Oxidant and antioxidant modulation of chloride channels expressed in human retinal pigment epithelium

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Weng, T. X., B. F. Godley, G. F. Jin, N. J. Mangini, B. G. Kennedy, A. S. L. Yu, and N. K. Wills. Oxidant and antioxidant modulation of chloride channels expressed in human retinal pigment epithelium. Am J Physiol Cell Physiol 283: C839–C849, 2002. First published June 13, 2002; 10.1152/ajpcell.00445.2001.—Retinal pigment epithelium (RPE) possesses regulated chloride channels that are crucial for transepithelial fluid and ion transport. At present, little is known about the molecular nature of chloride channels in human adult RPE (haRPE) or the effects of oxidative stress on membrane conductance properties. In the present study, we assessed CIC channel and cystic fibrosis transmembrane conductance regulator (CFTR) expression and membrane chloride conductance properties in haRPE cells. CIC-5, CIC-3, CIC-2, and CFTR mRNA expression was confirmed with RT-PCR analysis, and protein expression was detected with Western blot analysis and immunofluorescence microscopy. Whole cell recordings of primary cultures of haRPE showed an outwardly rectifying chloride current that was inhibited by the oxidant H2O2. The inhibitory effects of H2O2 were reduced in cultured human RPE cells that were incubated with precursors of glutathione synthesis or that were stably transfected to overexpress glutathione S-transferase. These findings indicate a possible role for CIC channels in haRPE cells and suggest possible redox modulation of human RPE chloride conductances.

immunocytochemistry; patch clamp; glutathione; glutathione S-transferase

RETNAL PIGMENT EPITHELIUM (RPE) comprises part of the blood-retina barrier and functions in several processes that are vital for the preservation of sight. Among the crucial roles of this epithelium is the regulation of the volume and electrolyte composition of the subretinal space. This function is achieved largely by regulated transepithelial transport of chloride from the subretinal space to the choroid with the obligatory movement of water. Disruption of RPE chloride transport can result in the accumulation of fluid in the subretinal space and subsequent retinal detachment (3, 26).

Chloride transport across the RPE is mediated in part by chloride channels that are stimulated by calcium and cAMP (Ref. 28; cf. Refs. 13, 14). In recent whole cell patch-clamp recordings in SV40-transformed cultured human fetal RPE (hfRPE) cells, we (39) identified an outwardly rectifying chloride current that was stimulated by cAMP but was inhibited by the chloride channel blocker DIDS, acidic bathing solutions, or low concentrations of the oxidative agent H2O2. The molecular identity of the chloride channel(s) responsible for this current was not determined. However, these cells expressed several candidate chloride channels including cystic fibrosis transmembrane conductance receptor (CFTR) and members of the CIC chloride channel family (CIC-2, CIC-3, and CIC-5; Ref. 39). At present, little is known about the expression of CIC chloride channels in the intact human RPE.

Recent evidence suggests that the CIC family of voltage-gated chloride channels may be crucial for retinal function. At least three members of this family are associated with retinal degenerations in mice. Specifically, transgenic mice that were deficient for CIC-3 (34), CIC-2 (6), or CIC-7 (17) were found to develop retinal degenerations and blindness within weeks after birth. The basis of these degenerations is not presently understood.

The finding of CIC channel expression in human fetal cells and the consequences of their deletion in mice indicate a possible functional role for these channels in the developing human RPE. To date, there have been no studies of CIC channel expression in the intact human adult RPE (haRPE). In addition, it is unclear

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whether regulation of RPE cell membrane conductances is similar in human fetal and adult cells.

In the present study, RT-PCR and immunocytochemical analysis methods were used to assess CFTR and CIC channel expression in haRPE cells. In addition, we determined whether an oxidant-regulated chloride conductance was present in primary cultures of haRPE cells and compared this conductance with those previously assessed in cultured hRPE cells. Finally, we assessed whether antioxidants can protect RPE cell chloride conductances from the inhibitory effects of oxidative agents.

METHODS

Immunocytochemistry. Postmortem eyes were obtained from the National Disease Research Interchange under local Institutional Review Board approval for the use of human tissue. For the present immunocytochemistry study, a donor eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem. RPE-choroid patches were dissected, and 6-H11011 eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem. For the present immunocytochemistry study, a donor eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem. RPE-choroid patches were dissected, and 6-H11011 eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem. RPE-choroid patches were dissected, and 6-H11011 eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem. RPE-choroid patches were dissected, and 6-H11011 eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem. RPE-choroid patches were dissected, and 6-H11011 eye from a 66-year-old Caucasian man was fixed in 4% formaldehyde in phosphate-buffered saline at −1°C post mortem.

Cultured hRPE cells. SV40-transformed hRPE cells (RPE 28 SV4; Coriell Institute, Camden, NJ) were grown in MEM (Sigma-Aldrich) supplemented with 1% penicillin and streptomycin and 10% FBS (HyClone Laboratories) as previously described (39). The cells were plated at subconfluent density and glass coverslips coated with poly-n-lysine. The cells were incubated in a humidified incubator at 37°C in 95% air and 5% CO2 overnight or were grown to confluence for a period of 3–7 days.

Stably transfected cells that overexpress human glutathione S-transferase (GST) A1.1 and control cells stably transfected with expression vector alone were produced by exposing 80–90% confluent hRPE cells to plasmid DNA and Transfast transfection reagent (Promega, Madison, WI) in DMEM at 37°C in a CO2 incubator for 1 h. Cells were subsequently overlaid with 4 ml of 10% FBS in DMEM and incubated another 48 h. Positive cells were then selected by using nontransfected SV40-transformed hRPE cells (RPE 28 SV4; Coriell Institute, Camden, NJ) as feeder cells. The culture medium was replaced three times per week. Individual clones were trypsinized, and single cells were transferred to a 96-well plate. Colonies grown from single cells were transferred to flasks and grown to confluence for subsequent use. In experiments investigating the effects of glutathione precursors, nontransfected SV40-transformed hRPE cells were incubated in fresh culture medium containing (in mM) 0.5 glutamate, 0.5 glycine, and 0.1 cysteine for 1 h before whole cell current measurements.

Whole cell patch-clamp recording. Membrane ionic currents were recorded with conventional tight-seal whole cell patch-clamp techniques (12) under conditions of symmetrical chloride concentrations in the absence of potassium ions. The composition of the solutions were as follows (in mM): bath, 130 tetramethylammonium chloride (TMA-Cl), 2 NaH2PO4, 2 calcium cyclamate, 1 MgSO4, 5 glucose, and 10 HEPES; pipette, 130 TMA-Cl, 0.2 calcium cyclamate, 3 MgSO4, 2 EGTA, 10 HEPES, and 3 Na-ATP. The pH of the solutions was 7.4, and the osmolalities were 300 and 270 mosmol/kgH2O, respectively. Borosilicate glass (cat. no. 18150F-3; WPI, Sarasota, FL) pipettes were pulled (model P-87; Sutter Instrument, Novato, CA) and fire-polished to a tip diameter of 1–2 μm (tip resistance = 3–6 MΩ). The pipette was connected to the head stage of a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA), and the bath was connected to ground with a 3 M KCl agar bridge.

Stable transfections with the expression vector containing the GST-A1.1 cDNA or the parental vector were carried out with a Pentium PC and analog-digital interface, using commercially available data acquisition and analysis software (DigData 1200 and pCLAMP 6.03 software; Axon Instruments). Electrode offset was balanced before forming a gigaseal. The electrode capacitance and series resistance were compensated with the amplifier’s analog circuitry. Solution junction potentials were negligible (<3 mV). Currents were low-pass Bessel-filtered at 5 kHz and digitized at 10 kHz for storage and analysis. Solution changes and drug delivery were achieved by a gravity drive superfusion system.

Drugs and antibodies. Glutathione, H2O2, glutamate, glycine and cysteine were obtained from Sigma-Aldrich. Unless otherwise noted, polyclonal antibody for CFTR was from

Hyclone Laboratories, Logan, UT. The cells were passaged between two and four times and grown for 2–5 days before whole cell patch-clamp investigations.
Alomone Labs and for ClC-5 was antiserum C1 raised against the COOH-terminal region of rat ClC-5 (21). Monoclonal CFTR antibody was from Genzyme (cat no.2503–01, now MAB25031, R&D Systems).

**Western blot analysis.** Human RPE was first homogenized then lysed in nonreducing Laemmli sample buffer following the methods of Marmorstein et al. (23). Cell lysates were electrophoresed on a precast 10% SDS-polyacrylamide gel (paler Gold; Cambrex, Bedford, MA). Alkaline-phosphatase-conjugated secondary antibodies and nitro blue tetrazolium/5-bromo-3-indolyloxophosphate (Western Blue Stabilized Substrate; Promega) were used for signal detection.

**RT-PCR.** Total RNA was isolated from primary cultures of human adult RPE cells with TRIzol (GIBCO) following the methods of Mangini et al. (22). For single-strand cDNA synthesis, 5 μg of total RNA from each sample was reverse transcribed with Superscript II reverse transcriptase (GIBCO, Gaithersburg MD), and oligo-dT priming. Amplification was performed with 2-μl aliquots of cDNA, High-Fidelity PCR Master (Roche Diagnostics, Indianapolis, IN), and specific primer sets for CIC-2, CIC-3, CIC-5, and CFTR. Primer sets were based on published sequences that spanned the lengths of the PCR products for ClC-2, ClC-3, ClC-5, CFTR1, and CFTR2. PCR reactions were carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C 30 s, 60°C 30 s, 72°C 1 min, and then 72°C 10 min (final extension). PCR products (5 μl) were electrophoresed with 2% agarose-1× TAE gels containing 0.5 μg/ml ethidium bromide. PCR DNAs were purified for direct sequencing with the Wizard PCR DNA purification system (Promega).

Data analysis and statistics. Steady-state currents were used to calculate the current-voltage (I-V) relationships. To calculate mean ± SE values, the raw data (contained in Axon binary files or and Axon XYM files) were exported to Excel spreadsheets. Paired t-tests or nonparametric tests were used to evaluate statistical significance, as appropriate (InStat; GraphPad Software, San Diego, CA). Graphs and further analysis were generated with SigmaPlot5 software (Aurora, CO).

**RESULTS**

Rationale for choice of experimental preparations. The following experiments used three different human RPE preparations. For haRPE, intact native epithelium was used to assess CIC chloride channel expression in Western blot analysis and in immunofluorescence microscopy experiments. Primary cultures of these cells were used to achieve whole cell patch-clamp recordings and for RT-PCR analysis to minimize contamination from other subretinal cell types. Finally, a continuous hRPE cell line (39) was used to enable genetic or environmental manipulation of antioxidative agents.

**PCR and Western blot analysis.** To obtain evidence for the presence of CIC and CFTR gene products in haRPE cells, RT-PCR analysis was performed with mRNA obtained from primary cultures of haRPE cells. As shown in Fig. 1, RT-PCR performed with specific primer sets for human CIC-2, CIC-3, and CIC-5 resulted in amplification of fragments of the predicted size and sequence for these channels. Similar experiments with CFTR-specific primers sets resulted in the amplification of cDNAs with the appropriate predicted sizes and sequences for CFTR (see Fig. 2). In addition, as shown in Fig. 3, Western blots of membrane proteins from intact haRPE (a gift of Dr. Alan Marmorstein) probed with antibodies raised against the NH2 terminus of CIC-5 (a gift of Dr. O. Devuyst; see Ref. 36) and CIC-3 (antibody 3A4, a gift of Dr. Thomas Jentsch; see Ref. 34) resulted in stained bands of the expected sizes for CIC-3 and CIC-5.

**Immunocytochemistry studies.** Figures 4–6 present images of cross-sections of native haRPE-choroid, oriented with the RPE cell layer at the top and the choroid below. Figure 4 presents a typical example of a differential interference contrast image. Bruch’s membrane is indicated by the triangle and the RPE apical microvillus membrane is at the top of the section. Figure 5 shows fluorescence images of two adjacent sections from the same eye that were processed using a deconvolution algorithm. The inverted white triangles indicate the apical surface of the RPE cells. (Cell nuclei were stained with DAPI and appear blue. Autofluorescence of the RPE cells and Bruch’s membrane appears red-orange.) In Fig. 5A, the cells were stained with primary antibody for CIC-5. Staining was most prom-
inent in the RPE near the apical membrane. Some faint staining is also evident in the choroid and capillary endothelial cells. In Fig. 5B, the section was treated identically except that primary antibody was incubated with ClC-5 peptide before incubation with the tissue. Note that a marked reduction of specific ClC-5 staining occurred. Figure 6A shows the results for a similar experiment using an antibody for ClC-3. As in the case of ClC-5 antibody, strong positive ClC-3 antibody staining was detected along the apical membrane. Preincubation of ClC-3 antibody with antigenic peptide blocked the staining (data not shown). Figure 6B illustrates tissue stained with an antibody against CFTR. Staining again was prominent along the RPE apical membrane but was also detectable along the lateral cell margins and on the basal RPE surface (Fig. 6B).

**Whole cell membrane properties.** To assess membrane chloride conductances, whole cell currents were recorded in primary cultures of haRPE cells using symmetrical chloride solutions with the absence of potassium and low sodium (see METHODS). Currents were stable within 2 min of obtaining a whole-cell configuration. Figure 7 compares membrane chloride current measurements recorded from primary cultured haRPE cells and SV40 transformed hfRPE cells (39). Figure 7, A and B, presents representative current recordings from haRPE and hfRPE cells, respectively, showing the response to voltage steps ranging from $-100$ to $+100$ mV in 20-mV increments. In both cases, the currents showed moderate outward rectification and little time dependence. The outward rectification is also evident in Fig. 7C, which presents average I-V relationships calculated from steady-state current values for both cell types (haRPE cells, n = 11; hfRPE cells, n = 8).

Figure 8 summarizes the effects of replacing chloride in the bathing solution with impermeant anions. As a first step in these experiments, TMA-Cl in the bathing solution was replaced by NaCl (Fig. 8). No significant changes were noted in the currents. In contrast, substitution of chloride by 130 mM cyclamate or iodide led to decreases in the membrane currents. Permeability ratios calculated with a modified version of the Goldman-Hodgkin-Katz equation (39) yielded a relative anion permeability sequence of $1:0.66\pm0.21:0.55\pm0.18$ for chloride:iodide:cyclamate, respectively (n = 4).

To further assess the ionic nature of the current, the chloride channel blockers chlorotoxin (1 µM) and DIDS...
(1 mM) were each applied to the bath. As shown in Fig. 9A, addition of chlorotoxin reduced the whole cell current by 34.5 ± 7% (at +100 mV) and the conductance by 46 ± 13% (n = 4) within 2 min (i.e., time required for complete change of the bath solution). As shown in Fig. 9B, addition of DIDS had a similar effect, reducing the conductance and current by 43.6 ± 11% and 37.2 ± 7%, respectively (n = 4).

Membrane conductance regulation. To test for possible modulation of the membrane chloride conductances in the primary cultured haRPE cells, several agents were used. Because CFTR is activated by cAMP and chloride conductances in hfRPE cells and RPE cells from other species have been shown to be increased by this agent, we assessed the effects of a cAMP cocktail that contained 250 µM 8-bromo-cAMP (8-BrcAMP), 100 µM IBMX, and 25 µM forskolin. As shown in Fig. 10, the current was increased by 39 ± 9% and the conductance was increased by 43 ± 17% mV (n = 4) within 3 min of the application of the cAMP cocktail.

In contrast to the effects of cAMP, acute exposure to H₂O₂ inhibited the chloride conductance of SV40-transformed hfRPE cells (39). As shown in Fig. 11, a similar inhibitory effect of H₂O₂ was observed for the chloride conductance of primary cultures of haRPE cells. Figure 11A shows the mean steady-state I-V relationships for these experiments in the absence of H₂O₂ and after addition of 100 µM H₂O₂ to the bathing solution. Outward currents (at +100 mV) were reduced by 38% ± 11%, and the slope conductance at positive potentials (between +60 and +120 mV) summarized in Fig. 11B was reduced by 37% ± 9% (n = 7). Currents and slope conductance at negative potentials were also significantly decreased by 35% ± 10% for currents at −60 mV and by 32% ± 11% for the slope conductance between −60 and −100 mV. The inhibitory effect typically occurred within 1 min of exposure and usually could be reversed by washing the cells with bath solution.

Effects of antioxidative agents. Glutathione, or GSH, is a naturally occurring substance that protects cells, tissues, and organs from toxic free radicals and disease and has been implicated in the modulation of the activity of several enzymes (2,32). Glutathione is a...
A tripeptide consisting of three amino acids, glycine, glutamate, and cysteine, and each of these precursors is necessary for the manufacture of glutathione within cells. Most intracellular glutathione is normally in the reduced state (GSH), where it plays a protective role by scavenging free radicals or by serving as a substrate for conjugation by enzymes that detoxify harmful substances such as lipid peroxides.

In preliminary experiments, we sought to test whether exogenous application of extracellular glutathione abolished the inhibitory effects of H$_2$O$_2$ on membrane currents. Although we initially observed that glutathione abolished the inhibitory effects of H$_2$O$_2$ on the membrane chloride conductance, this experiment proved to be confounded. Measurements of H$_2$O$_2$ concentrations revealed that glutathione addition directly decreased the quantity of H$_2$O$_2$ in the extracellular bathing solution. Therefore, we decided to test the effects of antioxidants by altering intracellular glutathione levels or by genetically manipulating the expression levels of antioxidant enzymes. Because a continuous RPE cell line was needed for the genetic manipulation experiments, these studies were carried out on the cultured hfRPE cell line used in our previous study (39).

To alter intracellular glutathione levels, cells were exposed to precursors of glutathione production (in mM: 0.5 glutamate, 0.5 glycine, and 0.1 cysteine) for 1 h before the experiments. As previously shown by Sternberg and coworkers (7, 33), these supplements significantly increase glutathione production and protect against oxidant-induced cell death. As shown in Fig. 12A, when the SV40-transformed hfRPE cells were previously exposed to culture medium supplemented with glutathione synthesis precursors, membrane currents were not significantly affected by subsequent H$_2$O$_2$ exposure ($\Delta I = -11 \pm 4\%$ at $+100$ mV; not significant (n.s.); $n = 4$). However, paired cells from the same batch that were bathed in normal culture medium (without supplementation) did show significantly decreased currents ($\Delta I = 38 \pm 8\%$; $n = 3$) in agreement with results from our previous study (39).

Figure 12B presents conductance data from the supplement-treated cells summarized in Fig. 12A. As in the case of the whole cell currents, the membrane conductance in the treated cells was not significantly altered by H$_2$O$_2$ treatment (12% ± 4%; n.s.). In contrast, the conductance decreased by 45 ± 11% ($P < 0.05$; t-test = 2.86) in cells that were not exposed to supplements.

Intracellular GSH is necessary for the detoxification of oxidant-induced toxins by the enzyme GST. To test the possible role of this antioxidant enzyme in protecting against the effects of oxidants, SV40-transformed hfRPE cells that were stably transfected with cDNA encoding human GST A1.1 were used. Godley and co-workers (unpublished observations) have demonstrated that GST activity is increased twofold in these cells. Figure 13 compares the effects of 100 $\mu$M H$_2$O$_2$ on the membrane chloride conductance in SV40-trans-
formed hfRPE cells that were stably transfected with vector alone (control) or with vector plus GST cDNA. As shown in Fig. 13A, currents in control cells were reduced by 36 ± 9% (n = 4), similar to the effects in both primary cultured haRPE cells (Fig. 11) and non-transfected SV40-transformed hfRPE cells (39). Figure 13B shows the results of identical experiments performed on SV40-transformed hfRPE cells that overexpress GST A1.1. The inhibitory effect of H2O2 on the membrane current was abolished (I = -4 ± 2%; n.s.; n = 4). The effect of H2O2 on the membrane conductance was also abolished as summarized in Fig. 13C. The change in conductance for the GST-transfected RPE cells was -3 ± 1% (n.s.; n = 4) compared with a decrease of -38 ± 12% for the vector-transfected RPE cells (P < 0.05; n = 4).

**DISCUSSION**

The results of these experiments provide new evidence for ClC channel expression in the intact haRPE. In addition, an outward chloride conductance was detected in primary cultures of these cells that was stimulated by cAMP and inhibited by H2O2. These data confirm and extend our previous results for cultured SV40-transformed hfRPE cells (39). In addition, the present results provide the first demonstration that the actions of oxidative agents on membrane currents and conductance properties of RPE cells can be prevented by antioxidants. Exposure of human RPE cells to glutathione precursors or overexpression of the antioxidant enzyme GST in these cells diminished the inhibitory effects of H2O2 on membrane chloride conductances. These results suggest that the membrane chloride conductance in human RPE cells is modulated by reactive oxygen species and can be protected by antioxidative agents.

**Chloride channel expression.** In the present study, RT-PCR analysis of primary cultures of haRPE cells resulted in the amplification of cDNA fragments with the predicted size and sequence for several known ClC channels, specifically ClC-2, ClC-3, and ClC-5. These findings are similar to our previous reports of ClC-5 and ClC-3 mRNA expression in cultured hfRPE cells (39). Similar evidence for CFTR mRNA expression was obtained in the same cells. These results agree with previous preliminary reports of CFTR mRNA expression in human RPE cells by others (4, 30).

The results of Western blot analysis and immunocytochemical experiments revealed positive reactivity with ClC channel antibodies that was blocked by antigenic peptides. These findings indicate the expression of ClC channel proteins in addition to mRNA expression. We previously reported (39) ClC-2, ClC-3, and ClC-5 protein expression in subconfluent fetal RPE cells. However, unlike the diffuse intracellular staining noted in fetal cultured cells, the staining of ClC-3 and ClC-5 in intact haRPE was predominantly located near the region of the apical membrane.

Recent studies in other organs such as the kidney and liver indicate that ClC-5 channels are mainly expressed in intracellular compartments (for review see Ref. 38). Although the channels were found in the apical region of the RPE cells in the present study, we cannot rule out an intracellular location. It is conceivable that channels could be contained both in the apical membrane and in a subcellular compartment below the apical surface. Schwake et al. (31) recently reported that ClC-5 can be inserted into surface membranes and is retrieved from the surface membranes via a mechanism that involves protein-protein interactions. These
interactions are mediated by a PY motif that, when mutated, abolishes retrieval of this channel from cell surface membranes. Further work is needed to determine whether these channels are trafficked to the apical membranes of RPE cells or are involved in phagocytosis or endocytosis.

ClC-2 and ClC-3 are widely expressed in most epithelial cells (38). Although strong staining was obtained for ClC-3 antibody, in preliminary experiments, ClC-2 immunoreactivity in haRPE was weak. Nonetheless, ClC-2 expression was recently confirmed in mouse RPE (6). Although ClC-3 is generally believed to be an intracellular ion channel (34), positive staining for ClC-3 was observed in the apical membrane region of haRPE cells and this staining was blocked by antigenic peptide.

Recently, controversy has arisen concerning the specificity of ClC-3 antibodies. Stobrawa et al. (34) reported that ClC-3 antibody from Alomone Laboratories demonstrated positive reactivity against an unidentified peptide in ClC-3 knockout mice. We cannot rule out the possibility that an unrelated peptide may have reacted with the ClC-3 antibody used in the present study. However, in Western blot studies, a positive reaction was obtained at the appropriate size for ClC-3. In addition, as noted above, PCR analysis provides evidence for mRNA expression for ClC-3. We note also that ClC-3 has been cloned from human fetal cells (39) and intact human adult retina (5).

As discussed above, CFTR expression in haRPE has been previously reported in preliminary studies by Quong and Miller (30). Peterson et al. (26) reported bright staining of CFTR predominantly near the basolateral membrane. In the present study, staining near both membranes was detected. Further experiments are needed to resolve this discrepancy and localize this channel. Nonetheless, the results of the present study are consistent with CFTR protein expression in haRPE cells and extend our previous findings of CFTR protein expression in cultured fetal RPE cells (39).

Chloride channel function. Previous functional studies of human RPE have identified two functionally distinct chloride channels located in the basolateral membrane, a cAMP-regulated channel and a calcium-regulated channel (28). In the present study, whole cell patch-clamp measurements in primary cultures of haRPE cells demonstrated an outwardly rectifying current that was reduced by replacement of chloride in the bathing solution with iodide or gluconate. In addition, the current was increased by cAMP. These properties are nearly
identical to those previously reported for cultured SV40-transformed hRPE cells (39).

At present, the relationship between regulated membrane chloride conductances in subconfluent RPE cells and specific ClC channels or CFTR channels expressed in RPE cells is unclear. Unfortunately, it was not possible to study the conductance properties of intact sheets of hâRPE in the present study. In the case of CFTR, it is generally believed that this channel is exclusively expressed on the apical membrane in CF-affected airway epithelia (26). However, previous reports of a cAMP-stimulated conductance (28) and the presence of CFTR near or in the basolateral membrane in RPE cells (26) raise the possibility that CFTR may at least partly mediate the basolateral membrane chloride conductance.

The putative apical and/or intracellular location of ClC-5 makes the participation of these channels in the basolateral membrane conductance unlikely. In other epithelial tissues such as the renal proximal tubule and hepatocytes, these channels are located in endosomes beneath the apical surface (8, 21, 31). In mice genetically engineered to be deficient in this channel, apical membrane endocytosis is impaired in proximal tubule cells but not for the sinusoidal membranes of hepatic cells (27). However, the function of these channels in endosomes is not well understood. Because the apical membrane of RPE undergoes a substantial amount of remodeling and endocytosis during phagocytosis, it would be interesting to determine whether the RPE shows defective endocytosis in ClC-5-deficient mice.

ClC-3-deficient mice show normal retinas at birth, but a striking photoreceptor degeneration occurs within the first 2 wk of life (34). The reasons for this loss are not understood. Previous investigators have suggested that ClC-3 may be a swelling-activated channel (9); however, no defects in swelling activated conductances were found in ClC-3-deficient mice (34). Moreover, Li et al. (20) demonstrated that the properties of ClC-3 currents expressed in heterologous systems were distinct from swelling activated currents. Therefore, ClC-3 channels may play a different, but presently unknown, role in the maintenance of photoreceptor viability.

Oxidative stress and antioxidants. Oxidative stress may play a role in RPE aging and in retinal degenerative diseases (2); however, the mechanisms by which oxidants cause damage to RPE and other epithelial cell membranes remain unclear (25). Addition of 100 μM H2O2 resulted in reversible inhibition of the membrane chloride conductance in primary cultures of hâRPE cells. We recently demonstrated (37) that similar concentrations of H2O2 inhibit ClC-5 currents expressed in mammalian somatic cells or Xenopus oocytes and membrane chloride conductances in SV40-transformed hRPE cells. CFTR is also known to be affected by oxidative agents (18, 35). These findings raise the possibility that membrane chloride channels, possibly including CFTR and ClC-5, may be modulated in RPE cells by redox-sensitive mechanism(s).

Role of antioxidants. Antioxidants are known to protect cells from the deleterious effects of oxidative stress. In previous studies, exposure of RPE cells to H2O2 was found to induce mitochondrial DNA damage (1) and apoptosis (15) and to produce an increase in the activity of the antioxidant enzyme GST (32). Preliminary studies using hRPE cells transfected to overexpress GST A1.1 showed protection from oxidant-induced DNA damage and loss of cell viability (B. F. Godley, unpublished observations). Using the same stably transfected cell line, we observed that GST overexpression protects against the immediate inhibitory effects of H2O2 on the membrane chloride conductances. Moreover, untransfected SV40-transformed hRPE cells in the present study were also protected...
against the inhibitory effects of $H_2O_2$ on membrane chloride currents and conductances when they were grown in media supplemented with precursors of glutathione synthesis according to the protocol of Sternberg et al. (33). It will be interesting to determine whether other oxidizing agents have similar inhibitory effects on membrane properties and, if so, whether other antioxidant mechanisms can protect against this inhibition.

In summary, haRPE cells express CIC and CFTR chloride channels, suggesting that the role of these channels is not limited to fetal development. In addition, these cells demonstrate a membrane chloride conductance that is sensitive to cAMP and inhibited by the oxidant $H_2O_2$. Overproduction of the antioxidant enzyme GST or exposure to amino acids that are precursors to glutathione production protected against the effects of $H_2O_2$. The deleterious effects of oxidants on the membrane chloride conductance and the protective effects of antioxidant systems raise the possibility that chloride channel function in RPE cells may be altered in diseases or conditions that increase oxidative damage such as diabetic retinopathy (19) or macular degeneration (2). Such disruptions in RPE chloride channel functions could provide one means by which oxidative stress contributes to pathophysiology associated with the retinal-RPE interface.

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