Characterization of the R162W Kir7.1 mutation associated with snowflake vitreoretinopathy

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A MEMBER OF THE INWARDLY rectifying potassium (Kir) channel family, Kir7.1 is encoded by KCNJ13, a gene mapped to chromosome 2 at 2q37. Formed by the coassembly of four Kir7.1 subunits, the channel is unique among members of the Kir channel family, in that it has a very low estimated single-channel conductance of 50–200 nS and a much larger macroscopic conductance to Rb+ than to K+ (4, 14, 26).

In the retinal pigment epithelium (RPE), Kir7.1 channels are localized at the apical surface and microvilli and account for the large K+ conductance of the apical membrane. From this strategic location, these channels mediate K+ secretion into the subretinal space, which serves to sustain Na+/K+ pump activity, buffer light-evoked decreases in subretinal K+ concentra-

tion, and help determine the direction and magnitude of transepithelial K+ transport (7). By establishing a large negative membrane potential, Kir7.1 channels also influence the driving forces and, thus, transport of other ions and solutes via channels and electrogenic transporters (28).

A mutation in KCNJ13 resulting in an arginine-to-tryptophan change at residue 162 (R162W) in the Kir7.1 amino acid sequence was recently found to be associated with snowflake vitreoretinal degeneration (SVD) (9), an inherited autosomal-dominant disease with vitreous degeneration, increased risk of retinal detachment, Fuchs corneal dystrophy, and mild retinal degeneration. Molecular modeling of the mutant protein predicted a disruption in the channel structure near the junction of the transmembrane pore domain and the intracellular vestibule formed by the NH2 and COOH termini, leading to the suggestion that this change might alter the stability and function of the closed state of the channel (9). Functional studies of Chinese hamster ovary cells transfected with green fluorescent protein (GFP)-tagged wild-type (WT) and mutant rat Kir7.1 constructs indicated that the mutation alters the channel’s selectivity and renders it permeable to Na+.

In the present study, we sought to confirm and extend these findings by investigating the properties of R162W mutant human Kir7.1 expressed in Xenopus laevis oocytes. We found that oocytes injected with mutant Kir7.1 cRNA exhibited currents that were more than seven times larger than those obtained in oocytes injected with WT Kir7.1 cRNA alone, demonstrating a dominant-negative effect of the mutant subunit. Similar to oocytes injected with WT Kir7.1 cRNA alone, coinjected oocytes exhibited inwardly rectifying Rb+ currents that were more than seven times larger than K+ currents, indicating that mutant subunits did not alter Kir7.1 channel selectivity. Immunostaining of Xenopus oocytes or Madin-Darby canine kidney cells expressing mutant or WT Kir7.1 demonstrated distribution of both proteins primarily in the plasma membrane. Our data suggest that the R162W mutation suppresses Kir7.1 channel activity, possibly by negatively impacting gating by membrane phosphatidylinositol 4,5-bisphosphate.

Materials and Methods

Plasmid constructs. The NH2-terminal GFP-tagged CMV-based WT rat Kir7.1 (GFP-tagged WT rat Kir7.1) and R162W mutant rat Kir7.1 (GFP-tagged mutant rat Kir7.1) expression vectors are described elsewhere (9). A full-length cDNA clone of human Kir7.1 (NM_002242.2) cloned in the pCMV6-XL5 eukaryotic expression vector (WT human Kir7.1) was obtained from Origene (Rockville, MD). A

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Expression of Kir7.1 in X. laevis oocytes. A commercially available cRNA capping kit (Message Machine T7 Ultra, Ambion, Austin, TX) was used to synthesize capped poly(A) cRNA from linearized plasmid cDNAs. cRNA was precipitated in LiCl, washed with 70% ethanol, dried, and redissolved in diethyl pyrocarbonate (DEPC)-treated water.

All procedures with X. laevis frogs were designed to minimize pain and suffering and conformed to the guidelines of the National Institutes of Health and approved by the University of Michigan Committee on the Use and Care of Animals. Xenopus oocytes were surgically removed from deeply anesthetized adult females and defolliculated by cutting small pieces of human retina-RPE-choroid from the eyes of a C441CHARACTERIZATION OF A DISEASE-LINKED Kir7.1 CHANNEL MUTATION

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484C

R162W mutant human Kir7.1 construct (mutant human Kir7.1) was created by site-directed mutagenesis (484C→T) using the WT human Kir7.1 plasmid; the mutation was confirmed by sequencing.

Transient expression of Kir7.1 in MDCK cells. MDCK cells (CCL34, passage 8) were grown in MEM (Life Technologies, Carlsbad, CA) supplemented with 10% FCS (Life Technologies) and antibiotics at 37°C in 5% CO2. Cells were replated on 12-mm Costar transmembrane inserts at a density of 2×10^4 cells/well. On the following day, when the cells had reached >90% confluence, the medium was changed to MEM without antibiotics, and MDCK monolayers were transfected by addition of plasmids (0.4 μg) mixed with Lipofectamine 2000 (1.25 μl; Life Technologies) in Opti-MEM (25 μl) to the apical side of the 12-mm insert. After 5 h of incubation with the plasmid-Lipofectamine 2000 mixture at 37°C, apical and basal compartments were replaced with fresh MEM without antibiotics.

Biotinylation. At 48 h after transfection, both sides of the MDCK monolayer-transmembrane complex were washed with 4°C PBS ++ (PBS with 1 mM Ca^{2+} and 1 mM Mg^{2+}) and then incubated with 1 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (catalog no. 1854210, Thermo Scientific, West Palm Beach, FL) dissolved in PBS ++ at 4°C for 30 min twice on the basolateral side. After biotin was removed, monolayers were thoroughly washed with freshly made 100 mM glycine (Bio-Rad, Hercules, CA) in PBS ++ at 4°C to stop biotinylation, fixed by incubation in 4% paraformaldehyde for 1 h at room temperature, and then washed three times for 5 min each in PBS at room temperature. For imaging biotinylated proteins, monolayers were incubated with Alexa Fluor 555-conjugated streptavidin (catalog no. S21381, Invitrogen; 1:200 dilution in PBS with 0.1% Triton X-100 and 2% BSA) in darkness for 2 h at room temperature. Antifreeze purified rabbit polyclonal antibodies raised against a synthetic peptide in the intracellular COOH-terminal portion of human Kir7.1 is described elsewhere (31). Monoclonal anti-glutamate synthetase antibody (MAB 302) was purchased from Millipore (Billerica, MA). The secondary antibodies used for indirect immunohistochemistry were fluorescence-conjugated goat anti-rabbit IgG (Alexa Fluor 488) and goat anti-mouse IgG (Alexa Fluor 555; both from Invitrogen).

Cell and tissue preparation for immunohistochemistry. Surgically isolated pieces of human retina-RPE-choroid from the eyes of a 72-yr-old woman and an 87-yr-old woman were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C overnight and then washed in chilled PB for 20 min three times. Tissues were obtained for research purposes with patient consent and University of Michigan Institutional Review Board approval. Tissues were cryoprotected before freezing by successive 1-h incubations in 5% and 10% sucrose solutions in PB and then in 20% sucrose in PB overnight at 4°C. Tissues were embedded in optimal cutting temperature embedding medium (Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Cryosections (8–10 μm) were cut using a cryostat (Leica, Deerfield, IL), collected on glass slides, dried at room temperature, and stored at −80°C until use.

At 48 h after cRNA injection, Xenopus oocytes were fixed in 4% paraformaldehyde at room temperature for 2 h, washed three times with PBS, and cryoprotected by successive 1-h incubations in 5% and 10% sucrose solutions in PBS and finally in 20% sucrose in PBS overnight at 4°C. Cryoprotected oocytes were embedded in optimal cutting temperature embedding medium and frozen in liquid nitrogen. Cryosections (8 μm) were cut, collected on positively charged glass slides, dried at room temperature, and stored at −80°C.

Immunohistochemistry. MDCK monolayers, Xenopus oocyte cryosections, or human retina cryosections were washed with PBS and incubated with blocking solution (PBS, pH 7.4, with 0.2% Triton X-100 and 10% goat serum) at room temperature for 1 h and then with rabbit anti-Kir7.1 antiserum (1:200 dilution) alone or together with mouse monoclonal anti-glutamate synthetase (1:5,000 dilution) in antibody solution (PBS, pH 7.4, with 0.1% Triton X-100 and 2% goat serum) at 4°C overnight. After they were washed with PBS three times, monolayers or sections were incubated at room temperature for 2 h in darkness with one or two mixed secondary antibodies diluted in PBS, pH 7.4, with 0.1% Triton X-100 and 2% goat serum (1:500 dilution). The specificity of the Kir7.1 antibodies was confirmed by preincubation with a twofold amount of antigenic peptide.

Specimens were analyzed on a scanning laser confocal microscope (SP5, Leica, Mannheim, Germany). Digital images were collected at 16-bit resolution in 0.29-μm z sections and analyzed by image analysis software (Leica LAS AF). The confocal immunofluorescence images in Figs. 4–6 are three-dimensional projections generated from 4–8 consecutive z sections and z-y projections generated from 30–60 z sections. Files were exported for additional processing by Photoshop CS2 (Adobe, San Jose, CA). In experiments comparing the expression of WT and mutant Kir7.1 expression or testing antibody specificity by preabsorption with antigenic peptide, laser settings, photomultiplier tube gain, exposure time, and brightness and contrast settings were identical for experimental and control images.

Solutions. The standard bath solution (ND96) for Xenopus oocyte recordings consisted of (mM) 96 NaCl, 2 KCl, 10 Na-HEPES, 1.0 CaCl2, and 1.0 MgCl2, pH 7.4. In experiments investigating the permeability or conductance of Kir7.1 to monovalent cations, the solution consisted of (mM) 98 X, 10 N-methyl-D-glucamine-HEPES, 1.0 CaCl2, and 1.0 MgCl2, where X was KCl or RbCl. Where indicated, 10 mm BaCl2 was added directly to the external solution. All chemicals were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO).

Electrophysiology. Whole cell currents in Xenopus oocytes were recorded 2 or 3 days after injection using the two-electrode voltage-clamp technique essentially as described previously (26). Microelectrodes, pulled from thick-wall borosilicate glass using a multistage programmable microelectrode puller (model P-97, Sutter Instruments, San Rafael, CA) and filled with 3 M KCl, were used as voltage-sensing and current-passing electrodes. The voltage drop across the bath solution and reference electrode that otherwise would have occurred during voltage clamping was eliminated using a two-electrode virtual ground circuit (catalog no. VG-2A X100, Axon Instruments, Union City, CA). Signals from the current-passing electrode were amplified using a GeneClamp amplifier (Axon Instruments) and stored on a computer hard drive for later analysis. Data acquisition and analysis were performed with pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). The resting membrane potential of each oocyte was measured in the open-circuit condition at the beginning of the experiment while it was bathed in ND96 solution. Current-voltage (I-V) relationships were determined from steady-state currents elicited by a voltage-step protocol in which the membrane potential was held at 0 mV and stepped for 1 s to voltages ranging from +50 to −150 mV in 10-mV increments. Oocytes exhibiting Ca^{2+}-activated Cl− currents were rejected for analysis.

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Selectivity for monovalent cations was determined under bi-ionic conditions in which Rb$^+$ or K$^+$ was the major monovalent cation in the bathing solution. The permeability ratio, $P_{Rb}/P_K$, was calculated according to a modified form of the Goldman-Hodgkin-Katz equation:
\[ E_{Rb} - E_K = (RT/F)\ln(P_{Rb}/P_K), \]
where $E_{Rb}$ and $E_K$ are the reversal potentials with 98 mM Rb$^+$ and 98 mM K$^+$ in the bath, respectively, and $T$, $R$, and $F$ have their usual meanings.

Statistics. Electrophysiological results are presented as means ± SE and represent measurements from three batches of oocytes. Statistical significance was calculated by ANOVA or the $t$-test as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Electrophysiology. As reported previously (26), oocytes injected with WT human Kir7.1 cRNA had a large negative membrane potential and exhibited inwardly rectifying currents. A representative family of whole cell currents recorded from a WT Kir7.1 cRNA-injected oocyte bathed in ND96 solution, which contains 2 mM K$^+$ and 106 mM Na$^+$, is shown in Fig. 1A, left. Prominent inward currents activated rapidly in response to hyperpolarizing voltage steps, whereas depolarizing voltage steps evoked smaller outward currents. In the presence of the K$^+$ channel blocker Ba$^{2+}$ (10 mM), most of the inward current was eliminated (Fig. 1A, middle). The Ba$^{2+}$-sensitive component of whole cell currents (Fig. 1A, right), obtained by taking the difference between the currents measured in the absence and presence of Ba$^{2+}$, largely reflected currents mediated by Kir7.1 channels (26). Results obtained in a total of 18 oocytes injected with WT Kir7.1 cRNA are summarized in Fig. 1, C and D, which plot the steady-state $I$-$V$ relationships for whole cell and Ba$^{2+}$-sensitive currents, respectively. WT Kir7.1 current (Fig. 1D) exhibited mild inward rectification, with substantial outward current at voltages positive to the zero-current potential and a prominent negative slope conductance at voltages positive to 0 mV. For 18 oocytes injected with WT Kir7.1 cRNA, the resting membrane potential ($V_m$) averaged $-91.4 ± 2.1$ mV and the amplitude of the Ba$^{2+}$-sensitive current at $-150$ mV ($I_{-150mV}$) averaged $-0.72 ± 0.03 \mu$A.

![Fig. 1. Wild-type (WT) and R162W mutant Kir7.1 currents. A: families of macroscopic currents recorded in a WT Kir7.1 cRNA-injected Xenopus oocyte bathed in ND96 solution in the absence (control) and presence of 10 mM Ba$^{2+}$ and their algebraic difference (right). Horizontal line to the left of each set of currents represents zero-current level. Voltage-clamp protocol used to evoke the currents is shown below currents in the Ba$^{2+}$ recording (middle). B: families of macroscopic currents recorded in a R162W mutant Kir7.1 cRNA-injected oocyte bathed with ND96 solution in the absence and presence of 10 mM Ba$^{2+}$ and their algebraic difference. Voltage-clamp protocol and scales as described in A. C: steady-state current-voltage ($I$-$V$) relationships in Xenopus oocytes injected with WT Kir7.1 cRNA (WT), R162W mutant Kir7.1 cRNA (MT), or water and bathed in ND96 solution. Values are means ± SE for 17–18 oocytes. D: $I$-$V$ relationships of Ba$^{2+}$-sensitive currents obtained in the same oocytes used for measurements in C.](http://ajpcell.physiology.org/)
In contrast, whole cell currents in oocytes injected with R162W (mutant) Kir7.1 cRNA (Fig. 1B, left) were considerably smaller, outwardly rectifying, and relatively insensitive to block by Ba\(^{2+}\) (Fig. 1B, middle and right). Results from 17 oocytes injected with mutant Kir7.1 cRNA are summarized in Fig. 1C and D, which show I-V relationships for whole cell and Ba\(^{2+}\)-sensitive currents, respectively. The Ba\(^{2+}\)-sensitive current exhibited outward rectification, a pattern that was similar to that seen in water-injected oocytes (Fig. 1D; n = 17) and was probably mediated by endogenous K\(^{+}\) channels. The average amplitude of the Ba\(^{2+}\)-sensitive current at -150 mV was similar for mutant Kir7.1 cRNA- and water-injected oocytes (Table 1), as was the average value of \(V_m\) [-31.7 ± 1.3 mV (n = 17) and -32.8 ± 2.4 mV (n = 17), respectively, \(P > 0.10\)]. Thus Xenopus oocytes expressing R162W mutant Kir7.1 appeared to exhibit predominantly endogenous currents.

It is possible that mutant Kir7.1 channels were present in the plasma membrane but that the macroscopic current they produced was small, because their abundance or open probability was relatively low. To increase the amplitude of Kir7.1 currents, we recorded from oocytes expressing WT or mutant Kir7.1 channels during superfusion with solutions containing 98 mM K\(^{+}\) or 98 mM Rb\(^{+}\). Previous studies showed that the macroscopic conductance of Kir7.1 has an unusually high dependence on extracellular Rb\(^{+}\) concentration, with the Rb\(^{+}\) conductance being about eightfold larger than the K\(^{+}\) conductance (26, 30). Figure 2A compares families of Ba\(^{2+}\)-sensitive currents recorded in an oocyte injected with WT Kir7.1 and bathed in solution containing 98 mM K\(^{+}\) or 98 mM Rb\(^{+}\). In both solutions, the Ba\(^{2+}\)-sensitive current was inwardly rectifying, but it was markedly larger in 98 mM Rb\(^{+}\). Results obtained in 18 oocytes injected with WT Kir7.1 cRNA are summarized by I-V plots in Fig. 2, C and D, and in Fig. 3. The Ba\(^{2+}\)-sensitive current at -150 mV was 9.19 ± 0.47-fold larger in the presence of 98 mM Rb\(^{+}\) than in the presence of 98 mM K\(^{+}\) (Fig. 3C). Substitution of Rb\(^{+}\) for K\(^{+}\) also produced a ~6.7-mV shift in the reversal potential of the Ba\(^{2+}\)-sensitive current (−12.2 ± 1.0 to −18.9 ± 1.2 mV, n = 16, data not shown), indicating a \(P_{K_{b}/P_{K}}\) of 0.77 ± 0.03. These results are similar to those reported previously for WT human Kir7.1 channels (26, 30).

When R162W mutant Kir7.1 was expressed in oocytes, steady-state whole cell currents were somewhat larger in 98 mM K\(^{+}\) solution than in ND96, but they were essentially unchanged when extracellular K\(^{+}\) was replaced with 98 mM Rb\(^{+}\) (Fig. 2, B–D). As summarized in Table 1, Ba\(^{2+}\)-sensitive inward currents in mutant Kir7.1 cRNA-injected oocytes were nearly identical in 98 mM K\(^{+}\) and 98 mM Rb\(^{+}\) but markedly smaller in those injected with WT Kir7.1 cRNA: ~10-fold difference in 98 mM K\(^{+}\) and ~100-fold difference in 98 mM Rb\(^{+}\). Similar results were obtained in water-injected oocytes (Table 1). The ratio of Rb\(^{+}\) current to K\(^{+}\) current (\(I_{Rb}/I_{K}\)) was similar for mutant Kir7.1 cRNA-injected and water-injected oocytes but much smaller than \(I_{Rb}/I_{K}\) for oocytes expressing WT Kir7.1 (Table 1). On the other hand, there was no significant difference in \(P_{Rb}/P_{K}\) between oocytes injected with mutant Kir7.1 cRNA (0.76 ± 0.04, n = 13), oocytes injected with WT Kir7.1 cRNA (0.77 ± 0.03), and oocytes injected water (0.72 ± 0.07, n = 12). Overall, these results provide additional support to the idea that the electrophysiological properties of oocytes expressing mutant Kir7.1 reflect the activities of endogenous channels and indicate that mutant Kir7.1 channels are not trafficked to the plasma membrane or are present in the plasma membrane but are not conductive under our experimental conditions.

The inheritance pattern of SVD, which has been associated with the R162W mutation in Kir7.1, is autosomal-dominant (9). To mimic the heterologous condition in affected individuals, we co-injected oocytes with a 1:1 mixture of WT and mutant Kir7.1 cRNAs. \(V_m\) of these oocytes averaged −58 ± 3.5 mV (n = 16), which is intermediate between \(V_m\) in oocytes injected with mutant Kir7.1 cRNA and \(V_m\) in oocytes injected with WT Kir7.1 cRNA, indicating that mutant Kir7.1 suppresses, but does not eliminate, WT Kir7.1 channel function. Figure 3A compares representative families of Ba\(^{2+}\)-sensitive currents recorded in an oocyte injected with 0.1 ng of WT Kir7.1 cRNA + 0.1 ng of mutant Kir7.1 cRNA and superfused with 98 mM K\(^{+}\) or 98 mM Rb\(^{+}\) solution. The Ba\(^{2+}\)-sensitive current exhibited strong rectification and was substantially larger in 98 mM Rb\(^{+}\) than 98 mM K\(^{+}\). Results obtained in 15 oocytes injected with a 1:1 ratio of WT to mutant Kir7.1 cRNA are summarized in Fig. 3B, which shows the I-V curves of Ba\(^{2+}\)-sensitive currents obtained in 98 mM K\(^{+}\) and 98 mM Rb\(^{+}\). As depicted in Fig. 3C and summarized in Table 1, the Ba\(^{2+}\)-sensitive K\(^{+}\) current at −150 mV averaged −0.31 ± 0.05 µA, which is 18.3% of the amplitude of the current measured in oocytes injected with WT Kir7.1 cRNA alone (−1.69 ± 0.22 µA). The Ba\(^{2+}\)-sensitive Rb\(^{+}\) current at −150 mV averaged −2.39 ± 0.41 µA, which is 16.6% of the amplitude of the current measured in oocytes injected with only WT Kir7.1 cRNA (−14.39 ± 1.50 µA). For oocytes injected with WT + MT Kir7.1 cRNAs that had well-resolved Ba\(^{2+}\)-sensitive currents [\(I_{(-150mV)}\) less than −1.0 µA], \(I_{Rb}/I_{K}\) averaged 7.21 ± 0.41, which was somewhat lower than \(I_{Rb}/I_{K}\).

### Table 1. Extracellular current dependence of currents at −150 mV in Xenopus oocytes injected with WT cRNA, mutant cRNA, water, and WT + mutant cRNAs

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<tr>
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<th>(I_{Rb}) &amp; µA</th>
<th>(I_{Rb + K}) &amp; µA</th>
<th>(I_{Rb + K}) &amp; µA</th>
<th>(I_{Rb + K}) &amp; µA</th>
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<td>Whole cell</td>
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<td>Whole cell</td>
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<tr>
<td>WT</td>
<td>−10.6 ± 0.15 (18)</td>
<td>−7.2 ± 0.12 (18)</td>
<td>−2.20 ± 0.29 (18)</td>
<td>−1.69 ± 0.22 (18)</td>
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<tr>
<td>Mutant</td>
<td>−0.33 ± 0.05 (17)</td>
<td>−0.06 ± 0.03 (17)</td>
<td>−0.50 ± 0.06 (17)</td>
<td>−0.57 ± 0.05 (17)</td>
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<tr>
<td>Water</td>
<td>−0.20 ± 0.02* (17)</td>
<td>0.01 ± 0.02 (17)</td>
<td>−0.38 ± 0.04 (17)</td>
<td>−0.08 ± 0.02 (17)</td>
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<tr>
<td>Water + mutant</td>
<td>−0.40 ± 0.10 (15)</td>
<td>−0.18 ± 0.10 (15)</td>
<td>−0.60 ± 0.06 (15)</td>
<td>−0.31 ± 0.05* (15)</td>
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Values are means ± SE of number of oocytes in parentheses. WT, wild-type; \(I_{Rb + K}\), current measured in the presence of 98 mM K\(^{+}\); \(I_{Rb + K}\), current measured in the presence of 98 mM Rb\(^{+}\); \(I_{Rb}/I_{K}\), the ratio of Ba\(^{2+}\)-sensitive currents measured in the presence of 98 mM K\(^{+}\) and 98 mM Rb\(^{+}\). Only oocytes for which Ba\(^{2+}\)-sensitive currents were equal to or less than −0.04 µA were included in calculation of \(I_{Rb}/I_{K}\). *\(P < 0.05\) vs. mutant. †\(P < 0.001\) vs. mutant, WT + mutant, and water. ‡\(P > 0.05\) vs. mutant.
in oocytes injected with WT Kir7.1 cRNA alone (9.19 ± 0.47, P < 0.01). This lower ratio may reflect a greater contribution of endogenous channels to Ba²⁺-sensitive K⁺ and Rb⁺ currents in oocytes injected with WT + mutant Kir7.1 cRNAs. After normalization of the Ba²⁺-sensitive Rb⁺ currents at each voltage to the current measured at −150 mV, the I-V curves for oocytes injected with WT Kir7.1 cRNA alone and oocytes injected with WT + mutant Kir7.1 cRNAs were nearly identical (Fig. 3D). These results provide additional evidence that the selectivity of Kir7.1 channels is unaltered by the presence of mutant Kir7.1 subunits.

Surface expression. To test the possibility that the R162 mutation impairs Kir7.1 trafficking to or stability in the plasma membrane, we performed indirect immunohistochemistry using a rabbit polyclonal anti-Kir7.1 antibody that we characterized previously (31) on cryosections of Xenopus oocytes injected with 0.1 ng of WT or 0.1 ng of mutant Kir7.1 cRNA. Preabsorption of the primary antibody with antigenic peptide eliminated the signal (Fig. 4, C and D), indicating that the immunoreactivity was specific to Kir7.1 protein. Similar results were observed in multiple other oocytes from the same frog and two other frogs, but variability within individual oocyte sections and among different oocytes precluded quantification of fluorescence intensity. These results suggest that there is no profound defect in the trafficking of mutant Kir7.1 protein to the Xenopus oocyte plasma membrane.

The processing of nascent proteins can be sensitive to temperature, leading to differences in their surface expression when expressed in Xenopus oocytes vs. mammalian cells. For example, the processing and delivery of mutant CFTR channels to the plasma membrane were defective in mammalian cells but normal in Xenopus oocytes or insect cells incubated at lower temperatures (2). To determine whether mutant Kir7.1 is trafficked to the plasma membrane in mammalian cells, we transiently overexpressed WT or mutant Kir7.1 in MDCK cells and grew them to confluence on permeable membrane supports for 2 days. To assist in the localization of Kir7.1 protein to the plasma membrane, MDCK cells were biotinylated on their basolateral surface before fixation with 4% paraformaldehyde and later tagged with Alexa Fluor 555-labeled streptavidin. As shown in Fig. 5, confocal microscopy revealed that WT and mutant Kir7.1 proteins localized primarily to the basolateral membrane. In addition, WT and mutant Kir7.1 proteins were detected at the apical and basal surfaces, although at lower densities. In other experiments, using constructs that were used in a previous study (9), we overexpressed GFP-tagged WT or mutant rat Kir7.1 in MDCK monolayers and observed by
confocal microscopy GFP fluorescence in the apical and basolateral membranes (results not shown). Taken together, the results indicate that surface expression of mutant Kir7.1 channels to the plasma membrane is normal in Xenopus oocytes and mammalian cells, suggesting that some other mechanism must account for their lack of function.

Immunolocalization of Kir7.1 in human retina. In a previous study on bovine retina using affinity-purified rabbit antibodies against human Kir7.1 (31), we found strong Kir7.1 immunoreactivity in the apical membrane of the RPE, as well as weaker immunoreactivity in the inner nuclear layer. We used the same antibodies to determine Kir7.1 distribution in frozen sections of adult human retina by indirect immunofluorescence and immunohistochemical labeling. Figure 6 shows confocal immunofluorescence images of a section of central retina obtained from a 72-yr-old woman and double-labeled with antibodies against Kir7.1 and glutamine synthetase, a Müller cell marker. Prominent Kir7.1 immunoreactivity was present in the RPE apical microvilli but was not evident in the neural retina or in the choroid. A similar expression pattern was observed in sections of the peripheral retina of the same individual and in sections of central and peripheral retina from an 87-yr-old woman. Control experiments with Kir7.1 antibodies preincubated with antigenic peptide were negative (not shown), indicating that the protein in human retina recognized by the antibodies was Kir7.1. In immunohistochemical experiments on retinal sections from the same individuals using the biotin-avidin complex/4,6-diaminido-2-phenylindole method, similar results were obtained, although in some specimens, weak immunoreactivity was also observed in nuclei in the ganglion cell layer, inner nuclear layer, and outer nuclear layer (not shown).

DISCUSSION

The present study demonstrates that the R162W mutation of Kir7.1 results in a nonfunctional channel when expressed alone and that, when expressed together with WT Kir7.1, the mutant protein exerts a dominant-negative effect. We found that Xenopus oocytes injected with R162W (mutant) human Kir7.1...
cRNA and bathed in ND96 solution exhibited electrophysiological properties that were indistinguishable from those of water-injected oocytes, suggesting that mutant Kir7.1 protein does not form functional channels in the plasma membrane. This conclusion was supported by the finding that replacement of extracellular K\(^+\)/H\(^+\) and Na\(^+\)/H\(^+\) with Rb\(^+\)/H\(^+\), which produces large inward currents through WT Kir7.1 channels (26, 30), failed to reveal currents in mutant Kir7.1-expressing oocytes.

In principle, the lack of channel activity could be caused by impaired surface expression, which can result from defects in a number of processes, such as protein folding, posttranslational modification, assembly, and membrane trafficking, or endoplasmic reticulum retention and degradation. We found, however, that mutant Kir7.1 protein was localized to the plasma membrane when expressed in Xenopus oocytes or MDCK cells. These results indicate that the loss of function in mutant Kir7.1 channels is not due to impaired processing or trafficking to the plasma membrane but, rather, point to some other mechanism, such as suppressed channel activity.

We determined that the R162W mutant had a dominant-negative effect on WT Kir7.1 channels, consistent with the autosomal-dominant inheritance pattern of SVD (9). Kir channels consist of four pore-forming subunits (8). If we assume that WT and mutant Kir7.1 subunits are expressed equally and coassemble randomly and that one mutant subunit is sufficient to block channel activity, then a 1:1 WT-to-mutant expression ratio should result in a current that is 6.25% of WT alone \( \frac{I}{I_0} = (1 - F_{\text{mut}})^4 \), where \( I \) is the current in oocytes injected with WT + mutant cRNA, \( I_0 \) is the current in oocytes injected with WT Kir7.1 cRNA alone, and \( F_{\text{mut}} \) is the fraction of subunits that are mutant (19). Our finding that a 1:1 mixture of WT and mutant Kir7.1 cRNAs resulted in a Rb\(^+\) current that was \( \sim 17\% \) of WT Kir7.1 cRNA alone implies that this model is not entirely correct. Perhaps a single mutant subunit only partially inhibits channel activity, or perhaps the translation, posttranslational processing, or oligomerization efficiency of the mutant subunit is reduced compared with the WT subunit (6). Future studies are required to distinguish between these possibilities.

What is the likely mechanism for the suppression of channel activity by the mutant subunit? Arginine in position 162 of Kir7.1 is one of a cluster of positively charged residues on the surface of the channel’s cytoplasmic domain that interacts with phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) in the plasma membrane to gate the channel open (18, 23). Site-directed mutagenesis of arginine or lysine at the equivalent positions in Kir1.1 and Kir2.1 to glutamine decreased channel-PIP\(_2\) interactions as measured by the rate of current inhibition by PIP\(_2\) antibody or poly-L-lysine (18). Recently, the X-ray crystal structure of a Kir2.2 channel in complex with a short-chain PIP\(_2\) derivative was determined (8). Structural analysis revealed that one PIP\(_2\) molecule binds to each of the four subunits at the interface between the transmembrane and cytoplasmic domains and that this produces conformational changes that position the inner helix gate in a preopen state. Furthermore, R186, which corresponds to R162 in Kir7.1, was shown to be positioned to interact directly with the 5\(^-\)-phosphate of PIP\(_2\). It is likely that the mutation of R162 in Kir7.1 to the bulkier hydrophobic residue tryptophan causes a significant disruption of subunit-PIP\(_2\) interactions, such that the homomeric mutant channel is not gated open by endogenous levels of membrane PIP\(_2\). In this scenario, heteromeric channels in the same membrane phospholipid environment might...
have a greater open probability due to a higher PIP2 binding affinity afforded by WT Kir7.1 subunits. Experiments involving the application of exogenous PIP2 to the cytoplasmic surface of excised membrane patches expressing mutant Kir7.1 channels may resolve this question.

It was previously reported that the overexpression of GFP-tagged R162W mutant rat Kir7.1 in Chinese hamster ovary cells produced a nonselective current that depolarized the membrane potential (9). It was noted that a high percentage of these cells had excessive “leak currents” and exhibited membrane fragility, which raises the possibility that the nonselective cation currents did not reflect mutant Kir7.1 channel activity. Two findings in the present study argue against the idea that the mutant subunit alters channel selectivity. 1) The whole cell current in Xenopus oocytes injected with mutant Kir7.1 cRNA was nearly identical to that in water-injected oocytes and exhibited a similar cation selectivity profile. 2) Oocytes injected with WT + mutant Kir7.1 cRNAs had inward currents that were substantially smaller than those in oocytes injected with WT Kir7.1 cRNA alone (indicating WT and mutant subunit interaction) but exhibited a cation selectivity profile and voltage dependence of Rb+ current that were nearly identical to those of homomeric WT channels.

Previously, using rabbit antibodies generated against a peptide sequence in the COOH terminus of human Kir7.1, our group performed immunofluorescence staining of frozen bovine retina and found specific and intense staining of the RPE apical membrane and more diffuse staining of the inner nuclear layer. In the current study, we used the same antibodies on frozen sections of human retina, and although we confirmed immunolocalization of the RPE apical microvilli, no significant labeling in any region of the neural retina was observed. These results differ from those reported previously by another group who used rabbit antibodies generated against a GST-Kir7.1 fusion protein on frozen sections of human cadaveric retina and reported Kir7.1 immunoreactivity in the RPE and inner nuclear layer, diffuse staining of the inner plexiform layer and nerve fiber layer, and intense staining of the internal limiting membrane (9). It was suggested by the authors of the study that the intense immunostaining of the internal limiting membrane...
could signify the presence of Kir7.1 channels in the vitreal end feet of Müller cells. In our study, Kir7.1 did not colocalize with glutamine synthetase, a Müller cell-specific marker. The reason for these disparate findings is unclear, but because control experiments using Kir7.1 antibodies preabsorbed with antigen were not performed in the previous study, it is possible that the retinal staining in human retina was nonspecific.

Physiological significance. Kir7.1 channels are abundantly expressed in the apical membrane of the RPE (15, 31) and account for most, if not all, of this membrane’s large K+ conductance (12). These channels mediate K+ secretion into the subretinal space, a narrow extracellular space that separates this epithelium from the rod-and-cone photoreceptor outer segments. By recycling K+ that enters the cell across the apical membrane Na+/K+ pump and Na+-K+-2Cl− cotransporter, Kir7.1 channels permit these transporters to operate at high levels of activity (13). Decreases in RPE apical K+ conductance reduce K+ recycling and dramatically enhance K+ absorption (20) and, secondarily, fluid absorption (5, 24). Light-evoked changes in photoreceptor activity decrease extracellular K+ concentration in the subretinal space from ~5 to 2 mM. This decrease in extracellular K+ concentration results in a large increase in the electrochemical driving force for conductive K+ exit across the RPE apical membrane (1), as well as an increase in apical membrane K+ conductance (16, 26), culminating in a substantial increase in K+ efflux (1). This K+ efflux across the apical membrane contributes to the movement of water out of the RPE cytoplasm (1) and, consequently, expansion of the subretinal space volume (11, 17). Additionally, by virtue of the accompanying hyperpolarization of the apical membrane potential (27), the driving forces on conductive Cl− and HCO3− transport pathways are also altered (7).

On the basis of the results of the present study, we predict that, in patients with SVD, the RPE apical membrane K+ conductance would be greatly reduced but not eliminated. A diminished apical membrane K+ conductance would tend to promote K+ and fluid absorption across the RPE when the retina is dark-adapted and dampen the efflux of K+ across the RPE apical membrane, as well as the accompanying changes in anion and water fluxes, at light onset. These alterations in RPE physiology could impact the subretinal space environment and RPE-photoreceptor interactions, adversely affecting photoreceptor function. Consistent with this idea, clinical features of SVD include elevated rod thresholds and reduced a-wave amplitude (10). Patients with SVD exhibit several other functional defects in the retina, including diminished photopic and scotopic b waves, photopic flicker electroretinogram, and oscillatory potential (10), but these changes could be secondary to defects in photoreceptor function. In addition to the progressive degeneration of the retina, patients with SVD also exhibit congenital abnormalities such as optic nerve-head dysmorphism and fibrillar degeneration of the vitreous, suggesting a possible role for Kir7.1 channels in ocular development and in other ocular tissues. Studies investigating the Kir7.1 expression pattern during ocular development are warranted. The mechanisms by which alterations in Kir7.1 lead to Fuchs corneal dystrophy should also be explored.

Recently, several recessive mutations of KCNJ13 associated with retinal degeneration have been identified (25). One these mutations, c.496C>T, leading to a nonsense mutation at R166X, was found in individuals diagnosed shortly after birth with Leber congenital amaurosis, a severe form of retinal degeneration with early onset. The resulting truncation of nearly the entire COOH terminus of Kir7.1 would be expected to cause loss of function, as the COOH terminus is required for Kir7.1 trafficking to the plasma membrane (29). A second homozygous mutation, c.722T>C (p.L241P), associated with a similar phenotype, was predicted to alter the orientation of a β-sheet in the COOH terminus of Kir7.1. On the basis of the severity of retinal degeneration, it was proposed that this mutation might also be associated with a loss of Kir7.1 function (25), but future patch-clamp experiments are necessary to confirm this hypothesis.

Outside the retina, Kir7.1 has been localized in iris pigment epithelium (32), choroid plexus, thyroid follicular cells, intestine (21), and kidney (3, 22), but among these tissues, robust Kir7.1-mediated currents have only been demonstrated in the choroid plexus, which shares developmental, structural, and functional similarities with the RPE. It will be interesting to
learn if patients with the R162W, R166X, or L241P mutations exhibit abnormal function in tissues other than the retina.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

W.Z., X.Z., H.W., A.K.S., and B.A.H. performed the experiments; W.Z., X.Z., H.W., and B.A.H. analyzed the data; W.Z., X.Z., H.W., A.K.S., A.O.E., and B.A.H. approved the final version of the manuscript; A.K.S. and B.A.H. are responsible for conception and design of the research; B.A.H. interpreted the results of the experiments; B.A.H. prepared the figures; B.A.H. drafted the manuscript; B.A.H. and A.O.E. edited and revised the manuscript.

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