely degraded by PDEs, because addition of IBMX augments the forskolin-stimulated chloride current (7/7 cell tested). Although this study does not provide a mechanism for this result, potentiation of forskolin-stimulated chloride current by IBMX suggests that PDE inhibitors may potentiate the forskolin-stimulated chloride current through the use of PDEs.
aim to address specifically which PDEs are present in tRCE cells, we conclude that they likely will play an important role(s) in chloride channel activation and thus in fluid transport in tRCE cells.

**Inhibition of Chloride Conductance by Glibenclamide**

The chloride channel blocker glibenclamide is widely used to define the CFTR chloride current.26,27 Thus, to provide further evidence that the chloride current we activated by the application of forskolin and genistein is CFTR mediated, we tested its effects. The whole-cell anion currents elicited by forskolin (10 µM) and genistein (20 µM) exhibited typical voltage-dependent inhibition by glibenclamide (250 µM). Figure 5A is a typical experiment demonstrating this voltage-dependent inhibition of the whole-cell cAMP-dependent chloride current that is activated by forskolin (10 µM) and genistein (20 µM). In nine cells, 250 µM glibenclamide inhibited the whole-cell chloride current by 24% and 57% at +100 mV and -100 mV, respectively (P < 0.05, Fig. 5B). In addition, we observed no effect of DIDS (500 µM) on this chloride conductance (n = 3, data not shown). These data are consistent with previously published observations and suggest that the chloride current activated by forskolin and genistein is attributed to CFTR.25–27

**Expression of CFTR mRNA in tRCE Cells**

We used RT-PCR methods to demonstrate expression of CFTR mRNA in tRCE cells (Fig. 6). Gene-specific primers were chosen from conserved regions on both rabbit and human CFTR sequences. Using these gene-specific primers to amplify reverse-transcribed cDNA, a major PCR product of ~457 bp was identified in tRCE cells, consistent with CFTR mRNA expression. This result was not due to DNA contamination because omission of RT produced no product. NIH3T3 cells stably transfected with wild-type (Wt) CFTR served as a positive control for CFTR expression. PCR products were extracted and purified from the gel and then sequenced. Sequence analysis confirmed the presence of CFTR.

**DISCUSSION**

Expression of the CFTR chloride channel has been well documented in a variety of epithelia (e.g., airway, intestine, sweat ducts, and pancreas), and its role in the debilitating lethal genetic disease cystic fibrosis was determined.28 However, the potential role(s) of the CFTR chloride channel in cornea epithelia is equivocal. Almost a decade ago, in freshly isolated rabbit cornea epithelial cells Marshall and Hanrahan11 discerned a chloride channel with characteristics similar to those reported for anion channels in other epithelia.11,24,25 Namely, a low conductance, linear I-V relationship. Unfortunately, this small conductance anion channel was observed on a mere 10 occasions, appeared to be relatively unresponsive to agonists that generate cAMP, and thus was not the recipient of much attention. More recently, the expression of CFTR in mouse cornea epithelium using RT-PCR and immunofluorescence techniques was established,24 and an important role was hypothesized for CFTR-mediated uptake of *Pseudomonas aeruginosa* by mouse corneal epithelial cells in experimental eye infections, namely murine keratitis. In other words, CFTR was proposed to act as a conduit for *P. aeruginosa*.24

The work presented in this study aimed to determine the presence of CFTR in tRCE cells and to resolve whether its biophysical and functional properties were similar to those observed in other epithelia. To date, no such studies have been performed. We have demonstrated in tRCE cells the presence of a PKA/ATP-dependent conductance (~9 pS), typical of CFTR. Using whole-cell patch-clamp, we found that the adenylate cyclase activator, forskolin (10 µM), elicited some small increase in chloride conductance that was potently potentiated by the known CFTR activator genistein. A potential question arising from this result is whether the concentration of forskolin used was sufficient to maximally activate the cAMP-PKA-dependent pathway in tRCE cells. A study by Illek et al.29 using NIH3T3 cells transfected with wild-type CFTR demonstrated that cAMP production increases in a dose-dependent manner over the range of forskolin concentrations tested (0.01–100 µM), saturating at forskolin concentrations ≥ 10 µM. In addition, we have previously demonstrated in Calu-3 cells, a cell line derived from a human pulmonary adenocarcinoma (endogenously expressing wild-type CFTR), that 10 µM forskolin was sufficient to maximally activate the cAMP-PKA pathway. In those studies, the application of the membrane permeant cAMP analog, CPT-cAMP, in the continued presence of forskolin, did not further enhance CFTR channel activity.29 Further-
more, we showed in those studies, that CFTR channel activity saturates at concentrations > 60 μM cAMP. From studies similar to those described above, we observed in tRCE cells that 10 μM forskolin maximally activates the cAMP-PKA pathway, because the addition of a saturating concentration of cAMP had no effect.

The observed increase in chloride conductance on application of genistein (20 μM) is consistent with data in the literature (approximately threefold increase in current). Additionally, genistein, has been demonstrated at a single-channel level to augment CFTR channel activity by increasing the Po, with a concomitant increase in the CFTR channel open time and a decrease in the CFTR channel closed time. Because part of genistein’s effect is via a prolongation of the channel open time, it is hypothesized that genistein acts through a direct binding to the CFTR protein. Direct biochemical evidence supporting this hypothesis that genistein interacts with CFTR (likely at a nucleotide binding domain) came from studies using a fusion protein comprising maltose-binding protein and the second nucleotide binding domain of CFTR. We found from single-channel kinetic analysis that genistein increases chloride channel conductance by increasing the CFTR channel Po, via an increase in channel open time and a decrease in channel closed time, which is consistent with previously published data.

It has been shown that selective PDE inhibitors modulate aqueous humor production, by fragmented and nonpigmented ciliary epithelium. Accordingly, we have demonstrated in airway epithelial cells (CaLu-3 and 16HBE cells, both endogenously expressing wild type-CFTR) that inhibitors of type III PDEs were the most specific and potent mediators of cAMP-dependent CFTR activation, whereas other classes of PDE inhibitors were without effect. We wanted to address whether PDEs were involved in the regulation of corneal epithelial chloride secretion. In this study, we demonstrate that IBMX (100 μM) augments the forskolin-stimulated chloride current, but only under specific conditions. For example, IBMX is ineffective in the presence of 10 μM forskolin; however, if the forskolin concentration is reduced (thus generating less cAMP), then IBMX is an effective activator of channel activity. These data are consistent with our previously published work. Furthermore, Marshall and Hanrahan described concern regarding the lack response of primary rat and rabbit cornea epithelial cells to cAMP-generating agonists. They hypothesized a “block distal to cAMP formation.” In actual fact, their data support our studies presented here, namely, that in cell-attached patches we observe CFTR chloride current, but in few patches the forskolin-stimulated CFTR current is small and is augmented by PDE inhibitors (suggesting that intracellular cAMP in corneal epithelium may encounter rapid degradation by PDEs). Although distinct profiles of PDE isoforms may be of significance in corneal epithelium, the goal of this study was merely to identify a potential role by PDEs in regulation of CFTR chloride channel activity.

In studies involving the identification of a chloride channel type, an additional tool with which to recognize the channel is the ability of chloride channel blockers to inhibit the current. For instance, the chloride channel blocker DIDS inhibits CFTR chloride current when applied to the intracellular surface but is ineffective when applied to the extracellular surface. We observed no effect of the extracellular application of 500 μM DIDS on the forskolin/genistein-stimulated chloride conductance, which was in agreement with those published data. Another chloride channel blocker, that is more widely used to inhibit CFTR is glibenclamide. We observed in tRCE cells a voltage-dependent inhibition of the forskolin/genistein-stimulated chloride conductance by glibenclamide (250 μM), consistent with this conductance being mediated by CFTR.

We conclude that the chloride conductance generated in these studies is mediated by CFTR; it is cAMP dependent, potentiated by genistein, influenced by PDE inhibitors, inhibited by glibenclamide, and DIDS insensitive, and moreover, these cells express CFTR-mRNA. The precise role of CFTR-mediated chloride secretion in corneal epithelium is unclear; however, its presence and the ability to pharmacologically manipulate it (i.e., via CFTR channel activators, PDE inhibitors) suggests that this conductance may have implications in a variety of corneal epithelia disease states where chloride secretion is dysfunctional.

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References


