UV-Induced Corneal Epithelial Cell Death by Activation of Potassium Channels

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PURPOSE. The purpose of the present study is to determine the role of K⁺ channel activity as an early event in UV-induced corneal epithelial cell apoptosis.

METHOD. Both cell-attached and nystatin-perforated patch-clamping were performed to record K⁺ channel activity in rabbit corneal epithelial (RCE) and primary cultured rabbit corneal epithelial (PRCE) cells exposed to UV irradiation. On exposure of corneal epithelial cells or intact corneas to UV-C irradiation or treatment of corneal epithelial cells with etoposide, cell apoptosis was determined by DNA fragmentation, ethidium bromide-acridine orange nuclear stain and TdT-mediated dUTP nick-end labeling (TUNEL).

RESULTS. In the present study, UV-irradiation-induced corneal epithelial cell apoptosis through activation of a K⁺ channel in the cell membrane was an early event in response to UV irradiation. UV-C irradiation (42 μJ/cm²) activated robust K⁺ channel activity in RCE and PRCE cells at both the single-channel and whole-cell levels, when measured with the cell-attached and nystatin-perforated patch clamps, respectively. Suppression of UV-irradiation-induced K⁺ channel activity with the specific K⁺ channel blocker 4-aminopyridine (4-AP) prevented UV-irradiation-induced apoptosis in the RCE and PRCE cells, loss of the superficial layer of corneal epithelium, and apoptosis in the basal layer corneal epithelium. However, suppression of K⁺ channel activity did not protect RCE and PRCE cells from etoposide, a topoisomerase II inhibitor, which induced cell death by bypassing the membrane. Furthermore, application of valinomycin, a K⁺ ionophore, to mimic the effect of mass activation of the K⁺ channel in RCE and PRCE cells caused cell apoptosis.

CONCLUSIONS. The results indicate that UV irradiation induces superactivity of K⁺ channels in the membrane is an early event mediating signaling transduction and resulting in corneal epithelial cell death in response to UV irradiation. (Invest Ophthalmol Vis Sci. 2003;44:5095–5101) DOI:10.1167/iovs.03-0590

Corneal epithelial renewal continuously replaces terminally differentiated cells in its superficial layer as they are being sloughed off. Their replacement assures the maintenance of corneal epithelial barrier function, which provides a protective mechanism against noxious agents and infection. Effective corneal epithelial barrier function is also dependent on the maintenance of the integrity of the epithelial cell membranes and the tight junctions between the cells in the outermost layer facing the tears. The physiological balance between the ability of the corneal epithelium to proliferate and programmed cell death (apoptosis) contributes to the maintenance of corneal deturgescence, transparency, and normal vision. There is definitive evidence that UV irradiation at wavelengths in the range from 280 to 310 nm induces apoptosis in the corneal epithelium based on TUNEL-positive staining in the intact rabbit cornea. Recently, it has been shown that exposure to UV is the leading cause of post-PRK corneal epithelial detachment and death. There is recent biophysical evidence showing that UV light penetrates and is differentially absorbed by ocular tissues. Their absorption pattern in the anteroposterior direction follows a wavelength-dependent decay. The corneal epithelium absorbs UV wavelengths shorter than 310 nm (UV-B between 290 and 320 nm and UV-C between 200 and 290 nm), thereby acting as a filter and protecting the lens and retina from UV-induced damage. In doing so the corneal epithelium and lens protect the retina from UV-irradiation-induced damage. In addition, repeated subthreshold or single-threshold UV exposures lead to inhibition of mitosis, nuclear fragmentation, and loss of the whole corneal epithelial layer. Disturbances in epithelial structure or function can lead to infection, development of corneal opacity, and loss of vision.

It has been shown that a hallmark of apoptosis is cell shrinkage. A significant increase in K⁺ efflux is another event that occurs before apoptosis-induced cell shrinkage. Recent evidence implies that apoptosis-induced K⁺ channel activation plays a critical role in eliciting excessive K⁺ efflux or intracellular K⁺ depletion. Blocking K⁺ efflux by a K⁺ channel blocker or by increasing extracellular K⁺ inhibits shrinkage and apoptosis in human eosinophil, human myeloid HL-60 cells, and mouse cortical neurons. On the contrary, increasing K⁺ efflux and intracellular K⁺ depletion activates interleukin (IL)-1β-converting enzyme in macrophages and monocytes. Lowering the intracellular K⁺ concentration activates caspase-3-like proteases and apoptosis. Furthermore, neuronal apoptosis induced by serum deprivation, staurosporine, β-amyloid peptide, or ceramide enhances an outward K⁺ current, which is inhibited by a K⁺ channel blocker and by increasing the extracellular concentration of K⁺. Recently, we found that UV irradiation induces apoptosis in human myeloblastic leukemia cells through activation of a membrane K⁺ channel. Thus, we investigated in corneal epithelial cells whether UV irradiation activates K⁺ channels and induces RCE cell apoptosis. Measurements of currents at the single-channel and whole-cell levels revealed that UV irradiation activates a K⁺ channel. Blockade of this UV-induced K⁺ channel activity with 4-aminopyridine (4-AP) completely prevented UV-induced apoptosis in corneal epithelial cells and in intact rat corneal epithelium.

METHODS

Culture of Primary and SV40-Immortalized Corneal Epithelial Cells

Primary cultured rabbit corneal epithelial (PRCE), SV40–transformed rabbit corneal epithelial (RCE), and human corneal epithelial (HCE)
cells were grown in DMEM/F-12 culture medium containing 10% fetal bovine serum, 5 μg/mL insulin, and 10,000 U/mL penicillin and 10,000 μg/mL streptomycin and maintained in an incubator supplied with 95% air and 5% CO₂ at 37°C. The medium was replaced every 2 days, and cells were passed by treatment with 0.05% trypsin-EDTA.

**Patch-Clamp Analysis**

Patchpipettes with a resistance of 3 to 4 MΩ when filled with 150 mM KCl solution were manufactured with a two-stage puller (PP-83; Narishige, USA, Inc., Greenvai, NY). For whole-cell K⁺ current recording, the nystatin-perforated-patch technique was used. This technique provides stable measurements without disrupting cytoplasmic concentrations of divalent cations or metabolites. The pipette tip was filled with a solution containing 140 mM KCl, 2 mM MgCl₂, 0.5 mM CaCl₂, 2 mM adenosine triphosphate (ATP), 0.05 mM guanosine triphosphate (GTP), 1 mM EGTA, and 10 mM HEPES (titrated with KOH to pH 7.2). The remainder of the pipette was backfilled with the same pipette solution and contained in addition 200 g/mL nystatin. The bath solution was composed of 140 mM NaCl, 2 mM KCl, 1 mM CaCl₂, and 10 mM HEPES (pH 7.4). A patch-clamp amplifier (200A; Axopatch; Axon Instruments, Inc., Foster City, CA) was used to make whole-cell current recordings. Data were collected and analyzed with the accompanying software (pCLAMP; Axon Instruments, Inc., for the cell-attached single channel patch clamp, solutions in both pipettes and in the patch and Po represents the open-channel probability. All experiments were performed at room temperature (21–23°C).

**Apoptosis Induction**

For UV irradiation experiments, confluent cells or corneas were placed in a tissue culture hood at a distance of 60 inches from the UV-C light source and exposed at an intensity of 45 μW/cm². For exposure to etoposide (an apoptosis inducer), a stock solution of 10 mg/mL etoposide was added to the culture medium at a final concentration of 20 μg/mL. After etoposide and UV treatments, cells were incubated at 37°C in 5% CO₂ for 15 to 24 hours, followed by measurements of cell viability and DNA fragmentation.

**Measurement of DNA Fragmentation**

RCE cells were washed twice with PBS. Lysis buffer (200 mM Tris-HCl [pH 8.0], 100 mM EDTA, 1% SDS, and 100 μg/mL proteinase-K) was added, and cells were then incubated for 4 hours at 55°C. The nuclear lysates were extracted twice with an equal volume of phenol and then extracted with an equal volume of phenol-chloroform-isooamyl alcohol (25:24:1). DNA was precipitated with 0.05 volume of 5 M NaCl and 2.5 volumes of absolute ethanol, incubated overnight at –20°C, and centrifuged at 13,000 g for 10 minutes at 4°C. The DNA pellet was dried and dissolved in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing 20 μg/mL RNase A and incubated for 1 hour at 37°C. The DNA was extracted with an equal volume of phenol-chloroform-isooamyl alcohol (25:24:1). DNA samples were analyzed by electrophoresis on 1.5% agarose gels, and the results were visualized by staining with 1 μg/mL ethidium bromide.

**Nuclear Staining with Ethidium Bromide and Acridine Orange**

Cell nuclear staining was performed to detect nuclear DNA condensation by adding a dye mixture containing 100 μg/mL of each ethidium bromide and acridine orange (EB/AO) to a cell culture dish at a final concentration of 3×10⁻⁵ M. Cell populations were scored according to color by using a UV-fluorescence microscope (Nikon, Tokyo, Japan). Nuclei staining green have not lost membrane integrity. In contrast, corneal epithelial cells in which the nuclei stain orange have lost membrane integrity. Apoptotic cells can be distinguished from non-apoptotic cells on the basis of the absence or presence of nuclear condensation and fragmentation.

**TUNEL Staining of Rat Cornea**

Intact corneas were rapidly excised from adult albino rats after decapitation. After a cold PBS wash, the corneas were transferred to 50:50 DMEM/F12 containing 1% FBS and preincubated in a CO₂ incubator at 37°C for 1 hour. After UV exposure, corneas were cultured for 8 to 12 hours. Corneas were fixed in 4% formaldehyde, embedded in paraffin wax and cut into 10-μm sections. Apoptotic cells in the cornea sections were detected by labeling 5'-OH DNA ends of fragmented DNA of apoptotic cells with a TUNEL kit (Promega, Madison, WI). Briefly, the cornea sections were rehydrated by immersion in a xylene series—100%, 95%, 85%, 70%, and 50% ethanol—and fixed in 4% paraformaldehyde. The sections were permeabilized with protease-K (20 μg/mL in PBS) for 20 minutes. After equilibration with a buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM dithiothreitol [DTT], 0.25 mg/mL BSA, and 2.5 mM cobalt chloride), apoptotic cells were labeled by incorporation of a biotinylated nucleotide DAN sequence using the TdT enzyme for 60 minutes. After incubation of tissue sections with 0.3% hydrogen peroxide for 5 minutes to block endogenous peroxidase, horseradish-peroxidase-labeled streptavidin was bound to the biotinylated nucleotides. Finally, peroxidase substrate, hydrogen peroxide, and diaminobenzidine (DAB), were used to detect nuclei of apoptotic cells. Conventional hematoxylin and cosin (H-E) staining was also performed on each cornea to that its histologic structures were intact.

**RESULTS**

**UV Irradiation-Stimulated K⁺ Channel Activity**

Changes in cell membrane K⁺ channel activity can mediate functional adaptation to a variety of chemical and physical stresses through membrane voltage stabilization and maintenance of salt and water balance. Using the nystatin-perforated whole-cell patch clamp, the whole-cell current was recorded from a holding potential of 60 to +60 mV in 20-mV increments. When the RCE cells were exposed to UV-C light for 1 minute, the amplitude of the K⁺ current was markedly increased. The UV-evoked K⁺ current was sensitive to 4-AP and was completely blocked by 1 mM 4-AP (Fig. 1A). The time course showed that the amplitude of the K⁺ current was doubled within 1 minute after exposure to UV light and reached its maximum amplitude within 5 minutes (data not shown). K⁺ currents in response to UV stimulation were plotted as a function of membrane potentials in the presence and absence of 1 mM 4-AP (Fig. 1B). Normalized K⁺ currents in the presence and absence of 1 mM 4-AP were determined by the fractions of UV-induced current amplitudes and currents measured in control patches (IUV / IC), where IUV and IC represent currents measured with and without UV irradiation, respectively (Fig. 1C).

To further confirm the effect of UV irradiation on single K⁺ channel activity, the cell-attached patch clamp was used. Single K⁺ channel activity (NPo) was recorded at a membrane potential of −60 mV in vivo. Exposure of cells to UV irradiation (~45 μJ/cm²) strongly evoked K⁺ channel activity (Fig. 2A). Single-
channel activity was suppressed by application of 100 μM 4-AP in the patch pipette. UV irradiation failed to activate K⁺ channel activity in the presence of 4-AP (Fig. 2B). Time courses of UV-induced increase of K⁺ channel activity were plotted in the presence and absence of 4-AP (Fig. 2C). UV induced a rapid increase in K⁺ channel activity with a time constant of 55 seconds, and 100 μM 4-AP completely blocked UV-induced K⁺ channel activity. Statistical analysis of NPo was performed in 18 independent patches, with and without 4-AP in the patch pipette. During UV irradiation, K⁺ channel activity was increased from 17% ± 3.6% to 67% ± 7.6% within 1 minute; however, K⁺ channel activity was markedly suppressed by application of 100 μM 4-AP in the patch pipette in the control and UV-stimulated cells (Fig. 2D). These results suggest that an early effect of UV irradiation is the direct stimulation of cell membrane K⁺ channel activity in RCE cells.

**Effect of Suppression of K⁺ Channel Activity**

To determine whether UV-induced K⁺ channel hyperactivity is a component of the cell signaling pathway that mediates UV-induced apoptosis, the effect of blocking K⁺ channel activity with the K⁺ channel inhibitor 4-AP was determined by measuring cell viability after UV irradiation in the presence or absence of 4-AP. The protective effect of 4-AP against UV- and etoposide-induced apoptosis was evaluated by measuring nuclear condensation based on the extent of nuclear EB/AO staining. Exposure of RCE and HCE cells to UV irradiation and etoposide resulted in decrease cell viability determined by orange-stained nuclei, which indicated nuclear death (Fig. 3). Suppression of K⁺ channel activity with 4-AP protected these cells from UV-irradiation-induced nuclear death, whereas it was ineffective in preventing etoposide-induced nuclear death. In the presence of 1 mM 4-AP, RCE cells were well protected from UV irradiation (98.7% ± 1.5% for control, 96.1% ± 2.0% for 4-AP alone, 36.3% ± 1.6% for UV-induced, and 91.9% ± 0.6% for UV+4-AP; Fig. 3A). Other groups of RCE cells were treated with another apoptosis inducer, etoposide (an inhibitor of topoisomerase II). 4-AP had no protective effect on etoposide-induced cell death. With etoposide alone, viability decreased to 45.0% ± 6.7%. This decline was indistinguishable from the effect of etoposide measured in the presence of 4-AP (42.7% ± 2.4%; Fig. 3A). In addition, after exposure of HCE cells to UV irradiation, the percentage of viable cells changed from 99.5% ± 0.5% for control to 99.1% ± 0.7% for 4-AP alone, 40.3% ± 2.3% for UV-induced, and 92.6% ± 1.6% for UV+4-AP (Fig. 3A). In contrast, 4-AP had no protective effect on HCE cells treated with etoposide alone, and viability decreased to 55.6% ± 2.7%. This decline was indistinguishable from the effect of etoposide measured in the presence of 4-AP (56.7% ± 0.4%; Fig. 3A).

The effects of blocking channel activity on UV- and etoposide-induced cell death were further confirmed by DNA fragmentation assays. Suppression of K⁺ channel activity with 4-AP completely prevented UV-induced DNA fragmentation in RCE and HCE cells, but did not prevent etoposide-induced DNA fragmentation (Fig. 3B). RCE cells were transformed with the SV-40 large antigen. To elucidate the effect of transformation, we used primary cultured RCE cells to confirm the effect of K⁺ channel on UV-induced cell death. UV-induced nuclear condensation and DNA fragmentation were also observed in primary cultured RCE cells (Fig. 3C). Suppressing K⁺ channel activity with 4-AP blocked UV-induced primary RCE cell apoptosis. The effect of 4-AP is the same as that from RCE cell lines. These results revealed that UV irradiation elicits K⁺ channel hyperactivity, which, in turn, mediates apoptosis. The ability of etoposide to induce apoptosis despite the presence of
4-AP is consistent with its known inhibition of topoisomerase II activity at the level of the nucleus.

**The K⁺ Ionophore and UV-Induced Apoptosis**

To confirm the hypothesis that increased K⁺ efflux may be an early event in UV-induced apoptosis, RCE cells were exposed to the K⁺ ionophore valinomycin (100 nM) for 24 and 48 hours to trigger apoptosis. It has been shown that valinomycin induces apoptosis in various cell types, including lymphocytes, tumor cells, and mouse neocortical neurons. Apoptotic responses by these cells to increases in K⁺ efflux were measured based on nuclear EB/AO staining (Fig. 4A). After exposure to valinomycin, nuclear condensation increased at both time points. Further experiments were performed to detect DNA fragmentation in cells incubated for 8 hours with 20 nM valinomycin. Valinomycin induced a clear pattern of DNA laddering, indicating internucleosomal DNA cleavage in RCE cells. The observation that an increase in K⁺ efflux elicits cell apoptosis supports the notion that intracellular declines in K⁺ due to UV-induced K⁺ channel hyperactivity may be a cause of cell death.

**Effect of Blockage of K⁺ Channel Activation in Rat Corneal Epithelium**

To further confirm that suppressing K⁺ channel activation prevents UV-irradiation-induced cornea epithelial apoptosis, we used the TUNEL technique in rat corneas in culture to determine whether 4-AP (1 mM) protects against UV irradiation. H-E staining revealed a normal corneal epithelial histologic structure (Fig. 5A). There was no positive TUNEL staining detected in the basal layer of the corneal epithelium, and there was slight positive staining limited to the superficial epithelial cells in the control cornea (Fig. 5B). However, UV irradiation induced nuclear condensation in all layers. Especially, there was a mass of positive staining in the basal layer of the corneal epithelium. In addition, UV irradiation markedly reduced the thickness of the corneal epithelium. In contrast, there was no positive staining in any epithelial layers of the cornea treated with 1 mM 4-AP, indicating that suppressing K⁺ channel with 4-AP prevents the corneal epithelium from UV-irradiation-induced cell apoptosis (Fig. 5D). These results further confirm that UV-irradiation-induced apoptosis of the corneal epithelial cells requires activation of membrane K⁺ channels.

**DISCUSSION**

We studied an interesting mechanism involving UV-irradiation-induced cell death in cultured corneal epithelial cells and intact corneal epithelium. UV-irradiation-induced corneal epithelial injury can affect corneal epithelial barrier function and in turn increase susceptibility to infection and to the development of...
corneal opacity. Healing in response to increased UV irradiation induces cell shedding into the tear pool, followed by replenishment of cells moving centrally from the limbus and anteriorly from the underlying basal layers of the epithelium. This response is an exaggeration of the normal growth-factor–induced cellular and subcellular events that mediate continuous corneal epithelium renewal. Results of our study demonstrated that a UV-irradiation-induced K⁺ channel activation might act as a UV sensor to trigger the subcellular events leading to apoptosis.

In the normal corneal epithelium and endothelium, it has been suggested that aromatic amino acids such as tryptophan,
are in part responsible for absorbing UV. In addition, ascorbate may effectively absorb UV in the cornea and lens epithelium. However, when the system is overwhelmed by too much UV irradiation, it may induce apoptotic damage. We provide evidence for the first time that an important early component of the signaling process mediating UV-irradiation-induced apoptosis is strong activation of cell membrane K⁺ channels. There is growing evidence showing that K⁺ channel activities are probably involved in programmed cell death in other cell types. Various investigations have shown that K⁺ channel activity can be affected by apoptosis inducers, including reactive oxygen species (ROS), Fas ligand and TNF, and anticancer drugs. The K⁺ channel blocker 4-AP can prevent cell shrinkage of human eosinophils undergoing apoptosis induced by cytokine withdrawal, and a combination of two K⁺ channel blockers, tetraethylammonium (TEA) and 4-AP, inhibited IL-1β release from lipopolysaccharide (LPS)-stimulated monocytes. Neurons undergoing apoptosis exhibited an upregulation of outward K⁺ currents by the K⁺ channel opener cromakalim. In the present study, we measured membrane K⁺ channel activity by using single- and whole-cell patch-clamp techniques. UV-irradiation-induced robust activation of these channels appeared to be responsible for K⁺ efflux and the consequent membrane hyperpolarization, thereby activating a particular intracellular signaling system(s) leading to corneal epithelial cell apoptosis. Stimulation of K⁺ channel activity can in fact result in the loss of intracellular K⁺ in addition to cell volume shrinkage. Either one of these alternatives-K⁺ efflux due to stimulation of the K⁺ channel activity or quick loss of K⁺ leading to cell shrinkage—could be an early upstream event in the signaling cascade in corneal epithelial cells leading to apoptosis. Alternatively, cell shrinkage that occurs as a result of a quick decline in intracellular K⁺ concentration, may trigger apoptosis. Accordingly, suppression of K⁺ channel activity may prevent a quick loss of intracellular K⁺ ions resulting from UV-induced K⁺ channel hyperactivity. This possibility is supported by recent findings that UV-irradiation-induced JNK activation can be mimicked by hypertonic stress in HeLa cells. In addition, cytokine receptors can be activated by either UV irradiation or hypertonic shrinkage. We speculate that cytokine receptor activation induced by hypertonic stress occurs as a consequence of cell shrinkage.

![Figure 4](image1.png)

**Figure 4.** Induction of RCE cell apoptosis by the K⁺ channel ionophore valinomycin. (A) RCE cell viability measured after treatments with different concentrations of valinomycin for 24 to 48 hours. Apoptotic cells were determined by EB/AO nuclear staining. (B) Valinomycin-induced DNA fragmentation of RCE cells. Cells were treated with 20 nM of valinomycin for 8 hours. Nuclear DNA was extracted and fractionated on 1.5% agarose gel.

![Figure 5](image2.png)

**Figure 5.** Effect of suppressing K⁺ channels on UV-irradiation-induced rat corneal epithelium apoptosis detected with TUNEL. Corneas were removed from rat eyes, washed three times with cold PBS, incubated 1 hour in DMEM/F12 (1:1) culture medium supplemented with 10% FBS and 5 μg/mL insulin, and then exposed to UV irradiation (40 μJ/cm²) for 3 minutes in the absence and presence of 4-AP. After incubation for additional 8 hours, corneas were fixed and cut into slides, and subject to H-E staining and TUNEL assay. Normal rat corneas were stained with H-E staining for the histologic structure (A). A TUNEL assay was performed to detect cell apoptosis in the control cornea (B) and UV-exposed corneas in the absence (C) or presence (D) of 1 mM 4-AP.

**References**


