Mechanism of Inhibitory Actions of Oxidizing Agents on Calcium-Activated Potassium Current in Cultured Pigment Epithelial Cells of the Human Retina

Shwu-Jiuan Sheu[1,2] *and Sheng-Nan Wu*[3,4]

Purpose. To identify the mechanisms by which oxidative stress with oxidizing agents alters the activity of ion channels in human retinal pigment epithelial (RPE) cells.

Methods. The effects of oxidizing agents on ion currents were investigated in human RPE R-50 cells with the aid of the whole-cell, cell-attached, and inside-out configurations of the patch-clamp technique.

Results. In the whole-cell configuration, *t*-butyl hydroperoxide (*t*-BHP; 1 mM), thimerosal (30 μM), and 4,4′-dithiodipyridine (DTDP; 30 μM) suppressed voltage-dependent K^+ current (I_K) that was sensitive to inhibition by iberiotoxin or paxillin, yet not by apamin or 5-hydroxydecanoate sodium. Meclofenamic acid or Evans blue, but not diazoxide, reversed the decrease in I_K caused by *t*-BHP. In cells dialyzed with ceramide (30 μM), neither *t*-BHP (1 mM) nor thimerosal (30 μM) had any effect on I_K, whereas DTDP (30 μM) slightly suppressed it. In cell-attached recordings, *t*-BHP (1 mM), thimerosal (30 μM), and DTDP (30 μM) suppressed the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. Dithiothreitol (10 μM) reversed DTDP-induced decrease in channel activity. Under current-clamp conditions, cell exposure to oxidizing reagents caused membrane depolarization. In cells dialyzed with ceramide (30 μM), membrane potential remained unaltered in the presence of *t*-BHP.

Conclusions. The results demonstrate that hydrophilic oxidants (e.g., *t*-BHP and thimerosal) suppress I_K and suggest that the underlying mechanism of this inhibitory action may involve the generation of intracellular ceramide. However, the inhibition of BK_{Ca} channels by DTDP, a membrane-permeable oxidant, in human RPE cells may result from the direct inhibition of BK_{Ca} channels and indirectly from an increase in the intracellular production of ceramide. (*Invest Ophthalmol Vis Sci.* 2003;44:1237–1244) DOI:10.1167/iovs.02-0330

The retinal pigment epithelium (RPE) is a transporting epithelium that helps regulate the volume and composition of the subretinal space surrounding photoreceptor outer segments.[1] Studies have demonstrated that both the apical and basolateral membranes of the RPE have high K^+ conductances.[2,3] A decrease in K^+ concentration in the subretinal space during light onset produces an apical membrane hyperpolarization and triggers a large efflux of K^+ from RPE cells. As a result, a reciprocal change in the volumes of the RPE cells and subretinal space may occur. It is thus important to elucidate the mechanisms through which both entry and exit of K^+ occur in RPE cells. Numerous types of voltage-dependent K^+ currents have been identified in the RPE.[4]

It has been reported that an excessive production of reactive oxygen species appears to be causally related in the etiology of age-related macular degeneration.[5,6] The RPE is thought to be the prime target of the early development of this disease.[5,7] It has been reported that different types of K^+ channels could be modified by reactive oxygen species.[8–10] Previous investigations have also demonstrated that the oxidizing agents affect the activity of Ca^{2+}-activated K^+ channels in several types of cells, including smooth myocytes, endothelial cells, and chemoreceptor cells.[11–13] Of particular interest, hydrogen peroxide has recently been shown to act on cell membranes to generate ceramide signaling in lung epithelial cells and RPE cells.[14,15] Ceramide is known to be a lipid second messenger that is implicated in the regulation of a variety of cellular responses to extracellular stimuli.[16] Moreover, it has been reported that ceramide, per se, suppresses the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels directly in coronary smooth myocytes and pituitary GH_3 cells.[17,18] A recent report has demonstrated that the oxidizing agents regulate the activity of Cl^- channels in fetal RPE cells.[19] However, to our knowledge, little information has been reported about the effects of the oxidizing agents on K^+ channels in RPE cells.

Therefore, in the present study, the electrophysiological effects of oxidizing agents (e.g., *t*-butyl hydroperoxide [*t*-BHP], thimerosal, and 4,4′-dithiodipyridine [DTDP]) were examined in human RPE R-50 cells. Our specific purposes were to investigate whether oxidizing agents affect the amplitude of ion currents in human RPE cells, determine the effect of these agents on the activity of BK_{Ca} channels, and determine whether the change in BK_{Ca}-channel activity induced by oxidizing agents is mediated by ceramide signal transduction in human RPE cells.

Materials and Methods

Cell Culture

The human RPE R-50 cell line was kindly provided by Dan-Ning Hu (The New York Eye and Ear Infirmary, New York Medical College, New York, NY).[20] Cells were routinely cultured in 5 mL Ham's F-12 medium (Life Technologies, Grand Island, NY) that was supplemented with 10% fetal calf serum and 2 mM L-glutamate (Life Technologies) in a 5% CO_2 atmosphere. The medium was consistently refreshed every 2 days to provide sufficient nutrition. Cell viability was assessed by a colorimetric method with the use of an enzyme-linked immunoadsorbent assay reader (Dynatech, Chantilly, VA). This method was used because

From the Departments of [1]Ophthalmology and [3]Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung City, Taiwan; the [2]National Yang-Ming University, Taipei City, Taiwan; and the [4]Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung City, Taiwan.

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Corresponding author: Sheng-Nan Wu, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, No. 386, Ta-Chung 1st Road, Kaohsiung City, Taiwan; snwu@isca.vghks.gov.tw.

trypan blue, similar to Evans blue, was found to enhance BK_{Ca}-channel activity.[21] The experiments were generally performed 5 or 6 days after cells were subcultured (60%–80% confluence).

Electrophysiological Measurements

Immediately before each experiment, human RPE cells were dissociated, and an aliquot of cell suspension was placed into a recording chamber affixed to the stage of an inverted microscope (Diaphot; Nikon, Inc., Tokyo, Japan). The microscope was coupled to a video camera system with a magnification of up to 1500×, to monitor cell size continually during the experiments. Cells were bathed at room temperature (20–25°C) in normal Tyrode solution containing 1.8 mM $CaCl_2$. Ion currents were measured as described previously.[21,22]—that is, using the whole-cell, cell-attached, and inside-out configurations of the patch-clamp technique, with the aid of a patch-clamp amplifier (RK-400; Bio-Logic, Claix, France). Patch electrodes (3–5 MΩ in bathing solution) were made from borosilicate glass tubing (Kimble Products, Vineland, NJ) using a two-stage vertical microelectrode puller (PB-7; Narishige Scientific, Tokyo, Japan), and the tips were heat-polished with a microforge (MF-83; Narishige).

The signals, consisting of voltage and current tracings, were displayed on a digital storage oscilloscope (model 1602; Gould, Valley View, OH) and a liquid crystal projector (AV600; Delta, Taipei, Taiwan). The data were also stored online in a laptop computer (Pentium III; Lemel, Taipei, Taiwan) via a universal serial bus port at 10 kHz with an analog–digital interface (Digidata 1320A; Axon Instruments, Union City, CA), controlled by commercially available software (pCLAMP 8.0; Axon Instruments). Voltage and current commands were also programmed with the software.

Single-Channel Analysis

Single-channel currents were analyzed using subroutines (Fetchan and Pstat) in the software (pCLAMP; Axon Instruments). Multi-Gaussian adjustments of the amplitude distributions between channels were used to determine unitary currents. The functional independence among channels was verified by comparing the observed stationary probability with the values calculated according to the binomial law. The number of active channels in a patch, n, was taken as the maximum number of channels simultaneously open under conditions of maximum open probability. When a sufficiently large number of independent observations appeared, the opening probabilities of unitary current were evaluated by an iterative process that was continued until the χ^2 result remained unchanged. The single-channel conductance was calculated by linear regression using mean current amplitudes measured at different voltages.

All data are expressed as the mean ± SEM. The paired or unpaired Student's t-test and ANOVA with a least-significant difference method for multiple comparisons were used for the statistical evaluation of differences between means. Differences between the values were considered statistically significant at $P < 0.05$.

Drugs and Solutions

Diazoxide, 4,4′-dithiodipyridine, dithiothreitol, t-BHP, Evans blue, ionomycin, meclofenamic acid, and thimerosal (mercury-[(*o*-carboxyphenyl)thio]ethyl sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). C_2-ceramide (*N*-acetylsphinganine) and 5-hydroxydecanoate sodium were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Iberiotoxin and paxilline were purchased from Alomone Laboratories (Jerusalem, Israel), and dequalinium dichloride was from Tocris (Bristol, UK). All other chemicals were of laboratory grade obtained from standard sources. The twice-distilled water that had been deionized through a filtering system was used in all experiments (Millipore-Q; Millipore, Bedford, MA).

The composition of normal Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES-NaOH buffer 5 (pH 7.4). The internal solution used to record K^+ currents or membrane potential contained (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 3, Na_2GTP 0.1, and HEPES-KOH buffer 5 (pH 7.2). In some experiments, ceramide (30 μM) was dissolved in the pipette solution and applied internally. For single-channel current recordings, high-K^+ bathing solution was composed of (in mM): KCl 145, $MgCl_2$ 0.53, and HEPES-KOH buffer 5 (pH 7.4), and pipette solution contained (in mM): KCl 145, $MgCl_2$ 2, and HEPES-KOH buffer 5 (pH 7.2).

Results

Inhibitory Effect of Whole-Cell K^+ Outward Current by t-BHP, Thimerosal, and DTDP in Human RPE Cells

In the first series of experiments, the whole-cell configuration of the patch-clamp technique was used to investigate the effects of these oxidizing agents on ion currents in these cells. Cells were bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$ and pipette solution contained a low concentration (0.1 mM) of EGTA and 3 mM adenosine triphosphate (ATP). As shown in Figure 1A, when the cell was depolarized from −40 mV to various potentials ranging from −30 to +30 mV in 20-mV increments, a family of outward currents were evoked. When the extracellular Ca^{2+} was removed, the amplitudes of outward currents were greatly reduced throughout the entire voltage step. For example, at the level of +30 mV, the removal of extracellular Ca^{2+} reduced current amplitude from 556 ± 42 to 131 ± 32 pA (n = 8). Figure 1B shows the average current–voltage (*I-V*) relationships for the current amplitude measured at end of voltage pulses in the absence and presence of extracellular Ca^{2+}. An increase in intracellular concentration of EGTA from 0.1 to 10 mM also almost abolished this outward current (data not shown). Iberiotoxin (200 nM) and paxilline (1 μM), two BK_{Ca}-channel blockers, suppressed this outward current effectively. However, neither apamin (200 nM) nor 5-hydroxydecanoate (30 μM) had any effect on it (Fig. 1C). Apamin is a blocker of small-conductance Ca^{2+}-activated K^+ channels, whereas 5-hydroxydecanoate can block ATP-sensitive K^+ channels.[23]

When RPE cells were exposed to t-BHP (1 mM), the amplitude of outward current was greatly reduced throughout the entire voltage-clamp step (Fig. 2). The averaged *I-V* relations for these currents in the absence and presence of t-BHP are illustrated in Figure 2C. Both thimerosal (30 μM) and DTDP (μM) also suppressed this outward current significantly (Fig. 1C). These results indicated that the oxidizing agents could produce an inhibitory effect on outward current in these cells.

To validate the nature of outward currents suppressed by t-BHP, another series of experiments were conducted in bath solution containing the different concentrations of extracellular K^+. The current obtained in the presence of t-BHP (1 mM) was subtracted from that in the control to yield t-BHP–sensitive current. The reversal potential for the t-BHP–sensitive current in each cell was measured. The data for t-BHP–sensitive currents were pooled and plotted as a function of extracellular K^+ concentrations (Fig. 2B). The results revealed a slope of 57 mV per 10-fold increase in extracellular K^+. This can be interpreted to mean that t-BHP–mediated inhibition of outward current in human RPE cells follows the Nernstian behavior of a K^+-selective channel, and rules out the possibility that the t-BHP–suppressed current is from the flow of Cl^- ions.

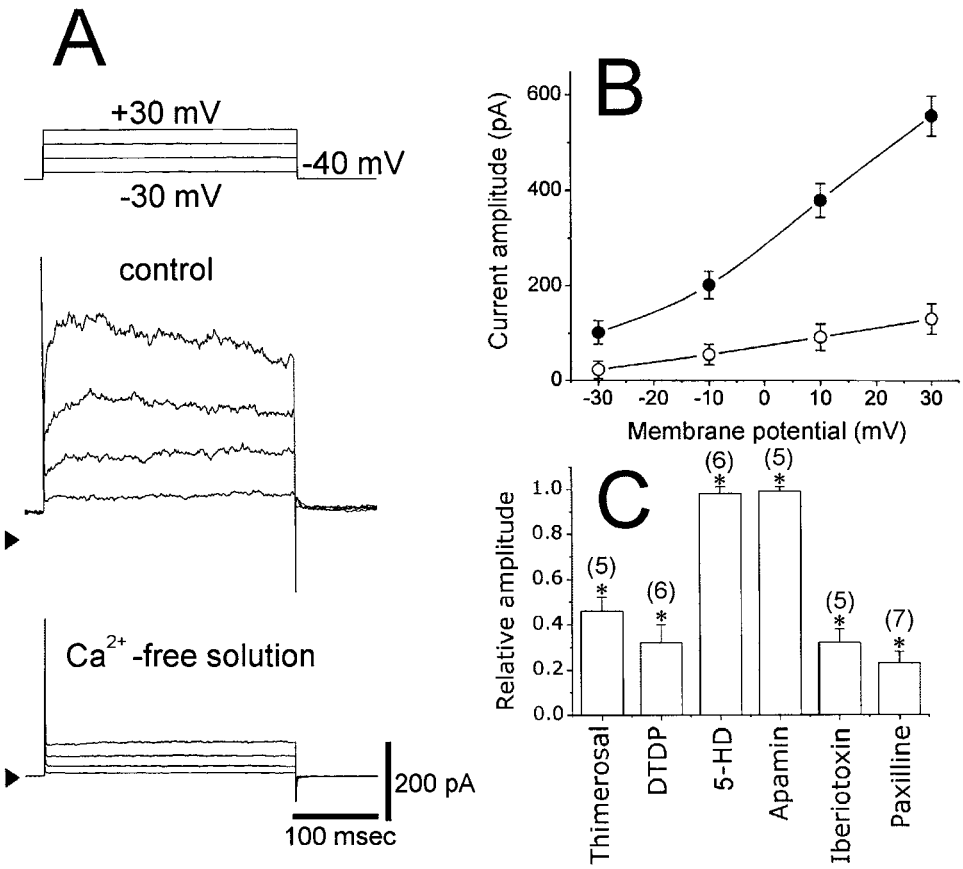

FIGURE 1. Effect of removal of extracellular Ca^{2+} on I_K in human RPE cells. The cells were bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$. The cell was held at −40 mV, and depolarizing pulses from −30 to +30 mV were applied in 20-mV increments. The patch pipettes contained 0.1 mM EGTA. (**A**) *Top*: voltage protocol. *Middle*: superimposed control current traces. *Bottom*: recordings obtained 2 minutes after cells were exposed to Ca^{2+}-free Tyrode solution containing 1 mM EGTA. *Arrowheads*: zero current level. (**B**) *I-V* relationships of I_K measured at the end of depolarizing pulses in the presence (●) and absence (○) of extracellular Ca^{2+} (1.8 mM). Each point represents the mean ± SEM (n = 6–9). (**C**) Effect of thimerosal (30 μM), DTDP (30 μM), 5-HD (5-hydroxydecanoate; 30 μM), apamin (200 nM), iberiotoxin (200 nM), and paxilline (1 μM) on the amplitude of I_K in these cells. Current amplitude measured at the level of +40 mV in the control was considered to be 1.0, and the relative amplitude in the presence of each agent was compared. *Significantly different from the control. The number of cells from which data were obtained is shown above the bars. Mean ± SEM.

Comparison of the Effects of *t*-BHP, Thimerosal, and DTDP on I_K in the Absence and Presence of Meclofenamic Acid, Evans Blue, Diazoxide, and Iberiotoxin

It was further examined whether the depressant effect of *t*-BHP on I_K was affected by the presence of meclofenamic acid or Evans blue, openers of BK_{Ca} channels,[21,24] and diazoxide, an activator of ATP-sensitive K^+ channels. As illustrated in Figure 3A, when the cells were held at −40 mV and ramped from −80 to +40 mV, *t*-BHP (1 mM) significantly reduced the amplitude of I_K. A subsequent application of diazoxide (30 μM) was found to have no effect on *t*-BHP–mediated reduction in current amplitude. In contrast, subsequent application of meclofenamic acid (30 μM) or Evans blue (30 μM) significantly increased the amplitude of I_K after suppression by 1 mM *t*-BHP (Fig. 3B). There was a significant difference in current amplitude between *t*-BHP and *t*-BHP plus meclofenamic acid (30 μM). However, when the cells were depolarized from −40 to +50 mV, no significant difference in current amplitude between *t*-BHP (1 mM) and *t*-BHP plus diazoxide (30 μM) was observed (201 ± 18 vs. 203 ± 21 pA, n = 6). Also, a subsequent application of iberiotoxin (200 nM) had no effect on the amplitude of I_K after suppression by *t*-BHP. Thus, taken together, these results suggest that the component of I_K that is sensitive to inhibition by *t*-BHP is Ca^{2+}-activated K^+ current, and that inhibition by *t*-BHP can be overcome by stimulation with meclofenamic acid or Evans blue.

Effect of *t*-BHP, Thimerosal, and DTDP on BK_{Ca}-Channel Activity in Human RPE Cells

To determine how these oxidizing agents act to affect I_K, their effects on BK_{Ca}-channel activity were further investigated. When cells were bathed in 145 mM K^+ solution and recorded in the cell-attached configuration, the activity of a channel with a unitary conductance of 168 ± 12 pS (n = 8) was observed at depolarized potentials. The application of ionomycin (10 μM), a Ca^{2+} ionophore, increased the open probability from 0.018 ± 0.002 to 0.037 ± 0.005 (n = 6). These properties confirm the identity of the channel as the BK_{Ca} channel.[4,10] When *t*-BHP (1 mM), thimerosal (30 μM), or DTDP (30 μM) was added to the bath, the channel activity decreased (Fig. 4). The open probability of BK_{Ca} channels at +60 mV in the control condition was found to be 0.018 ± 0.002 (n = 8). One minute after the cells were exposed to *t*-BHP (1 mM), the probability was significantly reduced to 0.011 ± 0.001 (n = 6). However, the amplitude of single-channel currents remained unaltered (10.0 ± 0.2 vs. 9.9 ± 0.2 pA, n = 6). Similarly, thimerosal (30 μM) and DTDP (30 μM) substantially decreased the probability of channel openings without altering single-channel amplitude. Thus, it is apparent that the challenge of cells with these oxidizing agents can suppress the activity of BK_{Ca} channels present in human RPE cells.

Lack of Effect of *t*-BHP on I_K in Cells Dialyzed with Ceramide

It has been reported that ceramide suppresses the activity of BK_{Ca} channels[17,18] and that the challenge of cells with oxidizing agents could be associated with an increase of intracellular ceramide.[15] Therefore, an additional series of whole-cell experiments were designed to examine the effect of *t*-BHP on I_K in cells that had been dialyzed with ceramide (30 μM). As shown in Figure 5, current amplitudes measured at the end of voltage pulses were gradually reduced immediately after rupture of the membrane. Intracellular dialysis with ceramide (30 μM) signif-

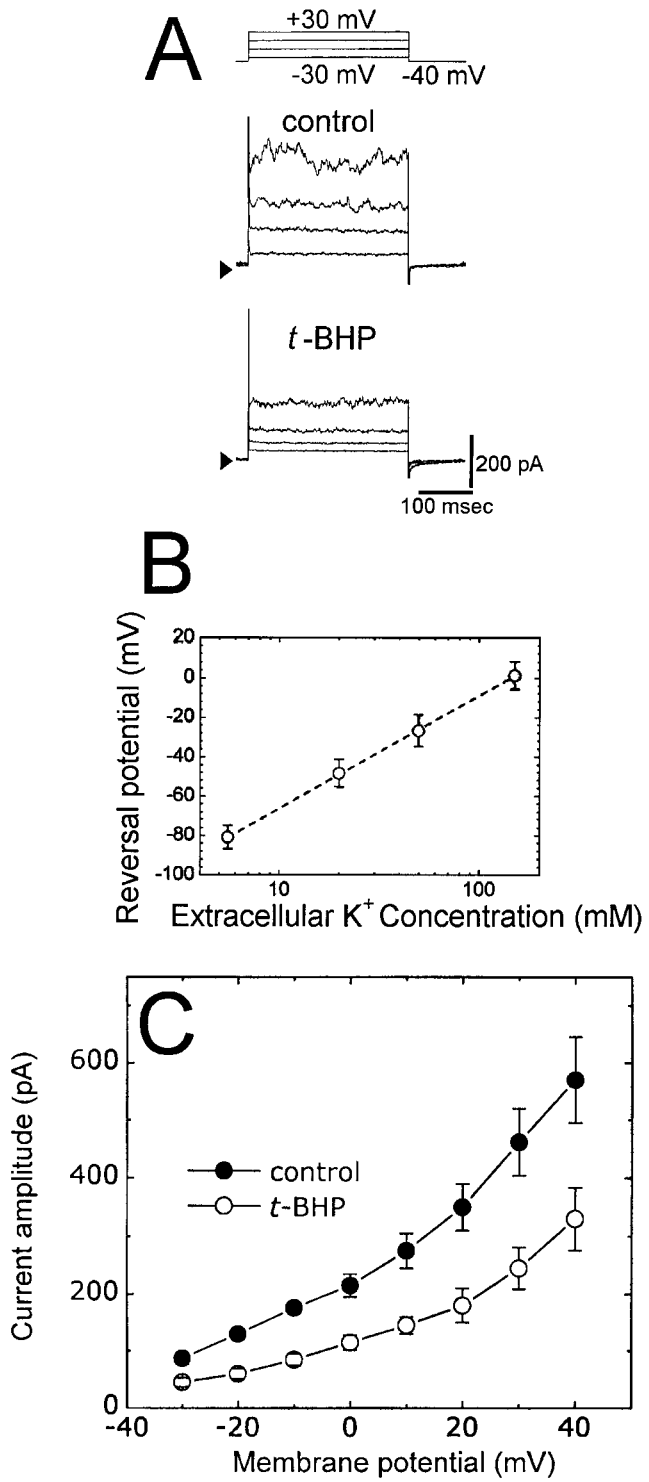

FIGURE 2. Inhibitory effect of *t*-BHP on I_K in human RPE cells. In these experiments, human RPE cells were bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$. (**A**) *Top*: voltage protocol. *Middle*: control traces. *Bottom*: Original current tracings obtained when the cell was held at the level of −40 mV and the voltage pulses were delivered from −30 to +30 mV in 20-mV increments. (**B**) The relationship between reversal potential of *t*-BHP–inhibited current and extracellular concentration of K^+ ions. Each cell was exposed to *t*-BHP (1 mM), and each patch pipette was filled with K^+-containing solution. Each point represents the mean ± SEM (n = 6–8). (**C**) *I-V* relationships of I_K in the absence and presence of *t*-BHP (1 mM). Each point represents the mean ± SEM (n = 7–11).

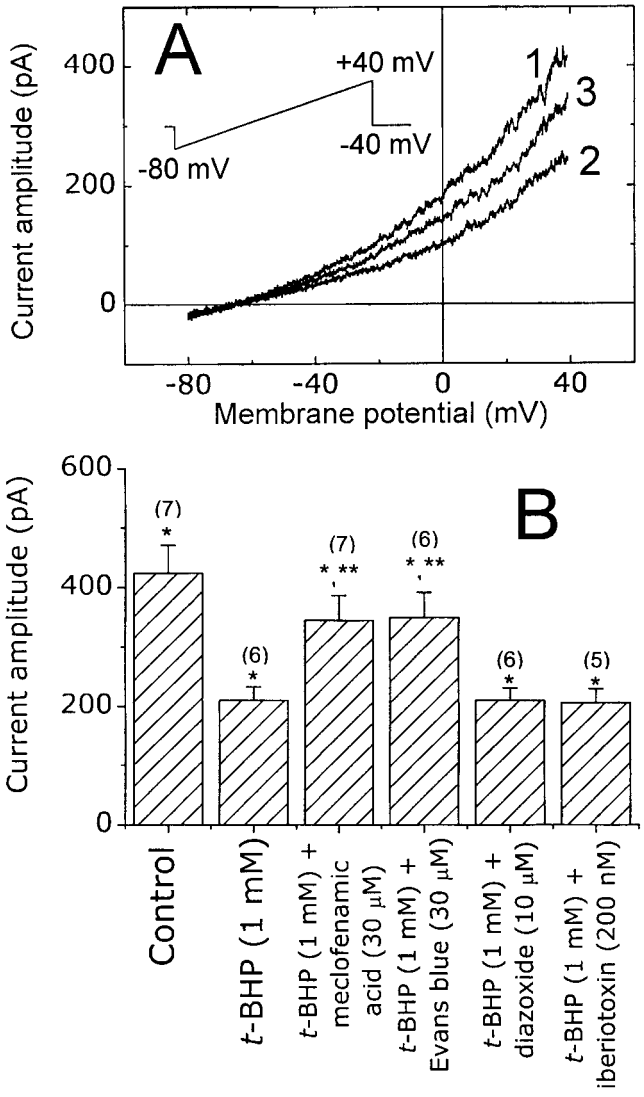

FIGURE 3. Effect of various related compounds on the inhibition of I_K caused by *t*-BHP in human RPE cells. In these experiments, cells were bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$. (**A**) Original current tracings obtained when the cell was held at −40 mV and the ramp pulses from −80 to +40 mV with a duration of 1 second were applied. 1: control; 2: *t*-BHP (1 mM); 3: *t*-BHP (1 mM) plus meclofenamic acid (30 μM). (**A**, *left*) Voltage protocol. (**B**) Effect of meclofenamic acid, Evans blue, diazoxide, and iberiotoxin on current amplitude decreased by *t*-BHP. Mean ± SEM. *Significantly different from control. **Significantly different from *t*-BHP alone group. The number of cells from which data were obtained is shown above the bars.

icantly decreased the amplitude of I_K at +40 mV from 538 ± 23 to 398 ± 19 pA (n = 5). When *t*-BHP (1 mM) or thimerosal (30 μM) was then applied to the cells, no further effects on the amplitude of I_K were seen, although subsequent application of DTDP (30 μM) caused a slight reduction of I_K from 387 ± 19 to 343 ± 18 pA (n = 5). Notably, both *t*-BHP and thimerosal are water-soluble sulfhydryl oxidizing agents, whereas DTDP is a membrane-permeable lipophilic oxidant. These results lead us to propose that the inhibitory effect of these oxidizing agents on I_K in these cells could be associated with alteration in the level of intracellular ceramide. The observed inhibition of I_K in the cells loaded with ceramide could be due to the direct effect of DTDP on I_K.

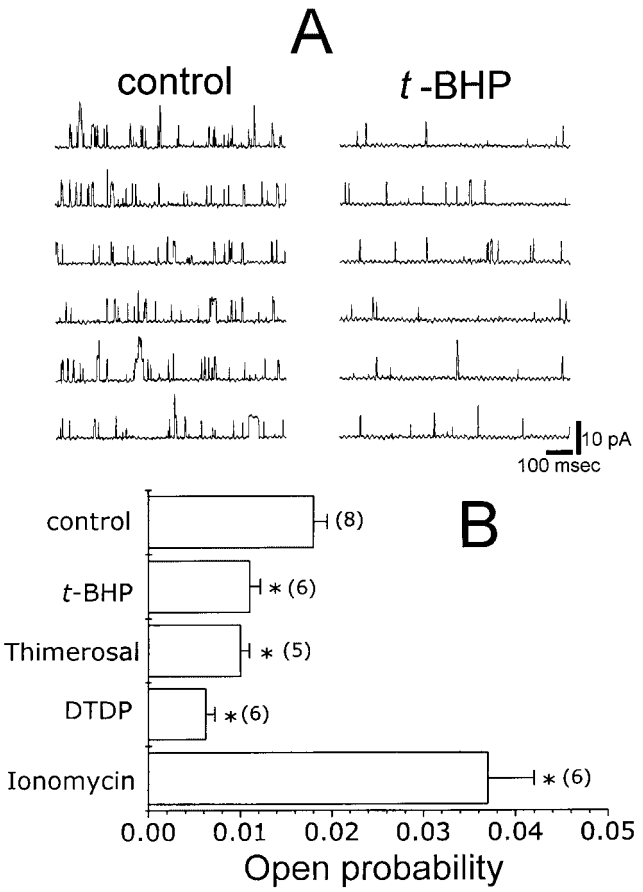

FIGURE 4. Effect of *t*-BHP on the activity of BK_{Ca} channels in cell-attached patches from human RPE cells. In these experiments, cells were bathed in 145 mM K^+ solution, and the holding potential was set at +60 mV. (**A**) Original current tracings obtained before (*left*) and during (*right*) cell exposure to *t*-BHP (1 mM). *t*-BHP was applied to the bath containing 1.8 mM $CaCl_2$. (**B**) Effect of *t*-BHP (1 mM), thimerosal (30 μM), DTDP (30 μM), and ionomycin (10 μM) on the activity of BK_{Ca} channels recorded from cell-attached patches. Each compound was applied to the bath. Mean ± SEM. *Significantly different from control. The number of cells from which data were obtained is shown next to the bars.

Effect of *t*-BHP, Thimerosal, DTDP, and Ceramide on BK_{Ca}-Channel Activity in Excised Membrane Patches of Human RPE Cells

To determine the direct effect of these compounds on BK_{Ca} channels, single-channel experiments in the inside-out configuration were also performed. In this configuration, bath application of DTDP (30 μM) suppressed the activity of BK_{Ca} channels. However, hydrophilic oxidants (e.g., *t*-BHP and thimerosal) and dequalinium dichloride, an inhibitor of small-conductance Ca^{2+}-activated K^+ channels,[25] had no effects on BK_{Ca}-channel activity, whereas bath application of ceramide (30 μM) and paxilline (1 μM) led to a significant decrease in the probability of channel openings by 66% and 85%, respectively (Fig. 5C). Moreover, meclofenamic acid (30 μM), an opener of BK_{Ca} channels,[24] or dithiothreitol (10 μM), a sulfhydryl-reducing agent, clearly reversed DTDP- or ceramide-induced decrease in channel activity. The relative open probability observed in the DTDP-alone group was significantly lower than that in the DTDP-plus-dithiothreitol group (0.45 ± 0.05 [n = 8] versus 0.82 ± 0.06 [n = 6]). On the basis of these observations, it is suggested that unlike *t*-BHP and thimerosal, DTDP and ceramide are able to suppress the activity of BK_{Ca} channels directly in human RPE cells. This inhibitory effect seems to be the result of a direct binding to the inner surface of the channel.

Effect of *t*-BHP on Membrane Potential in Human RPE Cells with or without Intracellular Dialysis of Ceramide

To determine whether these compounds affect the membrane potential of human RPE cells, experiments were conducted under current-clamp conditions. Cells, bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$, had a resting membrane potential of −46 ± 6 mV (n = 14). When cells were exposed to *t*-BHP (1 mM), the membrane potential depolarized to −34 ± 5 mV (n = 5). Subsequent application of meclofenamic acid (30 μM) hyperpolarized the cells back to −43 ± 5 mV (n = 5). The typical effect of *t*-BHP and meclofenamic acid on membrane potential in human RPE cells is illustrated in Figure 6.

Additional experiments were made in cells that were loaded with ceramide by including the compound (30 μM) in the filling solution of the recording electrode. In this condition, the membrane potential progressively depolarized after rupture of the membrane. Intracellular dialysis with ceramide (30 μM) significantly depolarized the cells from −43 ± 6 to −33 ± 5 mV (n = 5). Of note, 2 minutes after intracellular dialysis with ceramide, the application of *t*-BHP (1 mM) to the bath caused no effect on membrane potential (−33 ± 5 versus −33 ± 4 mV, n = 5). However, similar to the conditions in which cells were not dialyzed with ceramide, a subsequent application of meclofenamic acid clearly hyperpolarized the cells (Fig. 6B). Thus, dialysis with ceramide abolished the *t*-BHP–induced membrane depolarization but did not interfere with the ability of meclofenamic acid to hyperpolarize the membrane.

DISCUSSION

BK_{Ca} channels have been found in many tissues and can participate in a variety of cellular processes, including K^+ transport. These channels are made open both by binding of intracellular Ca^{2+} and by membrane depolarization.[26] In this study, the single-channel conductance of BK_{Ca} channels in human RPE cells measured with the use of 145 mM K^+ on both sides of the membrane was 168 ± 12 pS (n = 8). This value is similar to those of typical BK_{Ca} channels described previously in RPE cells.[4] More important, in this study, we also found out that the activity of BK_{Ca} channels present in human RPE cells could be modified by chemical injuries associated with oxidative stress.

There is immunohistochemical evidence showing that ATP-sensitive K^+ (K_{ATP}) channels are present in the retina.[27] These channels are blocked by 5-hydroxydecanoate, and stimulated by diazoxide.[28] It might be expected that the decrease in the amplitude of I_K caused by oxidizing agents in RPE cells is due to the blockade of K_{ATP} channels, as described previously in skeletal muscle cells.[29] However, 5-hydroxydecanoate had no effect on the amplitude of I_K, and the inhibition of I_K by oxidizing agents could be reversed by meclofenamic acid or Evans blue, yet not by diazoxide. Taken together, the decrease of I_K caused by oxidizing agents is mainly explained by their inhibition of BK_{Ca} channels, not K_{ATP} channels.

It has been reported that in an insulin-secreting cell line, hydrogen peroxide could cause an increase in intracellular Ca^{2+}.[30] In this study, by examining the effects of a direct application of DTDP to the intracellular surface of BK_{Ca} channels, we have shown that functional activity of these channels can be modulated by changes in their redox environment.

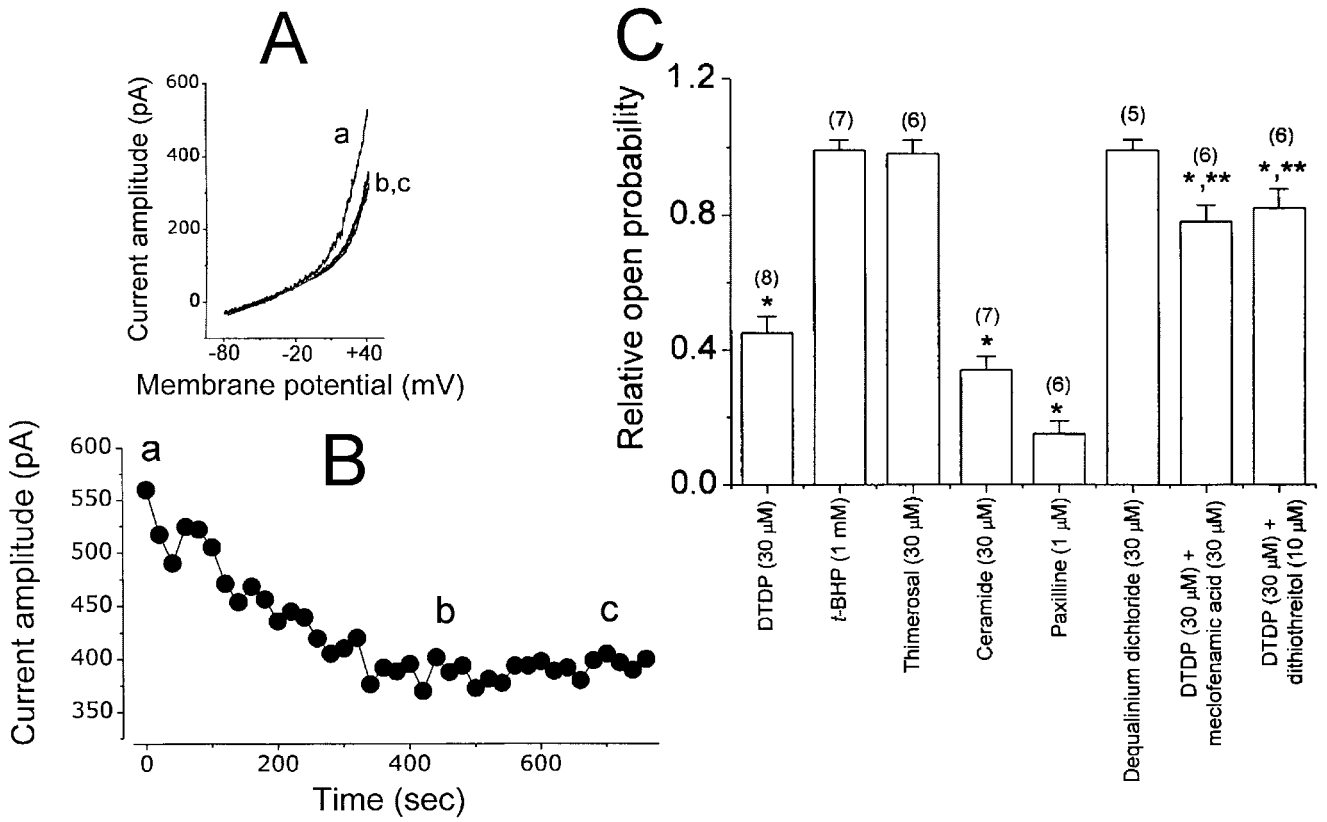

FIGURE 5. Effect of *t*-BHP on I_K caused by loading the cells with ceramide (30 μM) in the recording electrode (**A**, **B**) and effect of various related compounds on the activity of BK_{Ca} channels in excised membrane patches of human RPE cells (**C**). In these experiments, human RPE cells were bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$. The cells were held at −40 mV and the ramp pulses from −80 to +40 with a duration of 1 second were applied at a rate of 0.05 Hz. (**A**) Superimposed current tracings obtained in response to the ramp pulses correspond to the time points and amplitudes marked (a), (b), and (c) in (**B**). (**B**) Time course in the change of current amplitudes measured at the level of +40 mV in response to each ramp pulse. Current amplitudes were measured immediately after rupture of membrane. Note difference in *y*-axis scales in (**A**) and (**B**). (**C**) Effect of various related compounds on BK_{Ca} channels recording from excised membrane patches of human RPE cells. Cells were bathed in 145 mM K^+ solution. Each membrane patch was held at +60 mV, and bath medium contained 0.1 μM Ca^{2+}. The channel activity in the control was considered to be 1.0, and the relative open probability in the presence of each agent was compared. Mean ± SEM. *Significantly different from control. **Significantly different from DTDP (30 μM) alone group. The number of cells from which data were obtained is shown above the bars.

Consistent with previous observations,[11,12] DTDP, a lipophilic oxidizing agent, reduces the activity of BK_{Ca} channels without a change in single-channel conductance, whereas the reducing agent dithiothreitol can reverse DTDP-induced decrease in BK_{Ca} channel open probability. This finding may be interpreted to mean that the DTDP-mediated decrease in channel activity is unrelated to the reduced availability of intracellular Ca^{2+}. Although the mechanism of action of DTDP has not yet been determined, the fact that it can suppress BK_{Ca}-channel activity in inside-out patches suggests that this compound either directly modulates the channel or at least interacts with a closely associated structure.

Ceramide, a product of sphingomyelin turnover, is known to be a lipid second messenger that is involved in the regulation of several cellular responses to extracellular stimuli, including differentiation, growth suppression, cell senescence, and apoptosis.[16] Ceramide can be generated within the cell through the hydrolysis of sphingomyelin or de novo synthesis. Recently, an increase in ceramide level within RPE cell in response to oxidative stress has been found.[5,15] Of particular interest, the ability of ceramide to mimic the inhibitory action of *t*-BHP or thimerosal on I_K shown in this study led us to speculate that the observed effects of *t*-BHP, thimerosal, and ceramide on the inhibition of I_K in human RPE cells may involve intracellular signal transduction through a similar mechanism. Ceramide may be a mediator of *t*-BHP- or thimerosal-induced change in BK_{Ca}-channel activity. Sphingomyelin is thought to be preferentially concentrated in the plasma membrane of all mammalian cells.[31] Sphingomyelin hydrolysis can take place within seconds to minutes after the activation of sphingomyelin-specific sphingomyelinases. As a result, the level of endogenous ceramide may be increased. In contrast, de novo synthesis of ceramide always occurs over a period of several hours. The time course for the *t*-BHP- or thimerosal-induced decrease in channel activity observed in this study was noted be only seconds. Therefore, assuming that the inhibition of BK_{Ca} channels by hydrophilic oxidants is mediated by endogenous ceramide, it is likely that sphingomyelin hydrolysis, rather then de novo generation of ceramide, is a primary pathway responsible for its generation.

The data presented herein demonstrate that DTDP, a lipophilic oxidant, may exert a dual effect on the activity of BK_{Ca} channels expressed in human RPE cells. DTDP may act indirectly by stimulating sphingomyelin hydrolysis, leading to an elevation of intracellular ceramide. This mechanism is suggested by the observation that the effects of DTDP can be mimicked by intracellular dialysis with ceramide. DTDP, however, appears to interact with the channel protein(s) indepen-

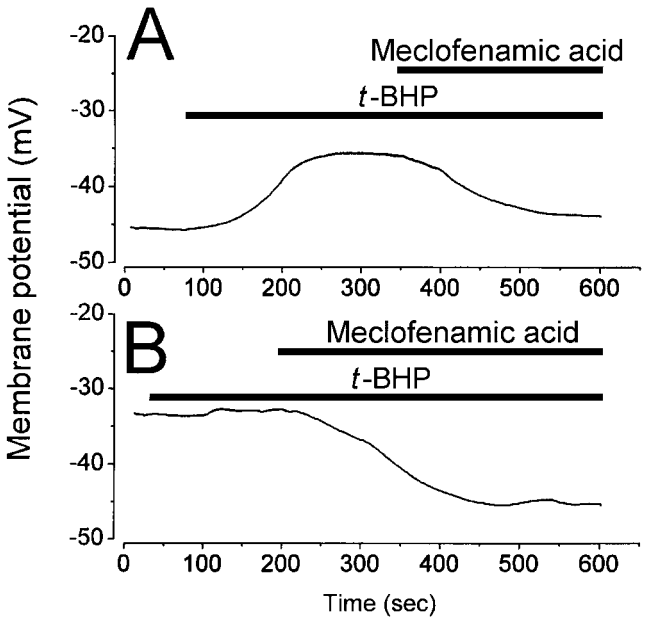

FIGURE 6. Effect of *t*-BHP on membrane potential in human RPE cells. In a current-clamp configuration, cells were bathed in normal Tyrode solution containing 1.8 mM $CaCl_2$. (**A**) Original tracing of a potential showing an effect of *t*-BHP (1 mM) and meclofenamic acid (30 μM) on the change in membrane potential of human RPE cells. (**B**) Original tracing of a potential showing no effect of *t*-BHP (1 mM) on membrane potential in a cell dialyzed with ceramide (30 μM). In this experiment, ceramide (30 μM) was included in the recording electrode, and membrane potential was measured 5 minutes after rupture of the membrane. *Horizontal bars*: time course of application of *t*-BHP (1 mM) or meclofenamic acid (30 μM).

dent of intracellular ceramide or Ca^{2+}. The modulation of thio groups may well constitute a physiologically relevant control mechanism of BK_{Ca} channel function in human RPE cells.

Oxidizing agents were also found to modulate the membrane potential of human RPE cells in this study. Sustained exposure to *t*-BHP resulted in depolarization. In addition, meclofenamic acid reversed *t*-BHP–induced membrane depolarization. These results are compatible with those from voltage-clamp experiments showing that meclofenamic acid counteracted the reduction of I_K induced by oxidative stress with *t*-BHP or thimerosal. Of particular interest, in cells dialyzed with ceramide, the level of resting potential remained unaltered in the presence of *t*-BHP. This result strengthens the idea that *t*-BHP and ceramide may involve a similar mechanism to suppress BK_{Ca}-channel activity. However, it has yet to be determined whether openers of BK_{Ca} channels are therapeutically beneficial in the treatment of age-related macular degeneration.[32]

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