Expression and Localization of the Inwardly Rectifying Potassium Channel Kir7.1 in Native Bovine Retinal Pigment Epithelium

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Purpose. The purpose of this study was to identify the molecular basis of the apical membrane K⁺ conductance in native bovine retinal pigment epithelium (RPE).

Methods. RT-PCR, Northern blot, and Western blot analyses were used to detect the expression of the inwardly rectifying K⁺ (Kir) channel subunits Kir7.1 and Kir4.1 in native bovine RPE and neural retina. The distribution of Kir7.1 protein was determined in frozen sections of bovine retina-RPE-choroid by indirect immunofluorescence analysis.

Results. RT-PCR analysis revealed Kir7.1 transcript in both RPE and neural retina, but Kir4.1 transcript only in the neural retina. In Northern blot analysis, Kir4.1 probe hybridized to an appropriately sized transcript in neural retina but not in RPE. Kir7.1 probe hybridized to a major transcript of ~1.5 kb in both RPE and neural retina, but with greater expression in RPE. In Western blot analysis, Kir7.1 antibody recognized a major band of ~53 kDa in RPE, whereas Kir4.1 antibody recognized a monomer of ~60 kDa in neural retina but not in RPE. Intense Kir7.1 immunolabeling was present on the apical surface of all RPE cells and appeared to extend over the length of the apical processes. Na⁺/K⁺-ATPase expression varied among RPE cells, but in highly expressing cells, it colocalized with Kir7.1.

Conclusions. These results indicate that the Kir7.1 channel subunit, but not Kir4.1, is a major component of the apical K⁺ conductance in bovine RPE. Kir7.1 is distributed over the length of apical processes, where it probably functions in the regulation of K⁺ transport and the electrical response of the RPE to light-evoked changes in subretinal K⁺ concentration. (Invest Ophthalmol Vis Sci. 2003;44:3178–3185) DOI:10.1167/iovs.02-1189

The retinal pigment epithelium (RPE) separates the distal retina from its major blood supply in the choroid. From this strategic position, it helps maintain an appropriate ionic environment for photoreceptor function by transporting fluid, ions, and metabolites into and out of the subretinal space—the small extracellular compartment bounded by the RPE apical membrane, Müller cell apical processes, and photoreceptor outer segments. It is well established that K⁺ channels in the RPE apical membrane play a central role in a wide range of RPE transport processes. For example, they provide a recycling pathway for K⁺ entering the cell across the apical membrane via the Na⁺,K⁺-adenosine triphosphatase (Na⁺,K⁺-ATPase) and Na⁺,K⁺,2Cl⁻ cotransporter, they influence anion transport by impacting membrane potential, and they determine the direction and magnitude of net K⁺ transport. In addition, apical K⁺ channels underlie the membrane hyperpolarization response of the RPE to light-evoked decreases in subretinal K⁺ concentration ([K⁺]ᵣ), which helps generate the c-wave of the direct-current electroretinogram.

Patch-clamp studies of native RPE cells from a variety of species have shown that the predominant membrane conductance in the physiological voltage range is an inwardly rectifying K⁺ (Kir) conductance with unusual permeation properties. In a study on the RPE-choroid preparation, we found that the sensitivity of the apical membrane K⁺ conductance to blockage by Ba²⁺ and Cs⁺ is similar to that of the Kir conductance, providing strong evidence that the Kir conductance is a major component of the apical K⁺ conductance. Over the past decade, molecular cloning has identified 15 structurally related Kir channel subunits that coassemble to form homo- or heterotetrameric channels. Recently, we presented molecular and functional evidence that Kir7.1 is the molecular basis of the apical membrane Kir conductance in bovine RPE. Kusaka et al., working in rat, reported that in addition to Kir7.1, the RPE expresses Kir4.1, a Kir channel subunit that is highly expressed in Müller cells. This group has also presented immunohistochemical evidence that these two Kir channel subunits are differentially distributed on the RPE apical membrane, with Kir7.1 localized to the root of apical processes and Kir4.1 distributed on more distal regions. The conductance properties of Kir7.1 and Kir4.1 differ dramatically from each other. Whereas Kir7.1 channels have a very low unitary conductance (~0.2 picosiemens [pS]) and a macroscopic conductance that is inversely proportional to extracellular [K⁺]₀, Kir4.1 channels have a moderate unitary conductance (21–23 pS) and a macroscopic conductance that is proportional to extracellular [K⁺]. We failed to observe Kir4.1 currents in patch-clamp recordings from isolated bovine RPE cells, but this could be explained if the portions of apical processes containing Kir4.1 channels were broken off during the mechanical separation of the neural retina from the RPE or as a result of enzymatic dissociation of RPE cells.

In the present study, we combined molecular biological, biochemical, and immunohistochemical approaches to assess the expression of Kir7.1 and Kir4.1 channel subunits in bovine RPE and to ascertain their subcellular distribution. We failed to detect Kir7.1 mRNA in the RPE, but confirmed that Kir7.1 is highly expressed at both the mRNA and protein levels. Moreover, we find that Kir7.1 is localized to apical membrane and is distributed along the length of the apical processes. The results support the idea that Kir7.1 is the major component of the K⁺ channel subunits Kir7.1 and Kir4.1 in native bovine RPE and neural retina. The distribution of Kir7.1 protein was determined in frozen sections of bovine retina-RPE-choroid by indirect immunofluorescence analysis.

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conductance in the RPE apical membrane, where it functions in the regulation of K⁺ transport and the generation of the electrical response of the RPE to light-evoked changes in sub-retinal K⁺ concentration. Some of these results have been reported in abstract form (Pan A, Yang D, Swaminathan A, Hughes BA, ARVO Abstract 2616, 2002; Yang D, Swaminathan A, Hughes BA, ARVO Abstract 4568, 2002).

METHODS

Preparation of Native Bovine RPE Sheets

Bovine eyes were obtained from a local abattoir and transported to the laboratory in ice-cold HEPES-buffered Ringer’s (HR; 135 mM NaCl, 5 mM KCl, 10 mM glucose, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 10 mM HEPES, titrated to pH 7.4 with NaOH). Adherent extracellular tissue was removed from the globes by dissection. Eyes were opened at the equator, and the vitreous body was removed from the eyecup. After the neural retina was gently peeled off, the eyecup was filled with 1% dispase-II (Roche Molecular Diagnostics, Indianapolis, IN) in HR and incubated for 60 to 90 minutes at 37°C. RPE sheets were then dislodged from Bruch’s membrane with a gentle stream of HR applied by a fire-polished Pasteur pipette. RPE sheets were collected and washed several times with HR to remove contaminating photoreceptors and choroidal cells.

Total and Poly(A⁺) RNA Isolation

Total RNA was isolated from freshly isolated bovine RPE sheets and neural retina (TRIzol reagent; Life Technologies, Inc., Rockville, MD), according to the manufacturer’s instructions. Bovine RPE and neural retinal poly(A⁺) RNA were isolated by applying total RNAs to an oligo(dT) column (Pharmacia, Piscataway, NJ). Rat retinal poly(A⁺) RNA was purchased from Clontech (Palo Alto, CA).

RT-PCR Analysis

Total RNA isolated from RPE sheets or neural retina was reverse transcribed with random decamers or oligo(dT) primers using reverse transcriptase (RetroScript Ambion, Austin, TX), by following procedures outlined in the manufacturer’s instructions. Control reactions lacking reverse transcriptase were performed in an identical manner. PCR was performed with a degenerated primer pair specific for human, mouse, and rat Kir4.1 or for human, rat, and guinea pig Kir7.1. The Kir4.1 forward and reverse primers were designed from the coding region corresponding to nucleotides 266-290 and 1522-1530 of human Kir4.1 (Genbank accession number: AF261085). The Kir7.1 forward and reverse primers were designed from nucleotides 113-131 of the human GAPDH sequence, and the reverse primer was designed from nucleotides 468-439 (Genbank accession number: AF261085). The primers (Table 1) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The hot-start method was used to initiate the PCR reaction. The PCR products were generated by adding DNA polymerase (SuperTaq-Plus; Ambion) and cycled 30 (GAPDH) or 40 (Kir4.1 and Kir7.1) times for 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C, followed by a 7-minute extension at 72°C. The RT-PCR products were separated by 1.4% agarose gel electrophoresis. The Kir7.1 and Kir4.1 RT-PCR products were directly confirmed by bidirectional sequencing with the same sets of primers used for PCR. DNA sequencing was performed by the DNA Sequencing Core Facility at the University of Michigan.

Northern Blot Analysis

Northern blot analyses were prepared by loading 2 μg of poly(A⁺) RNA or 30 μg of total RNA from bovine neural retina and RPE sheets. The BstXI-SalI fragment of rat Kir4.1 cDNA (gift of John Adelman, Oregon Health and Sciences University, Portland, OR), or the KpnI-BglII fragment of human Kir7.1 cDNA was used as the template for probe labeling. Probes were labeled by a DNA labeling system (RadPrime; Life Technologies, Inc.) with [α-32P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were sequentially hybridized with the α32P-labeled Kir4.1 and Kir7.1 cDNA probes at 62°C and the α32P-labeled GAPDH cDNA probe at 65°C in hybridization buffer (ExpressHyb; Clontech, Palo Alto, CA), with each followed by a posthybridization wash at 55°C in 0.2X SSC and 0.1% SDS. Blots were exposed to a phosphorescence screen (Storage Phosphor Screen; Molecular Dynamics, Sunnyvale, CA) overnight, scanned with a phosphorescence imager (Phosphorimager; Molecular Dynamics), and quantified with the accompanying software (ImageQuant; Molecular Dynamics).

Antibodies

Rabbit polyclonal anti-Kir7.1 antibodies were raised against a synthetic peptide corresponding to amino acid residues 330-350 (VK5PNRTDLDHINGQSIDN) in the intracellular carboxyl terminal portion of human Kir7.1. Antibodies were made using keyhole limpet hemocyanin (KLH)-conjugated peptide and affinity purified by Research Genetics, Inc. (Huntsville, AL). The affinity-purified Kir7.1 antibodies were eluted by a pH gradient, and stored in 0.125 M borate buffer in aliquots at −20°C until use.

Monoclonal anti-ezrin antibody (clone 3G12) and chicken polyclonal anti-Na,K-ATPase (α1B) antibodies were purchased from Sigma-Aldrich (St. Louis, MO) and Cortex Biochem (San Leandro, CA), respectively. Affinity-purified rabbit polyclonal anti-Kir4.1 antibodies were the generous gift of Paulo Koifish (University of Minnesota, Minneapolis, MN). The secondary antibodies for indirect immunohistochemistry were fluorescence-conjugated goat anti-rabbit IgG (Alexa Fluor 555), goat anti-mouse IgG (Alexa Fluor 488), and goat anti-chicken IgG (Alexa Fluor 488; all from Molecular Probes, Eugene, OR).

Transfection

Human embryonic kidney cell line HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL.
The expression construct pcDNA3.1(−)/Kir4.1 was generated by inserting a rat Kir4.1 coding sequence (GenBank accession number: X835885) in-frame into the pcDNA3.1(−). The expression construct pcS2+Kir7.1 was generated by inserting a human Kir7.1 coding sequence in-frame into the pcS2+. HEK 293 cells were transfected with each expression construct or vector using a lipofectamine transfection agent (Lipofectamine 2000; Life Technologies, Inc.), according to the manufacturer’s protocol. Whole-cell extracts were prepared for Western blot analysis 72 hours after transfection.

**Western Blot Analysis**

Western blot analysis was performed using the techniques described by Yang and Miller. Briefly, native RPE sheets and neural retinas were placed into RIPA buffer (150 mM NaCl, 25 mM Tris–HCl [pH 7.4], 2 mM EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS) containing complete protease inhibitor cocktail (Roche Molecular Biochemicals) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Kir subunit–transfected or vector-transfected HEK 293 cells in T-25 flasks were washed with PBS, scraped, and collected. Cell pellets were obtained by centrifugation (5 minutes at 1000g) and resuspended in RIPA buffer containing protease inhibitor cocktail and then sonicated on ice. Sonicated samples were centrifuged at 15,000g for 10 minutes at 4°C and the supernatants collected. The protein content of each sample was determined by a bichinchoninic acid (BCA) assay kit (Pierce, Rockford, IL), with bovine serum albumin used as a standard. One microgram (for transfected HEK 293 cells) or 20 μg of protein extracts (for RPE or neural retina) were solubilized in Laemmli sample buffer (62.5 mM Tris [pH 6.8], 25% glycerol 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol; Bio-Rad, Hercules, CA) heated to 70°C for 10 minutes, and then applied to 4% to 20% linear gradient Tris-HCl gel (Bio-Rad). After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and immunoblotted with Kir1.1 or Kir4.1 antibodies at a concentration of 0.5 μg/mL, followed by development with enhanced chemiluminescence (ECF) substrate (Amersham Pharmacia Biotech) and chemiluminescence visualization using a phosphorescence imager (Polaroid; Molecular Probes) and viewed with an epifluorescence microscope (model E800; Nikon, Melville, NY). Digital images were collected with a cooled charge-coupled device (CCD) camera and the allied software (ACT; Nikon). Figure panels were composed with image-management software (Photoshop; Adobe Systems, Mountain View, CA), for archival purposes.

**RESULTS**

**Kir7.1 and Kir4.1 mRNA Expression in RPE and Neural Retina**

We first performed RT-PCR analysis to examine the expression of Kir7.1 and Kir4.1 channel subunits in native bovine RPE and neural retina. The subunit-specific primer sequences and predicted sizes of the RT-PCR products are shown in Table 1. Figure 1 shows the results of RT-PCR analysis of mRNA isolated from bovine RPE and neural retina. Transcript for the Kir7.1 subunit was detected in both the RPE and neural retina, whereas Kir4.1 transcript was detected in the neural retina but not in the RPE. The identities of the RT-PCR products were confirmed by bidirectional DNA sequencing with the same sets of primers used for PCR. No band was observed in reactions of template and primers without reverse transcriptase, indicating that products were generated from mRNA rather than contaminating genomic DNA. RT-PCR with PCR primers for GAPDH also produced a band of the expected size (356 bp), establishing the integrity and relative abundance of the RNA. It is worth noting that Kir4.1 was undetected in bovine RPE after 40 PCR cycles, indicating that Kir4.1 subunit is not expressed in these cells.

To confirm this expression pattern of Kir subunits, we performed Northern blot analysis with poly(A) RNA from native bovine RPE cells and neural retina. As shown in Figure 2, Kir7.1 transcripts were detected in both RPE and neural retina, whereas Kir4.1 transcript was detected in neural retina but not in RPE. The Kir7.1 probe hybridized to a major transcript at 1.5 kb and a much weaker signal at 3.3 kb in both the RPE and neural retina. The Kir4.1 probe recognized a single 5.5-kb transcript in both bovine and rat neural retina, consistent with findings by other investigators working on rat retina. Similar results were obtained in another set of Northern blot experiments using total RNA (30 μg/lane) from native bovine RPE and neural retina (data not shown).

**Kir7.1 and Kir4.1 Protein Expression in RPE and Neural Retina**

To examine Kir4.1 and Kir7.1 expression at the protein level, we performed Western blot analysis using Kir4.1 and Kir7.1 antibodies. The specificity of the two antibodies was first determined by Western blot analysis of whole-cell lysates from HEK 293 cells transfected with recombinant Kir4.1 or Kir7.1...
construct using the appropriate antibodies. Figure 3 shows that Kir4.1 and Kir7.1 antibodies labeled appropriate-sized bands (~60 kDa for Kir4.1 and ~53 kDa for Kir7.1). Preincubation with the corresponding peptides either reduced or eliminated the signals (Figs. 3A, 3B, lane 2), indicating antibody specificity.

Western blot analysis of Kir7.1 and Kir4.1 expression in neural retina and RPE is shown in Figure 4. Kir4.1 antibodies labeled a band of ~60 kDa in neural retina but not in the RPE (Fig. 4A), whereas Kir7.1 antibodies labeled a major band of ~53 kDa and a weaker band of ~100 kDa in the RPE (Fig. 4B). Kir7.1 antibodies also labeled ~55- and ~100-kDa bands in the neural retina, as well as several additional bands of lower molecular mass. The higher molecular mass bands in RPE and neural retina likely represent oligomers of the Kir7.1 channel subunit, similar to what has been reported for other Kir proteins.\(^ {18,19}\) The apparent molecular masses of the Kir4.1 and Kir7.1 monomers were larger than those predicted for the unmodified proteins (42.5 and 40.5 kDa, respectively), consistent with other reports for Kir7.1 in rat thyroid and small intestine (54 kDa),\(^ {20}\) rat RPE (52 kDa),\(^ {13}\) and rat kidney (~52 kDa),\(^ {21}\) and are probably due to posttranslational modification.

The identity of the labeled protein band as Kir4.1 in the neural retina was confirmed by the absence of labeling when Kir4.1 antibodies were preincubated with Kir4.1 synthetic peptide (Fig. 4A, lane 4). Preincubation of Kir7.1 antibodies with its synthetic peptide eliminated labeling in the RPE but reduced labeling of only the major bands in neural retina. Thus, although the Kir7.1 antibodies may be specific when used in the RPE, in the retina, they may recognize other proteins as well. Taken together, the results of RT-PCR and Northern and Western blot analyses indicate that Kir7.1 is expressed in bovine RPE, but Kir4.1 is not.

**Distribution Pattern of Kir7.1 and Kir4.1 in Bovine Retina In Situ**

In a previous study, we made cell-attached recordings from the apical and basolateral poles of isolated bovine RPE cells to show that Kir7.1 channels are expressed predominantly in the apical membrane.\(^ {11}\) Although we speculated that Kir7.1 channels might also be distributed in RPE apical processes, we were
unable to test this idea electrophysiologically, because the small diameter of the processes precludes patch-clamp recordings. As an alternative approach, we used Kir7.1 antibodies to determine Kir7.1 distribution in sections of bovine retina by indirect immunofluorescence labeling.

Figure 5 shows Nomarski (Fig. 5A) and immunofluorescence (Figs. 5B, 5C) images from a section of bovine central retina labeled with Kir7.1 antibodies. Strong Kir7.1 immunolabeling was present in the apical membrane of the RPE, with weaker immunolabeling present in the inner nuclear layer of the neural retina (Fig. 5B). The immunolabeling was absent in both regions when the Kir7.1 antibodies were preabsorbed with the synthetic peptide (Fig. 5C), indicating that the protein recognized by the antibodies was Kir7.1.

We also investigated the expression pattern of Kir4.1 in retinal cryosections by immunolabeling with Kir4.1 antibodies (Fig. 6). Kir4.1 antibodies labeled various regions of Müller cells, particularly in the neuroblot layer and around blood vessels in the inner retina (Fig. 6B). There was also weaker Kir4.1 immunolabeling in the ganglion cell layer, inner nuclear layer, and outer limiting membrane. In contrast, the RPE was immunonegative for Kir4.1 antibodies. Control experiments with Kir4.1 antibodies preincubated with Kir4.1 peptide showed no immunolabeling in retinal sections (Fig. 6C). Thus, Kir4.1 was expressed in bovine neural retina as it is in other species,

Colocalization of Kir7.1 and Ezrin in Bovine RPE In Situ

To determine the expression pattern of Kir7.1 in the outer retina in finer detail, we performed double-labeling experiments using antibodies against Kir7.1 and ezrin, a linker protein that bridges actin microfilaments to plasma membrane proteins. Ezrin has been localized to the microvilli of a wide variety of polarized epithelia, including the RPE. Figure 7 shows Nomarski (Fig. 7A) and immunofluorescence (Figs. 7B–D) images of a frozen section of bovine central retina double labeled with Kir7.1 and ezrin antibodies. Strong ezrin immunolabeling was detected at the apical surface and processes of the RPE as well as the apical microvilli of Müller cells (Fig. 7C), consistent with the distribution pattern of ezrin reported for other species. In addition, there was weak ezrin immunolabeling of the RPE basolateral membrane, similar to the results of Bonilha et al. As shown in Figure 7B, strong Kir7.1 immunolabeling was present at the RPE apical surface and processes, where it colocalized with ezrin (Fig. 7D). No Kir7.1 immunolabeling was detected in the RPE basolateral membrane or in Müller cell apical microvilli. Sections of peripheral retina showed a similar expression pattern for Kir7.1 (Fig. 7B) and ezrin (data not shown). These results contrast with those reported for rat RPE, where Kir7.1 appeared to be localized to the root of apical processes.

Colocalization of Kir7.1 and Na+K+-ATPase in Bovine RPE In Situ

Numerous functional and immunohistochemical studies on the RPE from a variety of species have established that Na+,K+--
Kir Channels in the RPE

ATPase has a predominantly apical distribution. Recently, Burke et al.29 showed that in bovine central retina, individual RPE cells exhibit differences in Na⁺,K⁺-ATPase staining, with adjacent cells in some regions having high and low levels of immunolabeling. Because Kir7.1 and the Na⁺,K⁺-ATPase are thought to be functionally coupled, we were interested to learn whether there is correlation in the distribution of these two proteins. Figure 8 shows Nomarski and immunofluorescence images of peripheral (Figs. 8A–D) and central (Figs. 8E–H) retinal cryosections double labeled for Kir7.1 and Na⁺,K⁺-ATPase. In peripheral retinal sections, all RPE cells appeared to have Kir7.1 (Fig. 8B) and Na⁺,K⁺-ATPase (Fig. 8C) distributed in the apical processes, where the two proteins appeared to colocalize (Fig. 8D). In contrast, RPE cells in the central retina displayed intense Kir7.1 immunolabeling of their apical processes (Fig. 8F) but had a heterogeneous expression pattern of Na⁺,K⁺-ATPase, with high and low levels of Na⁺,K⁺-ATPase immunolabeling in adjacent RPE cells (Fig. 8G). Hence, in some RPE cells within the central retina, Kir7.1 and Na⁺,K⁺-ATPase colocalized in the apical processes, whereas in other cells Kir7.1 was present in the apical processes, but Na⁺,K⁺-ATPase was present at low levels or was undetectable (Fig. 8H).

**DISCUSSION**

Previous work in our laboratory indicated that the basis for the large K⁺ conductance of RPE apical membrane is a mild, inwardly rectifying K⁺ (Kir) conductance with unusual properties.7,8,30,31 More recently, we showed that the Kir7.1 channel subunit is highly expressed in the RPE and that the unique electrophysiological profile of native RPE Kir conductance closely matches that of heterologously expressed Kir7.1 channels but not other members of the Kir gene family.11 In this study, we combined molecular biological, biochemical, and immunohistochemical approaches to provide further evidence that Kir7.1 is the molecular basis for the apical K⁺ conductance of the RPE.

**Molecular Basis of Kir Channels in Bovine RPE**

Studies to date have reported the expression of both Kir4.1,12 and Kir7.1,11,13 channel subunits in the RPE. In the present study, we confirmed the presence of Kir7.1 transcript in RPE but failed to detect Kir4.1, even by RT-PCR analysis. Consistent with the findings in other species that Kir4.1 is expressed in Müller cells,14,16,22 we observed Kir4.1 transcript in bovine neural retina. For intronless genes such as Kir4.1,12 control RT reactions without reverse transcriptase are necessary to exclude false-positive readings resulting from trace genomic DNA contamination. Because Kir4.1 has been detected by RT-PCR analysis in rat RPE without this negative control,12 one cannot rule out the possibility that it resulted from trace genomic DNA contamination.

In a previous study, we performed Northern blot analysis on total RNA isolated from a variety of bovine tissues and detected a strong 1.6-kb signal in the RPE but not in the neural retina or any other tissue examined.11 In the present study, we performed Northern blot analysis on poly(A⁺) RNA and found that Kir7.1

**Figure 7.** Colocalization of Kir7.1 and ezrin in the apical membrane of bovine RPE. Nomarski (A) and immunofluorescence (B–D) images of a bovine central retinal cryosection (10 μm) double labeled with rabbit anti-Kir7.1 antibodies (B) and mouse anti-ezrin antibody (C), followed by fluorescence-conjugated goat anti-rabbit IgG (red) and goat anti-mouse IgG (green). (D) Merged image of Kir 7.1 immunofluorescence (red) and ezrin immunofluorescence (green). Abbreviations are as in Figure 5. (A, arrows) RPE basal membrane. Scale bar, 10 μm.

**Figure 8.** Colocalization of Kir7.1 and Na⁺,K⁺-ATPase in the apical processes of bovine RPE. Bovine peripheral (A–D, 10 μm thick) and central (E–H, 6 μm thick) retinal cryosections were double labeled with rabbit anti-Kir7.1 antibodies (B, F), and chicken anti-Na⁺,K⁺-ATPase (α1β1) antibodies (C, G), followed by fluorescence-conjugated goat anti-rabbit IgG (red) and goat anti-chicken IgG (green). (A, E) Nomarski images of bovine peripheral (A) and central (E) retinal sections; (D, H) Merged images of Kir 7.1 immunofluorescence (red) and Na⁺,K⁺-ATPase immunofluorescence (green). Abbreviations are as in Figure 5. (A, E, arrows) RPE basal membrane. Scale bars: (A–D) 10 μm; (E–H) 20 μm.
probe hybridized to transcripts of 3.3 and 1.5 kb in both the RPE and neural retina, with the expression level of 1.5 kb transcript much higher in RPE cells than that in neural retina (Fig. 2). Within the RPE, the relative abundance of the 1.5-kb transcript appeared to be much higher than that of the 3.3-kb transcript (Fig. 2). We may have failed to detect the 3.3-kb transcript in our previous study because of the lower amount (10 μg) of total RNA loaded and the lower expression level of the transcript. The presence of two transcripts of Kir7.1 in bovine RPE may be due to alternative usage of multiple promoters and polyadenylation signals, as has been shown for the rat Kir7.1 gene.32

To determine the expression of Kir7.1 and Kir4.1 channel subunits at the protein level, we performed Western blot analysis of RPE and neural retina. Consistent with the results of Northern blot analysis, only Kir7.1 protein was detected in the RPE, whereas in the neural retina, both Kir4.1 and Kir7.1 were detected. Despite the fact that Kir7.1 transcript was more abundant in the RPE than in neural retina, the RPE appeared to have less Kir7.1 protein than the neural retina. This apparent discrepancy could be explained if RPE microvilli were broken off during the mechanical separation of the neural retina from the RPE and remained associated with photoreceptor outer segments up to the point of protein isolation. If Kir4.1 expression in the RPE were localized to the microvilli, the loss of microvilli could also explain the absence of detectable Kir4.1 protein in Western blot analysis of RPE sheets. However, the absence of detectable Kir4.1 transcript in the RPE, together with the absence of Kir4.1 immunolabeling in the interphotoreceptor space of retinal sections, strongly argues against this possibility. Taken together, our results strongly support the idea that Kir7.1 is the molecular basis of Kir channels in bovine RPE.

Kir7.1 Distribution on RPE Apical Processes

Our immunocytochemical studies on bovine RPE suggest that the Kir7.1 channels are distributed over the length of the apical processes. This distribution pattern appeared in cells in both the central and peripheral retina. The basis for this conclusion comes from the observation that Kir7.1 colocalizes with ezrin (Fig. 7), a marker of RPE microvilli.25–28 Ezrin is detected in the apical microvilli of various epithelia,25,26,28–30,33–35 where it is thought to constitute a bridge between the plasma membrane and the actin cytoskeleton.25,28 The highly polarized RPE has abundant apical processes that possess an internal core bundle of densely packed actin microfilaments.26 High levels of ezrin were localized at microvilli and basal infoldings of adult rat RPE by immunoelectron microscopy.26,27 Cytoskeletal elements such as ezrin antibody indicate that the processes projecting apical surface of bovine RPE are mainly long villuslike processes, some of which may extend to the photoreceptor inner segments (Fig. 7). Bovine RPE basolateral membrane also exhibited weak ezrin immunolabeling, but displayed no significant Kir7.1 immunolabeling (Fig. 7).

Relationship between Kir7.1 and Na⁺,K⁺-ATPase

Burke et al.30 also showed by confocal microscopy that bovine RPE cells can differ from their neighbors in cell height and the extent of apical membrane elaboration and, furthermore, that this correlates with variations in Na⁺,K⁺-ATPase polarity and the level of E/P-cadherin expression. According to their model, cells with low E/P-cadherin levels tend to have a larger microvillar domain and predominantly apical distribution of Na⁺,K⁺-ATPase, whereas cells with high E/P-cadherin levels have a smaller apical microvillar domain, a taller lateral domain, and increased Na⁺,K⁺-ATPase immunolabeling of the basolateral membrane. In our study, we found that most bovine RPE cells in both central and peripheral retina had long apical processes (Fig. 7C). We did not observe tall RPE cells with short processes and strong Na⁺,K⁺-ATPase immunolabeling of the basolateral membrane, but the reason for this is unclear.

Previous studies have shown that Kir7.1 is colocalized with Na⁺,K⁺-ATPase in a variety of tissues outside the eye. For example, Kir7.1 and Na⁺,K⁺-ATPase colocalize on the basolateral membrane of thyroid follicular and intestinal epithelial cells, whereas in the choroid plexus, both are present on the apical domain.20 Our results in bovine RPE from peripheral retina showed a similar relationship, with Kir7.1 and Na⁺,K⁺-ATPase colocalizing on the apical processes. RPE cells from the central retina, however, showed a more complex pattern: although Kir7.1 was localized on the apical processes in every cell, Na⁺,K⁺-ATPase was expressed in the apical processes in some cells but not in others. This indicates that the expression and trafficking of Kir7.1 and Na⁺,K⁺-ATPase are differentially regulated. The functional consequences of this are uncertain, but they suggest that Kir7.1 has broader functions in the RPE than supporting Na⁺,K⁺-ATPase activity alone.

Based on immunohistochemical observations in rat outer retina, Kusaka et al.13,15 proposed a model for the RPE in which Kir7.1 and Na⁺,K⁺-ATPase are colocalized in the basal portion of apical processes and Kir4.1 is distributed on more distal regions. Our findings in bovine RPE clearly are incompatible with this model. In bovine RPE, Kir7.1 and Na⁺,K⁺-ATPase appeared to be distributed along the entire length of the apical processes, and Kir4.1 was not detected by RT-PCR, Northern blot, or Western blot analyses or by indirect immunofluorescence labeling. Although the differences between our results and those of Kusaka et al. might be explained by species differences, there appears to be some disagreement about the expression of Kir4.1 in rodent RPE. Nagelhus et al.35 performed immunohistochemical studies of rat retina using Kir4.1 antibodies that were apparently the same as those used by Kusaka et al.13,15 and failed to detect Kir4.1 immunolabeling in the basal portion of the RPE. Likewise, in a study of Kir4.1 in mouse retina, Kofuji et al.46 did not find Kir4.1 immunolabeling in the RPE. Further studies on rodent retina are needed to resolve this discrepancy.

Physiological Implications

It has been known for many years that the RPE apical membrane is highly elaborated into villuslike and sheetlike processes and that its passive properties are dominated by high K⁺ conductance.5,56 It has generally been assumed that the K⁺ channels underlying this conductance are broadly distributed in the RPE apical processes, because this is where most of the apical membrane surface area lies. The results of this study lend support to this idea by establishing that Kir7.1 is distributed on the apical membrane surface as well as the entire length of apical processes. Kir7.1 channels have a low unitary conductance of ~0.2 pS and are estimated to number roughly 61,000 per bovine RPE cell.11 These two factors may result in a K⁺ conductance that is evenly distributed on the RPE apical membrane, which would help minimize the development of local K⁺ gradients within the processes and the narrow extracellular space they share with photoreceptor outer segments.
The finding that Kir7.1 colocalizes with Na⁺,K⁺-ATPase in the apical processes of most RPE cells is consistent with the notion that an important function of this channel is to support Na⁺,K⁺-ATPase activity by providing a recycling pathway for K⁺. The capacity of the Kir7.1 channel to conduct relatively large outward currents makes it well suited for this function. Other properties of the channel allow it to function effectively in K⁺ homeostasis in the subtrenal space. A unique characteristic of native and cloned Kir7.1 channels is that their macroscopic outward conductance increases with decreases in extracellular K⁺ concentration ([K⁺])ₐ. In the dark-adapted retina, the onset of light triggers a transient decrease in subretinal [K⁺] from approximately 5 to 2 mM. This decrease in [K⁺] would tend to increase Kir7.1 conductance, thus enhancing the capacity of the RPE to secrete K⁺ and restore subretinal [K⁺] toward its dark-adapted level.

In conclusion, the present molecular biological, biochemical, and immunohistochemical results, coupled with our previous electrophysiological and pharmacological studies, suggest that Kir7.1 is the molecular basis of the apical membrane K⁺ conductance in bovine RPE.

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References


