

## Differential expression and distribution of Kir5.1 and Kir4.1 inwardly rectifying K<sup>+</sup> channels in retina

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Departments of <sup>1</sup>Pharmacology II and <sup>3</sup>Ophthalmology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871; and <sup>2</sup>Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Science, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan

Submitted 2 December 2002; accepted in final form 8 April 2003

**Ishii, Masaru, Akikazu Fujita, Kaori Iwai, Shunji Kusaka, Kayoko Higashi, Atsushi Inanobe, Hiroshi Hibino, and Yoshihisa Kurachi.** Differential expression and distribution of Kir5.1 and Kir4.1 inwardly rectifying K<sup>+</sup> channels in retina. *Am J Physiol Cell Physiol* 285: C260–C267, 2003. First published April 9, 2003; 10.1152/ajpcell.00560.2002.—Kir5.1 is an inwardly rectifying K<sup>+</sup> channel subunit whose functional role has not been fully elucidated. Expression and distribution of Kir5.1 in retina were examined with a specific polyclonal antibody. Kir5.1 immunoreactivity was detected in glial Müller cells and in some retinal neurons. In the Kir5.1-positive neurons the expression of glutamic acid decarboxylase (GAD<sub>65</sub>) was detected, suggesting that they may be GABAergic-amacrine cells. In Müller cells, spots of Kir5.1 immunoreactivity distributed diffusely at the cell body and in the distal portions, where Kir4.1 immunoreactivity largely overlapped. In addition, Kir4.1 immunoreactivity without Kir5.1 was strongly concentrated at the endfoot of Müller cells facing the vitreous surface or in the processes surrounding vessels. The immunoprecipitant obtained from retina with anti-Kir4.1 antibody contained Kir5.1. These results suggest that heterotetrameric Kir4.1/Kir5.1 channels may exist in the cell body and distal portion of Müller cells, whereas homomeric Kir4.1 channels are clustered in the endfeet and surrounding vessels. It is possible that homomeric Kir4.1 and heteromeric Kir4.1/Kir5.1 channels play different functional roles in the K<sup>+</sup>-buffering action of Müller cells.

inwardly rectifying potassium channel; heteromerization; glial Müller cells; amacrine cells; potassium siphoning

THE INWARDLY RECTIFYING POTASSIUM (Kir) channel family has more than 20 members, which have been classified into four major subfamilies (9, 11). Each subfamily is responsible for important cellular functions. Kir2.0 contributes to setting the deep resting membrane potential, whereas Kir3.0 and Kir6.0 play pivotal roles in the control of cell excitability by G proteins and by intracellular metabolic signaling, respectively. Kir1.1 and Kir4.0 are key molecules in transporting K<sup>+</sup> in epithelial and glial cells. The function of Kir5.1 has only recently been revealed. Kir5.1 and Kir4.1 form heterotetrameric channels in kidney (30, 31, 37). The

properties of the Kir4.1/Kir5.1 heterotetramer are quite different from those of Kir4.1 homotetramers. The single-channel conductance of the Kir4.1/Kir5.1 heterotetramer is larger than that of the Kir4.1 homomeric channel. The activity of Kir4.1/Kir5.1 heterotetramer channels is sensitive to a physiological range of intracellular pH (pH<sub>i</sub>), whereas Kir4.1 homomeric channels are inhibited only by strong intracellular acidification. Thus the Kir4.1/Kir5.1 heterotetrameric channel is supposed to be involved in pH<sub>i</sub>-dependent ion transport in kidney. On the other hand, although it was believed that Kir5.1 was nonfunctional when expressed alone (2, 26), we recently showed (29) that it can form a functional homotetramer with the aid of a PDZ-anchoring protein, PSD-95. Phosphorylation of Kir5.1 by protein kinase A (PKA) then disrupts the interaction between these proteins and abolishes channel activity. Thus we discover that Kir5.1 can be functional in two ways, 1) by heteromerization with Kir4.1 to form a pH<sub>i</sub>-sensitive Kir channel and 2) by homomerization through interaction with PSD-95 to form a PKA-regulated Kir channel.

Glial cells, which include retinal Müller cells, contribute to maintenance of the surrounding microenvironment that allows neurons to function properly. For example, glial cells absorb and transport accumulated extracellular K<sup>+</sup>, which if uncorrected would result in the loss of normal neural activity. This regulatory function was first proposed as a “spatial buffering” of K<sup>+</sup> accomplished by astrocytes in the optic nerve (23) and described as “K<sup>+</sup> siphoning” for retinal Müller cells (22). We previously reported (8) that the Kir channel Kir4.1 is expressed in retinal Müller cells in clusters in specific regions of the cells such as the endfoot and in processes surrounding blood vessels. This suggests that Kir4.1 may play a pivotal role in siphoning of K<sup>+</sup> by retinal Müller cells. This proposal was supported by the observation that ablation of the Kir4.1 gene caused a loss of normal visual activity (14).

In this study, we examined the expression and distribution of Kir5.1 in the retina. We have raised a

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specific rabbit polyclonal antibody against Kir5.1. The immunoreactivity of Kir5.1 was detected in some retinal neurons and in Müller cells. In Müller cells, the spots of Kir5.1 immunoreactivity were detected diffusely at the cell body and in distal portions of the cells, where it was largely colocalized with Kir4.1. In contrast, in the endfeet facing the vitreous surface or in the processes surrounding vessels, Kir5.1 immunoreactivity was not detected, whereas Kir4.1 was strongly concentrated. Immunoprecipitants from retina obtained with an anti-Kir4.1 antibody contained Kir5.1. Therefore, it is suggested that Kir4.1/Kir5.1 heterotetrameric channels and Kir4.1 homomeric channels exist and play different functional roles in retinal Müller cells.

## MATERIALS AND METHODS

Animals were treated in accordance with the guidelines for the use of laboratory animals of Osaka University Graduate School of Medicine.

**Antibodies.** Polyclonal anti-Kir5.1 antibody was raised in rabbit against the synthetic peptide corresponding to amino acid residues 8–30 (YRIVNVDSKYPGPPEHAIAEKR) in the NH<sub>2</sub>-terminal region of rat Kir5.1. The antiserum was purified with protein A-Cellulofine (Seikagaku, Tokyo, Japan) and antigenic peptide-coupled Sulfolink resin (Pierce, Rockford, IL). Polyclonal anti-Kir4.1 antibody was raised by the same methods with synthetic peptide EKEGSALS-VRISNV, which corresponds to amino acids 366–379 of Kir4.1 (10). Both immunoreactivities were prevented by their respective antigenic peptides. Rabbit polyclonal anti-aquaporin (AQP)4 antibody was purchased from Alomone Laboratories (Jerusalem, Israel). Mouse monoclonal antibodies against vimentin, the PSD-95 family, glutamic acid decarboxylase (GAD<sub>65</sub>), protein kinase (PKC)- $\alpha$ , and calbindin were purchased from Zymed laboratories (clone V9; San Francisco, CA), Upstate Biotechnology (clone K28/86.2; Lake Placid, NY), BD Biosciences Pharmingen (clone GAD-6; San Diego, CA), Santa Cruz Biotechnology (clone H-7; Santa Cruz, CA), and Sigma (clone CB-955; St. Louis, MO), respectively. Rat monoclonal anti-recoverin antibody was a generous gift from Dr. Grazyna Adamus (Oregon Health Science University, Beaverton, OR).

**Transfection and immunocytochemistry.** Green fluorescent protein (GFP)-tagged rat Kir5.1 was constructed by subcloning each coding region into a mammalian expression vector, pEGFP-C1 (Clontech, Palo Alto, CA). The plasmids were transiently expressed in a human embryonic kidney cell line, HEK293T, with LipofectAMINE Plus reagent (Life Technologies, Gaithersburg, MD). Cells expressing GFP-fused Kir5.1 were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate (PA solution), pH 7.4, at 4°C overnight. After the cells were washed with phosphate-buffered saline (PBS), they were incubated with Texas red-labeled anti-rabbit IgG (Alexa Fluor 568; Molecular Probes, Eugene, OR). Fluorescence was measured by laser scanning confocal microscopy (system LSM510; Carl Zeiss, Jena, Germany).

**Preparation of dissociated Müller cells.** Acute dissociation of Müller cells was performed as previously described (8). Briefly, male Wistar-Kyoto rats were deeply anesthetized by an overdose of pentobarbital sodium (100 mg/kg body wt ip), and eyes were enucleated. Pieces of dissociated retina were incubated with 0.0125% papain (Worthington, Freehold, NJ) in 2.5 mM EGTA, 2 mM L-cysteine, and Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hanks' balanced salt solution (HBSS) for 20 min at 37°C and

then rinsed in 1% bovine serum albumin (BSA; Sigma) and 300 U/ml DNase I (Sigma) in DMEM (Life Technologies). Cells were triturated gently with a Pasteur pipette and collected by centrifugation. Cells were then washed three times with DMEM. Dissociated cells were seeded on glass coverslips coated with poly-D-lysine and used for immunostaining.

**Immunohistochemistry.** Male Wistar-Kyoto rats weighing ~250 g were anesthetized deeply with pentobarbital sodium (100 mg/kg) and perfused with 250 ml of PA solution. After perfusion, eyes were enucleated, fixed again with PA solution for 3–48 h, dehydrated with 30% (wt/vol) sucrose, and then frozen with optimum cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetechnology, Tokyo, Japan). Sections (10  $\mu$ m) were cut on a cryostat and thaw mounted on gelatin-coated slides. Samples were washed twice with PBS containing 0.1% Triton X-100 (PBST) for 5 min each, treated with 5% (wt/vol) goat serum and 5% (wt/vol) BSA in PBST (IH solution) at room temperature (RT) for 60 min, and then incubated with first antibody, i.e., anti-Kir5.1 (0.8  $\mu$ g/ml), anti-Kir4.1 (0.9  $\mu$ g/ml), anti-vimentin (1:400), anti-PSD-95 family (1:400), anti-calbindin (1:400), or anti-PKC- $\alpha$  (1:400) in IH solution at 4°C overnight. The samples were then washed three times with PBST at RT for 30 min each and exposed to appropriate second antibody, i.e., fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Alexa Fluor 488; Molecular Probes) or Texas red-labeled anti-mouse or anti-rat IgG (Alexa Fluor 568; Molecular Probes) goat polyclonal antibodies. Double staining with the antibodies from the same species, i.e., rabbit polyclonal anti-Kir5.1 and rabbit polyclonal anti-Kir4.1 antibodies, was performed by the two-step method as previously reported (19). First, the samples were incubated with anti-Kir5.1 antibody (0.8  $\mu$ g/ml) in IH solution for 2 h at RT, followed by an excess (150  $\mu$ g/ml) of FITC-labeled goat F(ab) anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA), which was enough to saturate the epitopes of the precedent rabbit immunoglobulin (anti-Kir5.1 antibody). After being washed extensively three times, the samples were incubated with anti-Kir4.1 antibody (0.9  $\mu$ g/ml) in IH solution for 2 h at RT, followed by a normal amount (1  $\mu$ g/ml) of Texas red-labeled anti-rabbit IgG (Alexa Fluor 568) goat polyclonal antibody for 1 h at RT. The specificity of this method was confirmed by preliminary experiments (19). The stained samples were examined by laser scanning confocal microscopy. Specificity of the anti-Kir5.1 antibody was confirmed by preincubation with a 100-fold amount of antigenic peptides.

**Immunoblotting.** Retina and brain acutely dissected from rats, or cells transiently expressing GFP-Kir5.1 or GFP, were homogenized with a Physcotron (NITI-ON Medical Physical Instrument, Tokyo, Japan) and then with a tight-fitting glass-Teflon homogenizer in the extraction buffer containing (in mM) 150 NaCl, 50 Tris·HCl (pH 7.5), and 1 EDTA with protease inhibitor cocktails (Sigma). After centrifugation at 100,000 *g* for 30 min, the sedimented membrane fraction was suspended with extraction buffer with 2% (vol/vol) Triton X-100, 1% (wt/vol) CHAPS, and 0.5% (wt/vol) deoxycholate (DOC), homogenized with a glass-Teflon homogenizer, and then centrifuged at 100,000 *g* for 30 min. The supernatants (soluble membrane protein fractions) were separated by SDS-polyacrylamide gel (12%) electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated with anti-Kir5.1 at a concentration of 0.8  $\mu$ g/ml in *buffer A* containing 5% (wt/vol) skim milk and 0.2% (wt/vol) Lubrol PX in 50 mM Tris·HCl (pH 8.0) and 80 mM NaCl. After being washed three times with *buffer A* for 10 min each, the membrane was incubated

with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Wako Pure Chemical, Osaka, Japan) diluted to 1:2,000 (vol/vol) in *buffer A* for 2 h at RT, followed by three washes with 2% Lubrol PX and 0.2% (wt/vol) SDS in 50 mM Tris·HCl (pH 8.0) and 150 mM NaCl. Immunoreactive bands were developed with a SuperSignal chemiluminescence kit (Pierce) according to the manufacturer's instructions.

**Immunoprecipitation.** Protein G-Sepharose beads were pretreated with 1% (wt/vol) BSA in the extraction buffer for 2 h at 4°C. Then 20  $\mu$ l (bed volume) of the beads was incubated with 1  $\mu$ g of antibodies (anti-Kir4.1 antibody or nonimmune IgG) in the extraction buffer for 4 h at 4°C. The soluble membrane protein fractions of rat retinal tissues acutely dissociated from 30 eyes were prepared as described in *Immunoblotting* and incubated with nonimmune IgG-pretreated protein G-Sepharose beads for 4 h at 4°C. After centrifugation, the supernatant was incubated with anti-Kir4.1 antibody-pretreated protein G-Sepharose beads for 4 h at 4°C. The pellets of protein G-Sepharose beads were extensively washed five times with the extraction buffer. Immunoprecipitants were analyzed with SDS-PAGE and immunoblotting with anti-Kir5.1 antibody as described in *Immunoblotting*.

## RESULTS

**Characterization of anti-Kir5.1 antibody and localization of Kir5.1 in retinal cells.** We first confirmed the expression of Kir5.1 mRNA in retina with reverse transcriptase-PCR (RT-PCR) analysis of the total mRNA obtained from rat retinal tissues (not shown). To examine the expression and distribution of Kir5.1 protein in retina we developed a polyclonal antibody against a synthetic peptide corresponding to the NH<sub>2</sub> terminus of rat Kir5.1. After purification of the IgG fraction through protein A- and antigenic peptide-coupled resins from antiserum, we characterized the IgG (anti-Kir5.1 antibody) by immunocytochemical and immunoblotting analyses (Fig. 1, *A* and *B*). First, we tested the specificity of the antibody with immunocytochemistry (Fig. 1*A*). GFP-Kir5.1, transiently expressed in HEK293T cells, was detected with this antibody (Fig. 1*A*, *a–c*) but not with the antibody preincubated with antigenic peptides (Fig. 1*A*, *d–f*). We next used immunoblotting analysis. As shown in Fig. 1*B*, this antibody detected a single band at ~80 kDa in the lysate from HEK293T cells expressing GFP-Kir5.1 (*lane 1*). The band was not detected in the lysate from the cells expressing GFP (*lane 2*). Preincubation of the anti-Kir5.1 antibody with antigenic peptides completely abolished the immunoreactivity (not shown).

Immunostaining of rat retina against anti-Kir5.1 antibody was performed to examine the distribution of Kir5.1 (Fig. 1*C*). Kir5.1 immunoreactivity was broadly detected from the granular cell layer (GCL) to the outer nuclear layer (ONL), and relatively strong immunoreactivity was detected in the inner nuclear layer (INL) and the outer plexiform layer (OPL) (Fig. 1*C*, *a* and *c*). On the other hand, vimentin, which is an intermediate filament and a cell marker for retinal Müller cells (33), was perpendicularly distributed from GCL to ONL (Fig. 1*C*, *b*). The distribution of vimentin seemed to be associated with that of Kir5.1 in INL and OPL, where the cell bodies of Müller cells are found (Fig. 1*C*, *c*).

This result suggests that Kir5.1 may be expressed in Müller cells.

To confirm the expression of Kir5.1 in Müller cells, we examined Kir5.1 immunoreactivity in acutely dissociated retinal cells (Fig. 1*D*). Spotted Kir5.1 immunoreactivity was detected diffusely, but not homogeneously, on cell membrane throughout the aggregated Müller cells (Fig. 1*D*, *a* and *c*). The cells were also stained by anti-vimentin antibody (Fig. 1*D*, *b* and *c*), confirming that these cells were Müller cells.

We next checked the reliability of our anti-Kir5.1 antibody in immunoblot analysis of native tissue extracts. Unexpectedly, we noted that immunoblotting with the anti-Kir5.1 antibody showed two bands in rat brain extract but only a single band in mouse brain extract (Fig. 2*A*). The single band in mouse brain extract corresponded to the lower of the two bands in rat extract. Because the upper band in rat extract was also abolished by preincubation with antigenic peptides, it might represent Kir5.1 with posttranslational modifications, although we cannot completely exclude the possibility that the antibody recognizes an unknown protein endogenously expressed in rat but not in mouse tissue. However, there was practically no difference in the immunohistochemical staining of rat and mouse retinas (Fig. 2*B*). Therefore, even if our anti-Kir5.1 antibody recognizes an unidentified protein isolated from rat brain, the antibody specifically detected Kir5.1 protein in mouse and rat retina.

In the Western blot of rat retina extract, the upper band was so strong that we could not detect the lower band clearly. When we concentrated Kir5.1 proteins in rat retina by immunoprecipitating with anti-Kir5.1 antibody, however, we could clearly detect the lower band, as shown in Fig. 3*h*.

**Differential localization of Kir5.1 and Kir4.1 in retinal Müller cells.** Kir4.1 is expressed on Müller cell membranes and is involved in the K<sup>+</sup>-siphoning action of Müller cells (8, 14). To compare the subcellular distribution of Kir5.1 with that of Kir4.1, we next performed double staining of Kir5.1 and Kir4.1. Figure 3 depicts the distribution of immunoreactivities of Kir5.1, Kir4.1, and AQP4 in sections of rat retina. Both Kir5.1 and Kir4.1 immunoreactivities were detected throughout the retina (Fig. 3, *a–c*). Although the immunoreactivity of Kir4.1 generally overlapped with that of Kir5.1 throughout whole layers of retina (Fig. 3, *a–c*), strong immunoreactivity of Kir4.1 without Kir5.1 was noted at the endfoot region facing the vitreous surface (Fig. 3*e*). In INL, Kir5.1, but not Kir4.1, was stained in second-order neurons (arrowheads in Fig. 3*f*) whereas in the processes surrounding blood vessels Kir4.1, but not Kir5.1, was detected (Fig. 3*f*). The localization of Kir4.1 was quite similar to that of the water channel AQP4 (Fig. 3*d*), which was shown previously to be colocalized with Kir4.1 on Müller cell membranes (18). We could not detect any specific immunoreactivity of Kir4.1 in the retinal pigment epithelium layer in this study (17). Detailed examination showed that, in the distal portions (Fig. 3*g*) and microvilli of Müller cells in the extraneural spaces in IPL,

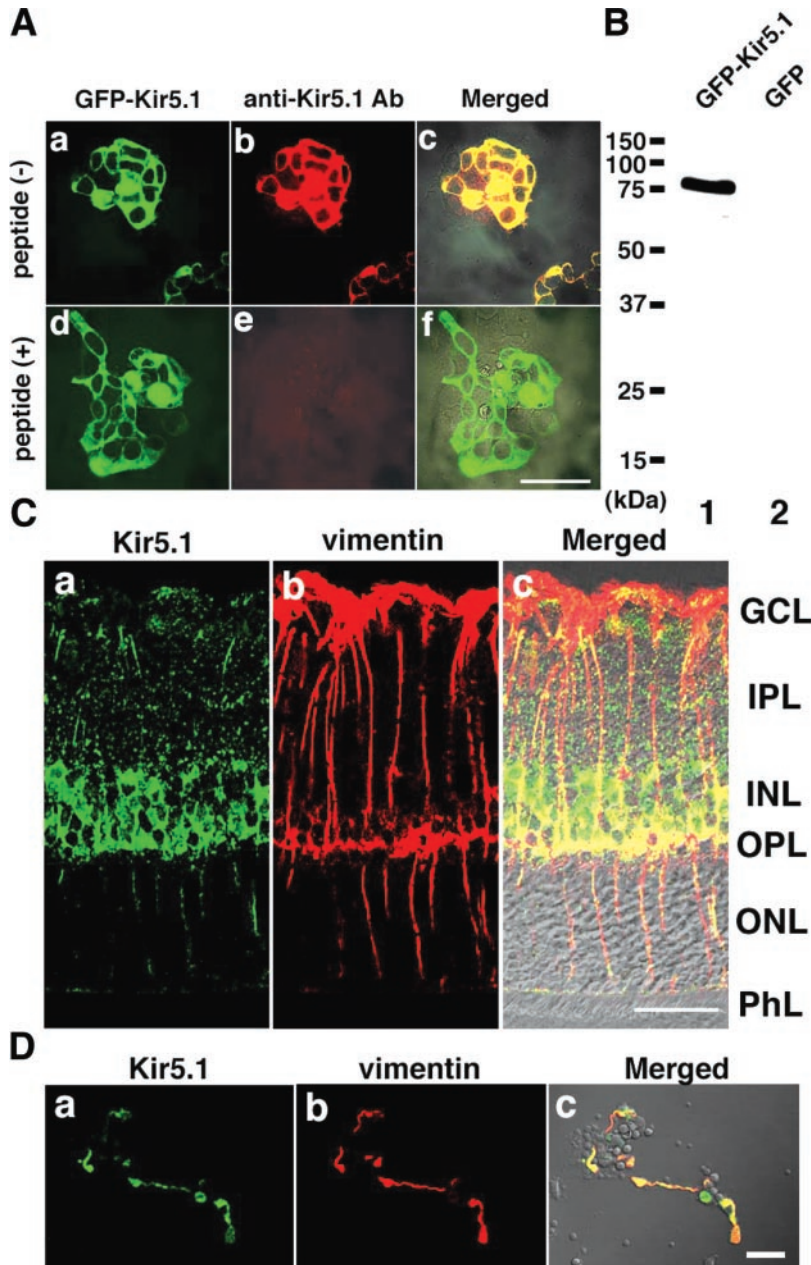


Fig. 1. Characterization of anti-Kir5.1 antibody and expression of Kir5.1 in retina. **A**: immunocytochemistry for Kir5.1 exogenously expressed in HEK293T cells. Cells transfected with green fluorescent protein (GFP)-Kir5.1 cDNA were fixed with 4% paraformaldehyde and detected with anti-Kir5.1 antibody (Ab). Immunoreactivity of anti-Kir5.1 antibody visualized with Texas red-conjugated anti-rabbit IgG antibody (red in *b* and *c*) completely overlapped with GFP (green in *a* and *c*). The immunoreactivity was absent when the antibody was preincubated with antigenic peptide (*d-f*). Scale bar, 50  $\mu$ m. **B**: immunoblotting analysis with anti-Kir5.1 antibody. Lysates of HEK293T cells with GFP-Kir5.1 (*lane 1*) or GFP (*lane 2*) were subjected to immunoblotting. Anti-Kir5.1 antibody specifically recognizes GFP-Kir5.1 (~80 kDa) in HEK293T cell lysates. Markers of Bio-Rad prestained precision standards (broad range; kDa) are shown on the left. **C**: immunohistochemical analysis of Kir5.1 and vimentin in retina. A section of rat retina (10  $\mu$ m thick) was double-stained with affinity-purified rabbit anti-Kir5.1 antibody (green in *a* and *c*) and with monoclonal anti-vimentin antibody (red in *b* and *c*). **c**: Triple exposures of immunostaining of anti-Kir5.1, anti-vimentin, and Nomarski images. Scale bar, 50  $\mu$ m. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PhL, photoreceptor layer. **D**: immunostaining of acutely dissociated retinal cells. Cells were double-stained with affinity-purified rabbit anti-Kir5.1 antibody (green in *a* and *c*) and with monoclonal anti-vimentin antibody (red in *b* and *c*). **c**: Triple exposures of immunostaining with anti-Kir5.1 and anti-vimentin antibodies and Nomarski image. Scale bar, 50  $\mu$ m.

INL (except around blood vessels), OPL, and ONL (Fig. 3, *e-g*), the distribution of Kir5.1 largely overlapped that of Kir4.1. It has been reported that Kir4.1 and Kir5.1 can coassemble to form a functional Kir channel in heterologous expression systems (20, 31, 37); it is therefore possible that Kir5.1 and Kir4.1 form heterotetrameric channels in these areas.

To examine whether Kir4.1/Kir5.1 heterotetramers were present in retinal Müller cells, we performed an immunoprecipitation assay (Fig. 3*h*). The immunoprecipitants obtained from retinal membrane fractions with anti-Kir4.1 antibody contained Kir5.1 (Fig. 3*h*, *lane 2*), whereas the precipitants with nonimmune IgG did not (*lane 1*). Immunoprecipitants with anti-Kir5.1 of whole retina (Fig. 3*h*, *lane 3*) and brain (*lane 4*) were also shown in the same gel. These results

indicate that Kir4.1/Kir5.1 heterotetrameric channels exist in retina.

**Distribution of PDZ-anchoring proteins and Kir5.1 in retina.** Kir5.1 can form functional homotetrameric channels with the aid of PSD-95 (29). To examine whether Kir5.1 interacts with PDZ-containing anchoring proteins in the retina we performed double immunostaining with anti-Kir5.1 and anti-PSD-95 family antibodies (Fig. 4*A*). The manufacturers state that the antibody used in this study against the PSD-95 family recognizes not only PSD-95 but also other PDZ domain-containing proteins such as SAP97 and Chapsin-110/PSD-93. Strong immunoreactivity of the anti-PSD-95 family antibody was detected only in OPL (Fig. 4*A*, *b* and *e*), which did not overlap with the distribution of Kir5.1 (Fig. 4*A*, *a*, *c*, *d*, and *f*). The distribution of

Fig. 2. Immunoblot and immunohistochemistry of rat and mouse tissues with anti-Kir5.1 antibody. **A**: immunoblot of rat and mouse brain tissue extracts with anti-Kir5.1 antibody. Two bands are detected in rat brain extracts (arrow and arrowhead), but only the lower band (arrowhead) was detected in mouse tissues. Markers of Bio-Rad prestained precision standards (broad range) are shown on the left. **B**: immunohistochemical study of rat and mouse retina with anti-Kir5.1 antibody. There seemed to be practically no difference between rat and mouse retina. Scale bars, 50  $\mu$ m.

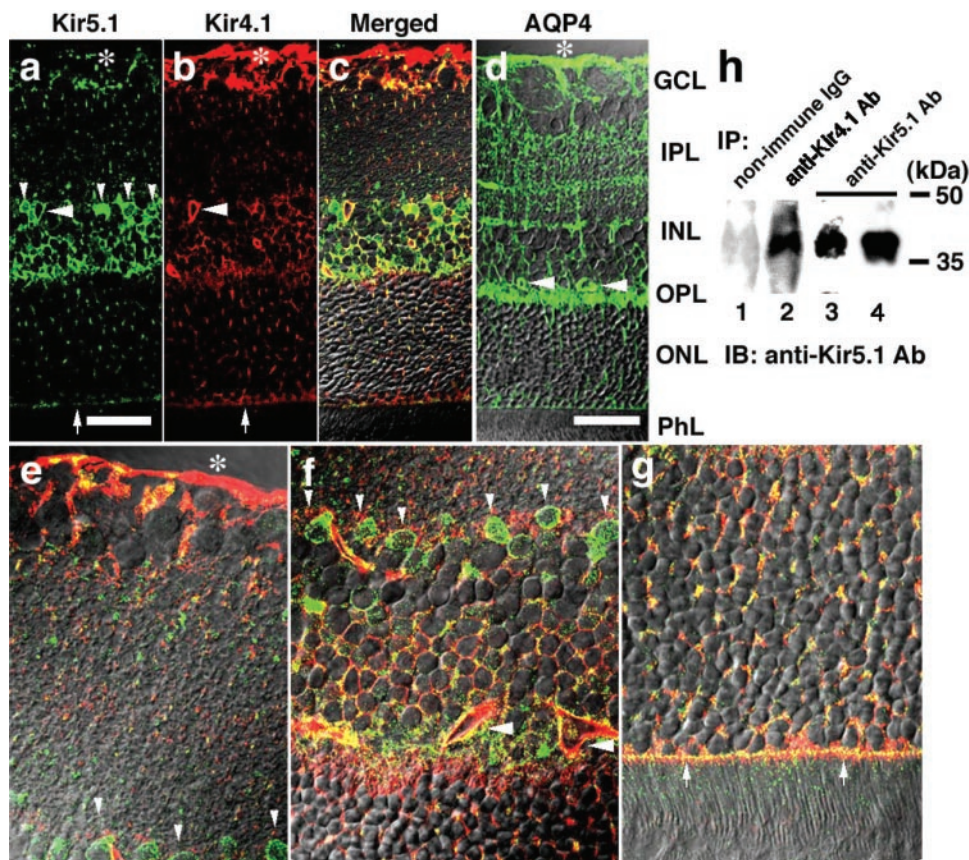
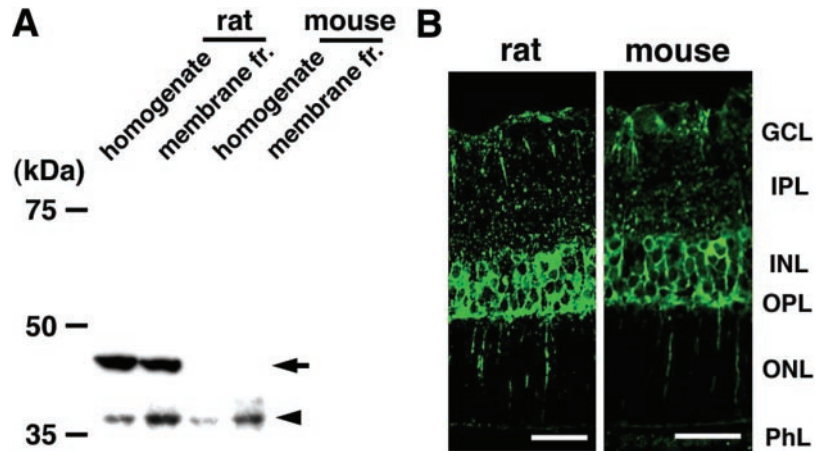


Fig. 3. Immunohistochemistry and immunoprecipitation analysis of Kir5.1, Kir4.1, and aquaporin (AQP4) in retina. **A** sagittal section was double stained with affinity-purified rabbit anti-Kir5.1 antibody (green in **a** and **c**) and with affinity-purified rabbit anti-Kir4.1 antibody (red in **b** and **c**). **c**: Merged image of both fluorescence images and Nomarski image of the same sagittal section as **a** and **b**. **d**: Immunostaining of affinity-purified rabbit anti-AQP4 antibody (green). **e-g**: Magnified images of triple exposures of immunostaining with anti-Kir5.1 and anti-Kir4.1 antibodies and Nomarski image in GCL (**e**), INL (**f**), and ONL (**g**). Kir5.1 was expressed in Müller cells and also in some types of neurons. Kir4.1 has already been revealed to be expressed in Müller cells, but its distribution is different from that of Kir5.1 while being quite similar to that of AQP4. Scale bars, 50  $\mu$ m. Asterisk, vitreous surface; horizontal arrowheads, blood vessel; vertical arrowheads, Kir5.1-positive neuron; arrows, outer limiting membrane. **h**: The existence of the Kir4.1/Kir5.1 heterotetramer in retina was confirmed by immunoprecipitation (IP) assay. The immunoprecipitants were immunoblotted (IB) with anti-Kir5.1 antibody. Although the immunoprecipitant with nonimmune IgG (**lane 1**) did not contain Kir5.1, the immunoprecipitant with anti-Kir4.1 antibody (**lane 2**) did contain Kir5.1. To show the position of Kir5.1, immunoprecipitants with anti-Kir5.1 of retina (**lane 3**) and brain (**lane 4**) were exposed in the same gel. Markers of Bio-Rad prestained precision standards (broad range) are shown on the right.

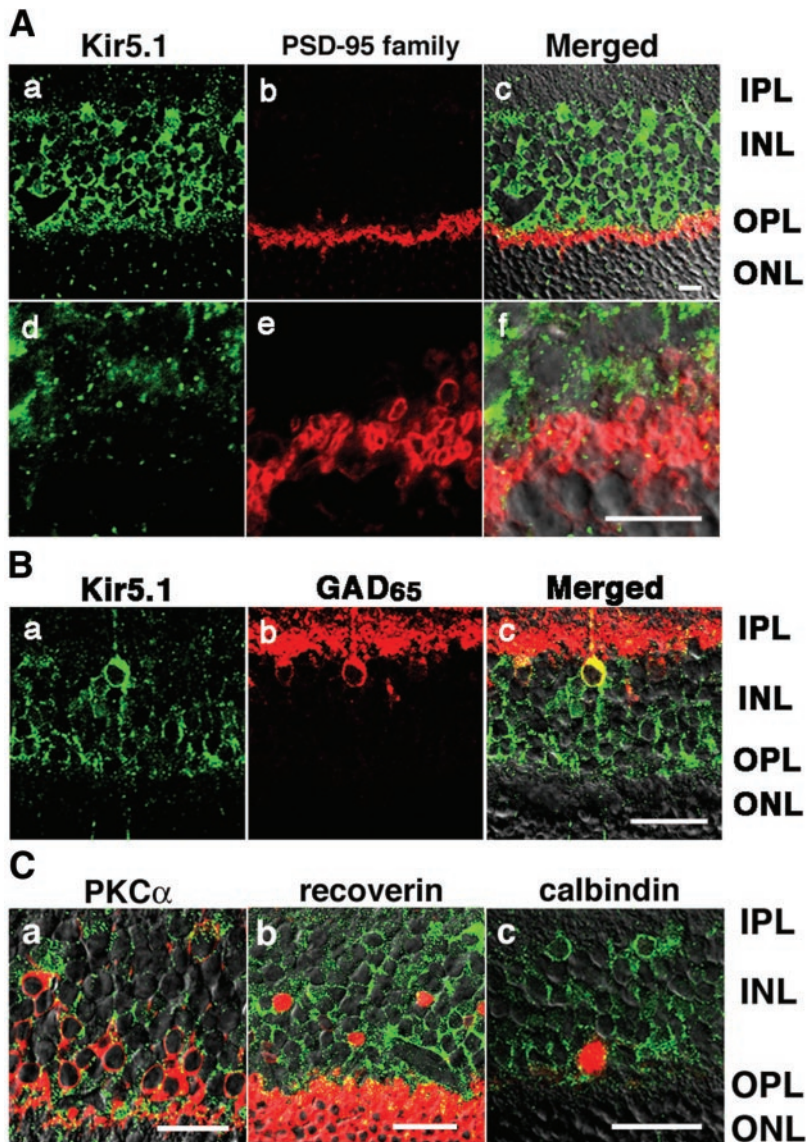


Fig. 4. Immunohistochemical analysis of Kir5.1, PSD-95 family proteins, and several retinal cell markers in retina. **A**: immunohistochemical analysis of Kir5.1 and PSD-95 family proteins. A section of retina was double stained with affinity-purified rabbit anti-Kir5.1 antibody (green in *a*, *c*, *d*, and *f*) and with mouse monoclonal anti-PSD-95 family antibody (red in *b*, *c*, *e*, and *f*). *c* and *f*: Triple exposures of immunostaining with anti-Kir5.1, anti-PSD95 family protein antibodies, and Nomarski of the same sagittal section as *a* and *b* and *d* and *e*, respectively. Scale bars, 10  $\mu$ m. **B**: immunohistochemical analysis of Kir5.1 and glutamic acid decarboxylase (GAD<sub>65</sub>). Retinal section was double stained with anti-Kir5.1 antibody (green in *a* and *c*) and with mouse monoclonal anti-GAD<sub>65</sub> antibody (red in *b* and *c*). *c*: Triple exposures of immunostaining with anti-Kir5.1 and anti-GAD<sub>65</sub> antibodies and Nomarski image. Scale bar, 50  $\mu$ m. **C**: immunohistochemical analysis of Kir5.1 and several retinal cell markers. Retinal section was double stained with anti-Kir5.1 antibody (green in *a*–*c*) and with mouse monoclonal anti-protein kinase C(PKC)- $\alpha$  antibody (red in *a*), rat monoclonal anti-recoverin antibody (red in *b*), and mouse monoclonal anti-calbindin antibody (red in *c*). None of these cell markers was colocalized with Kir5.1. Scale bars, 50  $\mu$ m.

PSD-95 family proteins was consistent with a previous report (16) in which PSD-95 was shown to be localized at the presynaptic membrane of photoreceptor cells. These results suggest that Kir5.1 is not colocalized with PSD-95 family proteins in retina.

**Identification of Kir5.1-expressing retinal neurons.** To identify the type of neurons expressing Kir5.1 in INL, retinal sections were stained by antibodies against various retinal cell markers, such as GAD<sub>65</sub> (Fig. 4B), PKC- $\alpha$  (Fig. 4C, *a*), recoverin (Fig. 4C, *b*), and calbindin (Fig. 4C, *c*). GAD, which is a rate-limiting enzyme in GABA synthesis from glutamic acid, is a marker of GABAergic neurons (12). In retina, it was reported that GAD is expressed in GABAergic amacrine cells and in a subset of horizontal cells (5, 34). As shown in Fig. 4B, the Kir5.1-positive neurons in INL were stained with a monoclonal antibody against GAD<sub>65</sub>, one of the isoforms of GAD. PKC- $\alpha$ , recoverin, and calbindin are the markers for rod bipolar cells (35, 36), photoreceptor cells and cone bipolar cells (6), and

horizontal cells (24), respectively. The Kir5.1-positive cells were stained by none of them. These results suggest that Kir5.1 is expressed in GABAergic retinal neurons that possibly belong to amacrine cells. Because amacrine cells are extremely heterogeneous, consisting of no less than 40 different species (15, 32), we could not determine whether Kir5.1 is expressed exclusively in GABAergic amacrine cells.

## DISCUSSION

This immunohistochemical study of the mammalian retina clearly shows that the inwardly rectified K<sup>+</sup> channel subunit Kir5.1 is expressed both in Müller cells and in some amacrine cells. However, two recent reports surprisingly showed no expression of Kir5.1 in retina (13, 27). The immunohistochemical analyses (13) showed that Kir2.1 and Kir4.1, but not Kir2.2, Kir2.3, or Kir5.1, were expressed in Müller cells. RT-PCR of Müller cells showed at the mRNA level the

expression of Kir2.1, Kir2.2, Kir2.4, Kir3.1, Kir3.2, Kir4.1, Kir6.1, and Kir6.2, but not Kir4.2 or Kir5.1 (27). Although we cannot explain the discrepancy between these results and ours, we can speculate that it may be attributable to differences of quality of the antibodies and the primer sequences for RT-PCR.

Kir5.1 can form heteromers with either Kir4.1 or Kir4.2 (25). In Kir5.1-expressing neurons Kir4.1 immunoreactivity was not detected, and because RT-PCR analysis showed that Kir4.2 was not expressed in the retina (not shown), Kir5.1 may here exist as homotetramers. Although PSD-95 family-anchoring proteins are a prerequisite for forming functional Kir5.1-homotetramers (29), immunoreactivity against these proteins was also absent from these neurons. PDZ-anchoring proteins that cannot be detected by the antibody used in this study might exist in neurons. It is also possible that a novel mechanism might be responsible for control of the localization and function of homomeric Kir5.1 channels in amacrine cells. Further studies are needed to clarify this point.

Müller cells display spatial buffering of extracellular  $K^+$  in the retina. It is thought that  $K^+$  accumulated in IPL on light-induced synaptic excitation is taken up by Müller cells through fine processes surrounding synapses.  $K^+$  may then be extruded from Müller cells via endfeet into the vitreous humor or via processes toward capillary blood vessels. Strong immunoreactivity of Kir4.1 without Kir5.1 was detected in the endfeet of Müller cells facing the vitreous body as well as in the processes surrounding blood vessels. This suggests that extrusion of  $K^+$  from Müller cells is exclusively carried out by homomeric Kir4.1 channels.

In the distal portions of Müller cells and in their fine processes in IPL, the immunoreactivity of Kir5.1 largely overlapped that of Kir4.1. Kir5.1 can form a heterotetramer with Kir4.1 in heterologous expression systems, and we found that the immunoprecipitant for Kir4.1 in retina contained Kir5.1. Therefore, although we cannot exclude the possibility that some Kir5.1 forms homomeric assemblies, it seems likely that Kir4.1/Kir5.1 heterotetramer channels exist in these areas of Müller cells and are responsible for uptake of  $K^+$  accumulated in IPL. The Kir4.1/Kir5.1 heteromeric channel is sensitive to physiological ranges of  $pH_i$ , whereas the homomeric Kir4.1 channel is more resistant (30, 31, 37). This functional characteristic of the Kir4.1/Kir5.1 heteromeric channel might be beneficial for uptake of  $K^+$  ions from extracellular solution. Glial cells, including retinal Müller cells, possess an electrogenic  $Na^+HCO_3^-$  cotransport system (1), and an increase in extracellular  $K^+$  concentration would depolarize them and intensify the inward transport of  $Na^+$  and  $HCO_3^-$ . The increased influx of  $HCO_3^-$  would cause an intracellular alkalization (20, 21). This should cause an increase of the open probability of Kir4.1/Kir5.1 heterotetrameric channels and may enhance  $K^+$  uptake into Müller cells. This indicates the possibility of a novel coupling mechanism between  $H^+$  regulation and  $K^+$  buffering by Müller cells.

In summary, it is suggested that for  $K^+$  siphoning retinal Müller cells differentially utilize at least two types of Kir channels, the Kir4.1 homotetramer for  $K^+$  extrusion and the Kir4.1/Kir5.1 heterotetramer for  $pH_i$ -dependent  $K^+$  uptake. In acutely isolated Müller cells, we could detect only a single population of Kir channel currents whose properties were identical to those of the cloned Kir4.1 homotetramer expressed in HEK293T cells (8, 28). The results shown here indicate that Kir4.1/Kir5.1 heterotetrameric channels are localized mainly at the distal portions and in fine processes of Müller cells and that the channel density is relatively low. The failure to detect Kir4.1/Kir5.1 channels in previous studies may be due to their low density and also to the loss of fine processes and microvilli during enzyme isolation of the cells.

Because Kir5.1 immunoreactivity is detected in astrocytes in the brain (unpublished observation), where we have previously shown (7) the expression of Kir4.1, the differential utilization of homomeric and heteromeric assembly of Kir4.1 and Kir5.1 subunits may be a general mechanism for glial  $K^+$  spatial buffering. The molecular mechanisms responsible for the differential distribution of Kir4.1 homomer and Kir4.1/Kir5.1 heteromeric channels within a glial Müller cell are unknown. Laminin is an extracellular matrix protein that is abundant in the retina and may play a pivotal role in the control of clustered distribution of Kir4.1 channels in Müller cells (8). Dystrophin Dp71, which associates with laminin via the dystroglycan complex (3), is also essential for the clustered distribution of Kir4.1 (4). Therefore, extracellular matrices seem to be involved in distribution of Kir4.1. Both Kir4.1 and Kir5.1 possess putative PDZ anchor protein binding motifs. Thus it is also possible that intracellular PDZ proteins might also be involved in the control of their distribution. Further studies are needed to clarify the whole view of the differential control of function and localization of Kir channels containing Kir4.1 and/or Kir5.1 in Müller and also other glial cells.

We thank Dr. Ian Findlay (Université de Tours, Tours, France) for critically reading this manuscript.

## DISCLOSURES

This work was supported by Grant-in-Aid for Specific Research on Priority Area (B) 12144207 (to Y. Kurachi), by Grant-in-Aid for Encouragement of Young Scientists 13770044 (to M. Ishii) from the Ministry of Education, Science, Sports and Culture of Japan, by a Japan Heart Foundation Dr. Hiroshi Irisawa Commemorative Research Grant (to M. Ishii), and by a Grant-in-Aid from Ichiro Kanehara Foundation (to M. Ishii).

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