Loss of Keratocyte Ion Channels During Wound Healing in the Rabbit Cornea

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Purpose. Corneal keratocytes are responsible for repairing the corneal stromal matrix after injury or infection. Recent work has characterized the primary voltage-gated ion currents in keratocytes from normal, uninjured corneas. The purpose of the present study was to examine and characterize keratocyte voltage-gated ion currents from freeze-wounded rabbit corneas.

Methods. Rabbit corneas were injured using a liquid nitrogen cooled brass probe. Keratocytes were isolated from control eyes, trephined buttons of stroma encompassing the wound area, and the stromal rim surrounding the button. Ionic currents were examined using the amphoterin perforated-patch variation of the whole cell patch clamp technique.

Results. The delayed rectifier K+ current, described previously as the primary voltage-gated outward current in keratocytes, was found in 100% of control cells, 91% of cells isolated from the corneal rim of wounded cells, and 33% of cells isolated from the wound region. Na+ currents were also seen with a lower frequency in cells from the wound area.

Conclusion. The majority of keratocytes migrating into a corneal freeze wound lose the voltage-gated K+ and Na+ ion channels present in cells from normal corneas. Ion channels from cells surrounding the wound site are minimally affected by the injury. Invest Ophthalmol Vis Sci. 1995;36:1095-1099.

Corneal keratocytes are the primary cellular component of the corneal stroma, accounting for approximately 10% of the stromal mass, with an estimated 2.4 million cells per human cornea. Keratocytes are responsible for repairing the corneal stromal matrix after injury or infection. After corneal wounding, quiescent keratocytes become activated, migrate to the wound area, and begin to secrete a host of substances required for wound repair. The cellular mechanisms responsible for keratocyte activation and migration have been examined to date through studies of substances capable of activating keratocytes. These activating substances include epithelial growth factor, fibroblast growth factor, interleukin-1, transforming growth factor-β, insulin, and retinoic acid.

Recent work has characterized the ionic currents in keratocytes from normal, uninjured rabbit corneas. It was found that the primary voltage-gated ionic currents in keratocytes are a delayed rectifying K+ current and an inwardly rectifying Na+ current. The present study was designed to examine voltage-gated ion currents in cells from wounded corneas.

MATERIALS AND METHODS

Wounding

Corneas from New Zealand White rabbits were injured by thermal trauma. This technique has been shown to kill all cell types (epithelium, stromal cells, endothelium) in the area of the freeze wound, with minimal disruption of the stroma or Descemet's membrane.

Freeze wounds were created in anesthetized rabbits by placing a liquid nitrogen cooled brass probe with a 3-mm diameter tip onto the epithelial surface of the cornea for 40 seconds (two 20-second applications). This resulted in an approximately 6- to 7-mm diameter transcorneal wound. Wounding was carried out with the animals under general (ketamine [35 mg/kg] + xylazine [5 mg/kg]) and topical (proparacaine [0.5%]) anesthesia. Corneas were allowed to heal for 24 to 90 hours, at which time the animals were anes-
thetized and killed in a carbon dioxide chamber. The eyes were enucleated, and keratocytes from the injured area were harvested by trephining out a 6-mm button encompassing the wound area and then isolating and dispersing the cells (see Cell Isolation). Cells were also isolated from the remaining corneal rim from several animals and from the corneas of unwounded control rabbits. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Isolation
Corneal keratocytes were isolated as described. Briefly, control corneas or buttons and rims from wounded corneas were isolated, and the corneal endothelium along with Descemet’s membrane was peeled from the stroma. The remaining stroma and epithelium was incubated at 37°C (5% CO₂) in 5 ml minimal essential medium plus 1.2 U/ml dispase (Sigma, St. Louis, MO) for 1 hour, after which the epithelium was wiped from the stroma with a surgical spear. The stroma was cut into 1-mm² pieces and incubated for an additional 1.75 hours in minimal essential medium plus 1 mg/ml collagenase (type 1A; Sigma). Cells were then gently triturated and centrifuged at 180g for 7 minutes. The resultant pellet was resuspended in the extracellular NaCl Ringer’s solution (see Electrophysiology) and repeatedly triturated with a fire-polished Pasteur pipette to obtain single cells.

Electrophysiology
To examine ionic currents in these cells, the amphoterin perforated-patch technique was used. Cells were isolated and dispersed as described, placed into an acrylic chamber filled with NaCl Ringer’s solution, and allowed to settle and attach to an uncoated glass coverslip. Currents were recorded using an Axon (Foster City, CA) 200A patch clamp amplifier. Voltage protocols and data analysis were performed using pClamp software (Axon). Records were capacity compensated with the amplifier circuitry, sampled at 2 kHz, and filtered at 1 kHz. Pipettes for perforated-patch experiments contained (in mM) 145 potassium methanesulfonate (KMeS), 2.5 NaCl, 2.5 CaCl₂, and 5 HEPES plus 240 mg/ml amphoterin b (Sigma). Unless otherwise stated, external solution contained (in mM) 149 NaCl, 5 KCl, 2.5 CaCl₂, 5 glucose, 5 HEPES. Electrodes were Sylgard (Dow Corning, Midland, MI)-coated and fire polished. KG-12 glass was used for all electrodes. Voltages were corrected for offset and perforated patch-induced potentials by subtracting the difference between the actual reversal potential of tail currents measured in symmetric K⁺ solutions and the 0 mV potential.

For activation protocols, cells were held at −9 mV or −69 mV to look for delayed rectifying K⁺ currents, and at −90 mV to look for Na⁺ currents. Na⁺ currents were also seen when using the −69 mV holding potential. Voltage clamp test potentials within the K⁺ current protocol ranged from −90 or −69 mV to +121 mV using 15-mV steps (Fig. 2, inset). Voltage clamp test potentials within the Na⁺ current protocol ranged from −40 mV to +60 mV using 5-mV steps (data not shown).

RESULTS
There were no visible morphologic differences between cells from the different groups as viewed using Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY). Cells were small, fairly round, and had either a relatively smooth cell membrane or a ruffled cell membrane. Figure 1 shows a group of keratocytes from a wounded button. A cell with a smooth cell membrane is shown attached to a patch pipette surrounded by several cells with ruffled cell membranes.

The primary finding of this study was the loss or reduction of the primary delayed rectifier K⁺ current and Na⁺ current in keratocytes isolated from the wound area. Figure 2 shows a typical whole cell current from a cell isolated from a corneal button isolated from a wound area. Figure 2 shows a typical whole cell current from a cell isolated from a corneal button isolated from a wound region. It can be seen that the rim cell had both types of voltage-gated currents, whereas the cell isolated from the wounded but-
Figure 2. Whole cell activation currents from keratocytes of wounded corneas. (A) Whole cell current from a keratocyte isolated from a corneal rim surrounding the wound site. Cell was held at −69 mV (inset: protocol). Arrowhead points to the inward Na⁺ current. (B) Whole cell current from a keratocyte isolated from a corneal button encompassing the wound site. Cell was held at −69 mV (inset: protocol). (C) Current–voltage relationship from the current records in A and B. ◯ = the rim cell; • = cells from the wound area.

ton had only a small outward current with kinetics different from the delayed rectifier. Figure 2C shows the current–voltage relationship (I–V) taken from the activation area of the curves in Figure 2A and 2B. This I–V clearly shows different activation features for the currents in the rim cell and the cell from the wound area. Whole cell currents did not appear to differ between cells with smooth or ruffled cell membranes.

Figure 3 shows tail currents from the same cells in Figure 2. Current–voltage relationships from the instantaneous portion of the tail currents are pictured in Figure 2C. The tail current from the rim cell reverses close to −60 mV (−85 mV expected for a purely K⁺-selective current), whereas the small current seen in the cell from the wound area reverses at approximately −5 mV. This demonstrates the different ionic selectivity of the channels in the two groups. It is expected that the −5 mV reversal potential for the cell from the wound area represents a nonselective leak current or possibly an anion current with weak selectivity between Cl⁻ and methanesulfonate.
Table 1 lists the frequency of occurrence of the different currents in each experimental group. In control keratocytes, 5 of 5 cells (from 4 corneas of 3 rabbits) had the delayed rectifying K⁺ current and Na⁺ current seen in the initial keratocyte ion channel characterization study. Of 11 keratocytes examined from the rim portion of wounded corneas (from 11 corneas of 8 rabbits), 10 cells had the delayed rectifying K⁺ current and 4 had the Na⁺ current. Of the 45 keratocytes examined in wounded corneal buttons (from 35 corneas of 32 rabbits), only 15 had the delayed rectifier and 10 had the Na⁺ current. It is noteworthy that 6 of 11 (54%) cells from the wound area with quantifiable delayed rectifier currents had peak amplitudes < 100 pA, whereas only 1 of 6 (17%) rim cells and 0 of 5 control cells had peak amplitudes < 100 pA. Because the average capacitance value for each group of cells (listed in Table 1) was similar (P = 0.28, analysis of variance), this points to a decreased number of delayed rectifier channels in more than half the cells in the group from the wound area that did display delayed rectifier currents. Mean (±SE) delayed rectifier peak amplitudes for the wounded, rim, and control cells equaled 138 ± 28 pA, 205 ± 55 pA, and 284 ± 56 pA, respectively. Healing duration and cell morphology appeared to have no effect on the presence or absence of voltage-gated channels within the freeze-wound group.
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References


DISCUSSION

This study reveals the loss or reduction of the normal voltage-gated ion channels in keratocytes migrating into a thermally induced corneal wound. This was unexpected in light of recent results reporting the expression of a new or previously dormant channel in the endothelial cells of wounded and healing corneas.15

It was shown9 that the high density of Na⁺ channels in keratocytes from normal corneas could be stimulated to fire action potentials. It was postulated that the delayed rectifier and Na⁺ channels may act to send electrical signals through gap junctions to keratocytes widely distributed through the corneal stroma, possibly through action potentials. It is possible that gap junctions are lost or modified as cells migrate into the wound area and that this creates a signal or the loss of a signal required for the presence of these channels. Alternatively, the simple activation of the keratocytes after injury might lead to the loss of channel protein message or expression. Because the physiological significance of these channels is only speculative, the reason for their disappearance is impossible to gauge. Given the heightened physiological activity of these cells in the wound healing cornea, it is probable that ion channel activity is important. Although the voltage-gated channels are lost, it is likely that ligand or mechanically gated ion channel activity comes into play. The absence of any appreciable voltage-gated current in these cells should allow the wounded keratocyte model to become an important tool for the study of other channel types in activated keratocytes.

Key Words
cornea, keratocyte, ion channel, wound healing, patch clamp

Table 1. Frequency of Occurrence of Different Currents

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>Number of Cells With ( \text{Na}^+ ) Current</th>
<th>Number of Cells With Delayed Rectifier</th>
<th>Capacitance (mean ± SE)</th>
</tr>
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<tbody>
<tr>
<td>Button</td>
<td>45</td>
<td>10</td>
<td>15</td>
<td>16.9 ± 0.4</td>
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<tr>
<td>Rim</td>
<td>11</td>
<td>4</td>
<td>10</td>
<td>15.3 ± 0.0</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>22.1 ± 3.0</td>
</tr>
</tbody>
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* \( n = 37; \) † \( n = 10. \)