Molecular characterization of an inward rectifier channel ($I_{\text{Kir}}$) found in avian vestibular hair cells: cloning and expression of pKir2.1

Manning J. Correia, Thomas G. Wood, Deborah Prusak, Tianxiang Weng, Katherine J. Rennie, and Hui-Qun Wang. Molecular characterization of an inward rectifier channel ($I_{\text{Kir}}$) found in avian vestibular hair cells: cloning and expression of pKir2.1. Physiol Genomics 19: 155–169, 2004. First published August 17, 2004; doi:10.1152/physiogenomics.00096.2004. —A fast inwardly rectifying current has been observed in some of the sensory cells (hair cells) of the inner ear of several species. While the current was presumed to be an $I_{\text{Kir}}$ current, contradictory evidence existed as to whether the cloned channel actually belonged to the Kir2.0 subfamily of potassium inward rectifiers. In this paper, we report for the first time converging evidence from electrophysiological, biochemical, immunohistochemical, and genetic studies that show that the Kir2.1 channel carries the fast inwardly rectifying currents found in pigeon vestibular hair cells. Following cytoplasm extraction from single type II and multiple pigeon vestibular hair cells, mRNA was reverse transcribed, amplified, and sequenced. The open reading frame (ORF), consisting of a 1,284-bp nucleotide sequence, showed 94, 85, and 83% identity with Kir2.1 subunit sequences from chick lens, Kir2 sequences from human heart, and a mouse macrophage cell line, respectively. Phylogenetic analyses revealed that pKir2.1 formed an immediate node with hKir2.1 but not with hKir2.2–2.4. Hair cells (type I and type II) and supporting cells in the sensory epithelium reacted positively with a Kir2.1 antibody. The whole cell current recorded in oocytes and CHO cells, transfected with pigeon hair cell Kir2.1 (pKir2.1), demonstrated blockage by Ba$^2^+$ and sensitivity to changing K$^+$ concentration. The mean single-channel linear slope conductance in transfected CHO cells was 29 pS. The open dwell time was long (~300 ms at ~100 mV), and the closed dwell time was short (~34 ms at ~100 mV). Multistates ranging from 3–6 were noted in some single-channel responses. All of the above features have been described for other Kir2.1 channels. Current clamp studies of native pigeon vestibular hair cells illustrated possible physiological roles of the channel and showed that blockage of the channel by Ba$^2^+$ depolarized the resting membrane potential by ~30 mV. Negative currents hyperpolarized the membrane ~20 mV before block but ~60 mV following block. RT-PCR studies revealed that the pKir2.1 channels found in pigeon vestibular hair cells were also present in pigeon vestibular nerve, vestibular ganglion, lens, neck muscle, brain (brain stem, cerebellum and optic tectum), liver, and heart.

Pigeon; potassium channel

Over fifty years ago Katz (27), described a potassium (K$^+$) conductance showing “rectification anomale” in frog skeletal muscle. That is, the inward ionic current rectification was abnormal since it was in a direction opposite to the outward rectification normally seen in K$^+$ delayed rectifiers. Since then, inwardly rectifying conductances have been studied in numerous cells including glia and neurons in the brain, cardiac cells, skeletal muscle cells, renal and pancreatic cells, sensory and other epithelial cells, as well as various immortal cell lines. Those studies are reviewed elsewhere (3, 10, 25, 46).

Inwardly rectifying conductances have been measured in sensory epithelial cells (hair cells) of the inner ear of several species. These conductances have been studied in the developing and the mature cochlea (15–17, 37, 38, 48, 62) and in the vestibular labyrinth (4, 23, 39–42, 47, 58, 65).

In 1993, Ho et al. (21) and Kubo et al. (32) first reported the cloning, by expression, of an inward rectifier channel. Since then, using expression cloning and homology screening, a novel family of inward rectifier potassium channel genes (Kir) has been studied revealing seven subfamilies designated, using the nomenclature of Chandy and Gutman (5) and Gutman et al. (19), as Kir1.0 through Kir7.0.

Previously, only one study (45) has cloned an inward rectifier found in cochlea or vestibular hair cells. In that study, a gene named cIRK1 was isolated from sections of different regions of the auditory papilla (including hair cells, supporting cells, and nerve terminals) of the chick which was subsequently expressed in oocytes. Single-channel and whole cell patch clamp recordings from oocytes revealed a current with a single-channel conductance of 16 pS and a barium block sensitivity of EC$_{50} = 12$ µM. This single-channel conductance is only 70% (32, 34), and the EC$_{50}$ is roughly five times that determined previously for other cloned Kir2.1 channels carrying $I_{\text{Kir}}$ currents (61, 67).

Herein, we describe a series of experiments in which we isolated for the first time a gene (pKir2.1) coding for an inward rectifier channel in single vestibular hair cells of the pigeon. We present evidence from electrophysiological, pharmacological, immunohistochemical, and biochemical studies suggesting that the gene encodes a Kir2.1 potassium ion channel.
MATERIALS AND METHODS

All of the procedures described below were approved by the University of Texas Medical Branch (UTMB). Institutional Animal Care and Use Committee and conform to the Guiding Principles for Research Involving Animals and Human Beings as set forth by the National Institutes of Health and The American Physiological Society. The experimental animals used in these studies were male and female white king pigeons, Columba livia (Double T Farms, Glenwood, IA). The animals were 10–52 wk old and weighed from 200 to 400 g.

RT-PCR Cloning of pKir2.1

Total RNA was isolated from pigeon vestibular semicircular canal ampullae (including hair cells, supporting cells, and nerve terminals) using guanidinium thiocyanate (7). First-strand cDNA was synthesized in a final volume of 20 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 10 mM dithiothreitol, 50 μM of each dNTP, 0.5 μg oligo(dT)₁₂–₁₈, and 200 U of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplification was performed using a 100-μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 1–10 μl cDNA, 5 U of AmpliTaq (PerkinElmer, Wellesley, MA), and 100 pmol of each primer. Each reaction was denatured for 2 min at 95°C prior to 30–35 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 4 min. The final primer extension was continued for 10 min at 72°C to enhance complete synthesis of the amplified target DNA. The initial PCR amplifications were performed using primers C₁–C₄ listed in Table 1, which were designed based upon the nucleotide sequence of the potassium inward rectifier channel, cIRK₁, cloned from the chicken basilar papilla (45).

The amplified DNAs (567 and 997 bp) shared a common sequence overlap of 282 bp. These DNA fragments were subcloned into pCR 2.1 (Invitrogen) and sequenced. Internal primers were designed (P₁ and P₂, Table 1) based upon the pigeon inward rectifier potassium channel (pKir2.1) sequence and used in a second set of PCR amplifications in combination with primers C₁ and C₂ (Table 1). The amplified DNAs from these PCR reactions were denatured, annealed for 1 min at 40°C and then incubated at 72°C for 10 min in a final volume of 100 μl containing 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 50 mM KCl, 200 μM of each dNTP, and a mixture of AmpliTaq (PerkinElmer) and Vent (New England Biolabs, Beverly, MA) 1:1. DNA containing the full-length open reading frame (ORF) for the pigeon pKir2.1 was then amplified following the addition of primers C₅ and C₆ (Table 1). These primers introduce unique EcoRI and BamH restriction sites at the respective 5’ and 3’ ends of the amplified DNA. pKir2.1 cDNA was subcloned in PCR 2.1 (Invitrogen) and confirmed by DNA sequence analysis. The sequence for the pKir2.1 ORF can be found in GenBank under accession number AF192507.

Table 1. PCR primers for pKir2.1 ORF

<table>
<thead>
<tr>
<th>Primer</th>
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ORF, open reading frame.

Single cell RT-PCR

Because it was known that pKir2.1 was in hair cells and surrounding supporting cells (39) but unknown whether pKir2.1 was expressed in abutting vestibular nerve terminals (calyces and boutons) and/or unmyelinated nerve fibers, all of which can be found in excised vestibular end-organs (ampullae and maculae), single type I and type II hair cells in slices through the pigeon ampullary and utricular epithelium (39, 65) were identified and whole cell patch clamped. Inwardly rectifying whole cell JKᵢᵣtonic currents were obtained from type II hair cells (see Figs. 1C and 3A); the cytoplasm was aspirated (Fig. 3B, a–c), and single-cell quantitative RT-PCR was carried out using primers C₃ and C₄ (see Fig. 3C).

Single Cell Quantitative RT-PCR (qRT-PCR)

Following the recording of ionic currents, the contents of the pipette containing hair cell cytoplasm and pipette intracellular solution were ejected into a sterile, silanized microcentrifuge tube. Cytoplasm was gathered from 70 different cells. The tube was stored immediately at –80°C and subsequently assayed for pKir2.1 using quantitative RT-PCR.

First-strand cDNA synthesis was performed using SuperScript II (Invitrogen) and conditions recommended by the manufacturer. PCR amplification was performed in a 100-μl reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 5 U of AmpliTaq (PerkinElmer), and 100 pmol of each primer (5’ AGACATCAATGATGGTTGACGC 3’ and 5’ AGATGGGTGATTGGACACTGCG 3’). These primers were specific for the pKir2.1 ORF sequence. End-labeled (Δ[32P]ATP) primers (4–6 × 10⁶ cpm/pmol) were added (1–3 × 10⁶ cpm/pmol) for both primers to the PCR reaction. Following a denaturation step at 95°C, for 2 min, reactions were incubated for 35 cycles of 94°C for 15 s, 65°C for 30 s, and 72°C for 2 min. The final primer extension was continued for 10 min at 72°C. An internal standard RNA representing a 59-base deletion in the pKir2.1 target sequence (ΔpKir2.1) was added (100 molecules) to each RT-PCR assay. This reference RNA was used to quantify the pKir2.1 transcripts in each sample (2). A 20-μl sample from each reaction was electrophoresed through a 5% polyacrylamide gel (Fig. 3D) and analyzed using a PhosphorImager (Molecular Devices, Sunnyvale, CA).

Multiple Tissue RT-PCR

Total RNA was isolated from pigeon ampullae, maculae, vestibular ganglia, lens, neck muscle, brain stem, cerebellum, optic tectum, liver, and heart using the ToTALLY RNA kit (Ambion, Austin, TX). First-strand cDNA synthesis was performed using the RETROscript kit (Ambion) following the manufacturer’s protocol. PCR amplification was performed using the same primers (specific for pKir2.1 ORF) as were used for single cell qRT-PCR. Also, primers specific to the L19 ribosomal protein gene, were used as a positive control (sense 5’-TGAGAGGATTCATCATTGCACC-3’, antisense 5’-TGCGCT-AATGTTTTTGCGTGCG-3’). The reaction mixture contained 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μM of each dNTP, 5 μl cDNA, 50 pmol of each primer, and 5 U AmpliTaq DNA polymerase (PerkinElmer) in a total volume of 100 μl. The cycle conditions for the PCR were as follows: denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s and primer extension at 72°C for 2 min for a total of 40 cycles. The final primer extension was expanded to 10 min to allow for full completion of incomplete products. Amplification products were analyzed by electrophoresis using 5% nondenaturing polyacrylamide gels and visualized by ethidium bromide (1%) staining.

pKir2.1 Expression in Oocytes

Oocyte vector. To develop a functional assay for pKir2.1, we cloned the pKir2.1 ORF into pOCYT7, a vector modified from pTLN.
and 5% CO2. The complete media contained Dulbecco's modified Eagle's medium at UTMB and maintained but split once a week in complete (CHO) cells.

0.33 Ca(NO3)2, and 0.41 CaCl2. The oocytes were then washed with Barth solution and then stored for 3–4 h at 17°C. Fifty nanograms of cRNA was microinjected (Drummond Scientific, Broomall, PA) into stage II oocytes following the methods of Mo and colleagues (43). The injected oocytes were stored in Barth solution, and every day the solution was changed and dead oocytes were removed.

Oocyte electrophysiology. At 24–48 h postinjection, ionic currents were recorded from the oocytes using two-electrode voltage clamp methods. The recording pipettes were filled with 3 M KCl and had impedances of ~1 MΩ. The bath was ND96 containing (in mM) 2 KCl, 98.0 NaCl, 1.0 MgCl2, and 5.0 HEPES plus amikacin (100 μg/ml) and streptomycin (100 μg/ml). The amplifier that was used was a Heka EPC-7 (Instrutech, Port Washington, NY).

While the pKir2.1 ionic currents and their response to blockers in oocytes were qualitatively similar to those recorded in native hair cells, the time course of the inactivation of the pKir2.1 currents in oocytes was an order of magnitude longer than the I Kir currents in native hair cells. To obtain a heterologous expression system (HES) with physical dimensions more like hair cells, we developed techniques to transfect pKir2.1 into mammalian Chinese hamster ovary (CHO) cells.

CHO Cell Culture and Plasmid Transfection

CHO-S cells were obtained from the Recombinant DNA Laboratory at UTMB and maintained but split once a week in complete media in T75 flasks in an incubator at 37°C, 95% relative humidity and 5% CO2. The complete media contained Dulbecco's modified Eagle's medium (DMEM; Invitrogen/GIBCO, Carlsbad, CA), 10% fetal bovine serum (heat-inactivated, Invitrogen/GIBCO), 1× nonessential amino acids (Sigma, St. Louis, MO), and 1× penicillin/streptomycin/neomycin (Invitrogen/GIBCO). Following trypsinization, CHO cells were split from T75 flasks, counted with a hemocytometer, and plated into 35-mm petri dishes at a density of 3 × 105 cells/dish (2 ml). The cells were maintained up to 72 h in 2 ml of complete media. After 25 passages the cells were discarded, and new cells were obtained.

When the cells were 40–80% confluent, they were transfected, using liposome-mediated methods, with plasmids encoding pKir2.1 ORF and enhanced green fluorescent protein (EGFP). In some experiments, pKir2.1 and EGFP were cloned into separate vectors (pC3DNA3 and pEGFP-N1; BD Biosciences/Clontech, San Jose, CA). These plasmids were cotransfected into CHO cells. In other transfection experiments, both pKir2.1 and EGFP were expressed in the same vector (pCMS-EGFP, BD Biosciences/Clontech), or pKir2.1 was cloned in-frame with the COOH terminus of EGFP (pEGFP-C1, BD Biosciences/Clontech) or the NH2 terminus of EGFP (pEGFP-N1, BD Biosciences/Clontech).

Lipofectamine 2000 (Invitrogen/GIBCO) and Polyfect (Qiagen, Valencia, CA) were used as transfectants. In the former case, 10–12 μg of DNA was used per 10 μl of Lipofectamine 2000; in the latter case, 1.5–3.0 μg of DNA/10 μl of Polyfect was used. The remaining procedures were according to the manufacturers instructions. The transfection efficiency was 60–80%. The cells were incubated post-transfection for 24, 48, and 72 h. The cells were removed from the 35-mm petri dishes using a nonenzymatic dissociation solution (Specialty Media, Phillipsburg, NJ) and centrifuged at 500 g. After the supernatant was removed, the cells were resuspended in the extracellular bath solution and plated on the coverslip bottom of the recording dish, which had previously been coated twice with poly-D-lysine (0.5 μg/ml). In other cases, cells were cultured on 25-mm circular coverslips. Surface tension held the coverslips to the recording dish bottom.

CHO Cell Electrophysiology

Electrodes, pulled on a P-2000 puller (Sutter Instruments, Novato, CA), were made from blanks with an internal filament. The glass was either quartz (0.75 ID, 1.50 OD; Sutter Instruments) or borosilicate (0.75 ID, 1.50 OD, no. 1B150F-3; World Precision Instruments, Sarasota, FL). To prohibit mRNA from adhering to the walls of the pipettes during cytoplasm extraction, the borosilicate or quartz blanks were initially washed with Chromogel (Fisher Scientific, Hampton, NH), silanized with 5% dimethyldichlorosilane (Sigma) in chloroform (Electron Microscopy Sciences, Hatfield, PA), then subsequently washed multiple times and dry heat sterilized at 240°C. The whole cell recording electrode impedance was typically 2–5 MΩ. The single-channel electrode impedance was typically 2–5 MΩ, but often to isolate a single channel, electrodes were used with impedances as high as 30 MΩ. Series resistance and capacitance were compensated between 80 and 98% with a typical value of 95%. No online leak subtraction was performed. In some cases, leak subtraction was performed digitally. Whole cell currents were amplified 1× or 0.5×, and single-channel currents were amplified 100–200×, using an Axopatch 200 amplifier (Axon Instruments, Union City, CA). Signals were acquired using a Digidata 1200 interface (Axon Instruments) or a 16-bit analog-to-digital interface (National Instruments, Austin, TX). The whole cell and single-channel currents were filtered at 2 kHz and sampled at 5 kHz. For the single-channel recordings, the system noise was 0.5 pA.

Stimulus waveforms for single-channel cell-attached on-cell recordings included voltage ramps and pulses over the range from 40 to 180 mV (Δ = 20 mV). Positive shifts in applied voltage were hyperpolarizing, and positive current was inward. For whole cell recordings the same applied voltages were used but with inverted sign and the current polarities were opposite. Current clamp whole cell recordings were made using the Axoclamp 2A amplifier (Axon Instruments), and pulse stimulation ranged from 20 to −100 nA (Δ = 20 nA).

Cells that showed green fluorescence, when viewed using FITC epifluorescence and differential-interference contrast (DIC) transmitted light, were selected for patch clamp recording. The cells were viewed through the optics of an Axophot microscope (Carl Zeiss, Oberkochen, Germany). During single-channel on-cell patch clamp and during whole cell recording, the bath consisted of (in mM) 130.0 KMeSO4, 20.0 KCl, 2.0 CaCl2, 5.0 HEPES, and 5.0 glucose. The pipette solution contained the same substances as the bath. In some experiments, the bath (extracellular) and pipette solutions used were the same as used for native cells in the labyrinthine slice (see below).
Native hair cell Electrophysiology

Hair cells in labyrinthine slices were patch-clamped in whole cell mode using methods previously described (39, 65). Briefly, amniopallae and utricles were harvested and dissected free of each other. The tissue was then incubated in DMEM augmented with 24 mM NaHCO₃, 15 mM PIPES, 50 mg/l ascorbate, and 2 mg/l sodium pyruvate. The bath solution was titrated to a pH of 7.4 with NaOH and HCl and to an osmolality of 320 mosmol/kgH₂O, as described previously (39, 65). The tissue was sliced, stained with Mayer's hematoxylin (Poly Scientific, Bay Shore, NY), mounted on a microscope stage (Zeiss Axioskop), and the cells were viewed using DIC microscopy optics (Zeiss Plan-Apochromat 63×/1.2 oil objective). The temperature of the bath was 23°C, and the superfusion pipette (100 μm bore) was delivered at a rate of 1.2 ml/min. The patch pipettes contained (in mM) 140.0 KCl, 1.0 CaCl₂, 1.0 MgSO₄, 15 HEPES, 10 glucose, and 50 mg/l ascorbate, and 2 mg/l sodium pyruvate. The bath solution was titrated to a pH of 7.4 with NaOH and HCl and to an osmolality of 315 mosmol/kgH₂O.

Native Hair Cell and CHO Cell Pharmacology

Channel blockers were superfused onto the cell using a pressurized computerized superfusion system (model DAD-12; ALA Scientific Instruments, Westbury, NY). The superfusion pipette (100 μm bore) was placed ~30 μm from the cell, and the flow rate varied from 5 to 1 μl/s.

Immunocytochemistry

Pigeons were perfused with 10% buffered formalin using transcardiac cauterization as previously described (11). The pigeon heads were placed in 10% EDTA in 10% buffered formalin (Sigma) that was changed twice a week. The progression of decalcification was monitored by weekly skull X-rays. After 30 days, the skulls were hemisected and blocked to include the labyrinths. Paraffin infiltration and embedding was performed on a Shandon Pathcentre instrument (Thermo Electron, Waltham, MA). The decalcified embedded and blocked tissue was serially sectioned at 6 μm and placed in triplicate on albuminized SuperFrost slides (Fisher Scientific). Subsequently, the sections on slides were deparaffinized and rehydrated by passage through xylene and graded ethanol solutions. The slides were then treated with 3% hydrogen peroxide with 0.03% sodium azide in PBS for 10 min. Next, the slides were treated with protease XXIV (BioGenex, San Ramon, CA) for 8 min at room temperature. Following sequential 15-min incubations with 0.1% avidin and 0.01% biotin (Vector Laboratories, Burlingame, CA), to block endogenous avidin and biotin, the slides were incubated in 0.05% casein (Sigma)/0.05% Tween-20 (DAKO)/PBS for 30 min to block nonspecific protein binding. Primary rabbit polyclonal anti-Kir2.1 (no. APC-026; Alomone Labs, Jerusalem, Israel) was applied to sections at dilutions ranging from 1:90 to 1:120 for 60 min. Rabbit serum (InnoGenex, San Ramon, CA) was applied as a negative control. Biotinylated F(ab')₂ fragments of swine anti-rabbit immunoglobulins (DAKO) supplemented with 10% pigeon serum served as the secondary antibody and was detected by streptavidin-HRP and colorized by DAB (DAKO). Slides were counterstained with Mayer's modified hematoxylin (Poly Scientific, Bay Shore, NY) before mounting and viewed under an Olympus BX51 microscope, and images were recorded by an RT Slider Digital Camera (Diagnostic Instruments, Sterling Heights, MI).

Confocal Microscopy

CHO cells were grown on 25-mm coverslips in DMEM (Invitrogen/GIBCO) at 37°C, 75% humidity and 5% CO₂. The cells were transiently transfected with a plasmid whose pKir2.1 and EGFP ORFs were under the control of the same promoter or with either of two fusion proteins: one with pKir2.1 in frame with EGF and more proximal to the amino end of the protein; and the other with pKir2.1 more proximal to the carboxyl end. At 48 and 72 h posttransfection, the coverslips with plated cells were placed in a viewing chamber, and L-15 culture medium was substituted for the DMEM. The cells were imaged using a confocal microscope (Zeiss model LSM 510) using a Plan-Apochromat 63×/1.4 NA objective. The cells were optically scanned using a wavelength of 488 nm and optically sectioned at either 0.5 or 0.9 μm.

Bioinformatics

Sequence analysis was performed using two programs: DS Gene v1.15 (Accelrys, San Diego, CA) and Lasergene v5.07 (DNASTAR, Madison, WI). Probabilities that serine, threonine, and tyrosine were phosphorylation sites were obtained by submitting the ORF for pKir2.1 to the NetPhos 2.0 Server at the Center for Biological Sequence Analysis, Technical Univ. of Denmark (http://www.cbs.dtu.dk).

Data Analysis

Single-channel recordings were acquired and analyzed using either QuB (50, 51) or Clampex and Clampfit v9.0 (Axon Instruments) software. Whole cell macroscopic currents were acquired and analyzed using Clampex and Clampfit v9.0 (Axon Instruments). Data reduction was preformed using Origin v7.5 (OriginLab, Northampton, MA), PowerPoint 2002 (Microsoft, Redmond, WA), Photoshop v7.0 (Adobe, San Jose, CA), and Excel 2002 (Microsoft).

Throughout the text, unless otherwise specified, average results are expressed in the following format: mean ± standard error of the mean (SE), with number of samples (n). In the figures, error bars indicate ± 1 SE.

RESULTS

The inwardly rectifying current Iₖ is found singly in about 30% of pigeon vestibular hair cells in the ampullae of the semicircular canals and in the maculae of the utricle (otolith organ) (65). About 16% of the time Iₖ is accompanied by a second inward rectifier current, Iₖ. Figure 1A shows a macroscopic current (recorded in voltage clamp mode) from a vestibular hair cell that contains both Iₖ and Iₖ. In Fig. 1C, Iₖ is blocked by 500 μM barium and Iₖ remains (shown in inset). Subtraction of Iₖ from the macroscopic current (Fig. 1A) unmasks Iₖ (shown in Fig. 1C). Figure 1B shows that the resting membrane potential for this cell (at t = 0) is ~90 mV. Positive current pulses cause the membrane to depolarize ~35 mV. Negative pulses of the same amplitude hyperpolarize the membrane ~20 mV. However, when Iₖ is blocked by 500 μM barium, the resting membrane potential depolarizes 30 mV to ~60 mV and the membrane hyperpolarizes ~60 mV in response to negative currents. Thus Iₖ holds the membrane potential near Eₖ (~98 mV) and limits the hyperpolarization of the membrane until Iₖ is unmasked.
Figure 2 shows the nucleotide sequence for pKir2.1 ORF cloned from total RNA from six semicircular canal ampullae (GenBank accession no. AF192507). The translated amino acids are shown above each nucleotide codon. The transmembrane domains M1 and M2 of the protein are indicated. Between domains M1 and M2 is the extracellular loop, the pore helix, and the pore domains. The M1-to-pore region and the pore-to-M2 region, comprising the extracellular loop do-

cisons are indicated by larger letters representing the amino


molecule. The highly conserved pore helix and pore re-


ded as those involved in determining the open


hains, are demarked by parentheses and identi-


pyramidal current from the same cell. Figure 3


n that pKir2.1 channel block depolarizes that cell and releases the mem-


brane potential clamp of hyperpolarizing po-


/n that pKir2.1 is found in native hair cells, we


ducted single cell RT-PCR using single hair cell cytoplasm from seven individual cells following patch clamp recording of current from the same cell. Figure 3A shows ionic currents recorded from a type II hair cell (cell C92). The test voltage protocol involved hyperpolarizing pulses from a negative holding potential of −63 mV. It can be seen that the peak amplitudes of the ionic currents increase with hyperpolarization and inactivate with time typical of $I_{\text{Kir}}$ currents. Following acquisition of ionic currents, cytoplasm was extracted from the cells as shown in Fig. 3, A–C, and single cell RT-PCR was carried out. Figure 3C shows amplified product from 3/5 positive reactions (cells C66, C73, and C92) and 2/2 negative reactions (cells C91 and C93). It can be seen in Fig. 3, A and C, that cell C92 produced $I_{\text{Kir}}$ currents and amplified product with a fragment size of ~281 bp.

$I_{\text{Kir}}$ and $h$ are expressed with different frequencies in different regions of the ampullary and macular epithelium (39, 65). Moreover, the magnitudes of the currents are different in different regions of the epithelium. Figure 3D presents preliminary data suggesting that the expression and magnitude of $I_{\text{Kir}}$ in a given cell in a given region may be transcriptionally regulated. Seventy cells were taken from different regions of the ampullary and utricular epithelium. Figure 3D results from an analysis of the cytoplasm from three of these cells. The cell
Fig. 2. Open reading frame (ORF) for pKir2.1. One-letter amino acids are shown above the line, and three-letter nucleotide codons are shown below. Transmembrane regions M1 and M2 are identified as are the subregions membrane-to-pore (MP) and pore-to-membrane (PM). These regions are enclosed by parentheses. The pore helix and pore are identified by large amino acid letters. The pore helix is enclosed with square brackets. Serines, threonines, and tyrosines that have a probability of ≥0.5 of being a phosphorylation site are identified by the {$\text{F}$}, {$\text{I}$}, and {$\text{T}$} symbols, respectively. At or near these sites, posttranslational, casein II (Ck2), protein kinase C (PkC), and protein kinase A (PkA) site motifs are demarked. A tyrosine kinase (Tk) site motif is also indicated. The signature sequence (PKKR) for binding phosphatidylinositol-4,5-bisphosphate (PIP2) is denoted by a line over the residues surrounding the symbol “PIP2.”
that produced the band indicating 44 transcripts in its cytoplasm expressed an $I_{\text{Kir}}$ current whose peak magnitude was $-1.3$ nA at a test voltage of $-123$ mV. On the other hand, cells showing four and seven pKir2.1 transcripts (lane 1 and lane 3), respectively, expressed no $I_{\text{Kir}}$ current, only $I_h$ (data not shown).

Table 2 summarizes a ClustalW alignment analysis of nucleotides in the ORF of pKir2.1 (AF192507) against comparable sequences for cIRK1 found in chick auditory papilla and Kir2.1 sequences from rhesus monkey lens, human midbrain, guinea pig heart, chick lens, and a mouse macrophage cell line. The cells of Table 2 contain the percent identity of the tissue referenced in the cell column and row. By viewing down the first column of Table 2, it can be seen that pKir2.1 ORF has high identity to the Kir2.1 subfamily of inwardly rectifying potassium channels across species and tissues.

Differences between amino acid residues in the avian inner ear (auditory and vestibular) are minimal. Only 3/427 residues are different between pKir2.1 (Fig. 2) and cIRK1 obtained from of the chick auditory papillae (45). These differences are all on the carboxyl side of the ion channel pore and transmembrane segments. The differences are Q at 247, N at 383, and M at 398. When pKir2.1 nucleotide sequence was compared with nucleotides 7974861–7976144 on chromosome 18 of the sequenced chick genome (UCSC Genome Bioinformatics, http://genome.ucsc.edu), the nucleotide iden-

Table 2. Identity matrix for nucleotides in the ORF for pKir2.1 compared with other species and organs

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GenBank accession numbers are listed in row and column headings. Pig VHC, pigeon vestibular hair cell; Ck A Pap, chick auditory papilla cells; Rm lens, Rhesus monkey lens cells; Ck lens, chick lens cells; Hu heart, human heart cells; Gp heart, guinea pig heart cells; Mm cell line, mouse macrophage cell line.

Fig. 3. Single cell electrophysiology and quantitative RT-PCR. A: patch clamp recordings of currents from native vestibular hair cell C92. B: steps in extraction of cytoplasm following recording session were as follows. B-a: access to the inside of cell during whole cell recording. Arrowheads point at cilia protruding from apical surface of hair cells. B-b: collapsing cell body (pointed at by arrowhead) resulting from extrusion of cytoplasm when negative suction was applied to the micropipette. B-c: small arrow points to bleb of cytoplasm in pipette. Remnants of cell (nucleus, cuticular plate, and cilia) can be seen following cytoplasm extraction. C: PAGE gel showing pKir2.1 product fragments (281 bp) from 3 cells (including C92) following whole cell patch clamp recording, cytoplasm extraction, and reverse transcription and amplification. D: PAGE gel showing migration of pKir2.1 from three different cell cytoplasms. An internal standard, ΔpKir2.1 (100 molecules), was included in RT-PCR reactions L1–L3. L4 is a no-template control. Equal volumes of the reactions were analyzed by PAGE and subsequently further analyzed using a PhosphorImager. The estimated number of molecules of pKir2.1 is shown at the bottom of each lane. The cell whose cytoplasm contained 44 molecules of pKir2.1 transcripts produced a peak inward ionic current of $-1.3$ nA at $-123$ mV (data not shown) with a waveform similar to the one shown in A. Cells whose product is shown in L1 and L3 demonstrated no pKir2.1 current.
Fig. 4. A: a phylogram relating the Kir2.1 ORF sequence in tissue from avians, rodents, and primates. The accession numbers are identified in Table 2. Distances are scaled in absolute differences in number of nucleotides. The bar represents an absolute difference of 15 nucleotides. B: a phylogram comparing the amino acid sequences of the ORFs for pKir2.1 with human Kir2.0 subunit sequences (hKir2.1 to hKir2.4). The GenBank accession numbers used in this phylogenetic analysis are in parentheses. The bar represents an absolute difference of 7.5 amino acids.

Since the Kir2.1 ion channel protein determines a cell’s excitability by regulating the cell’s resting membrane potential, plays a role in regulation of K+ flux across the cell membrane, and is associated with the terminal repolarization phase of action potentials, its mRNA is found in many cell types and many tissues. Figure 5 presents a PAGE gel showing RT-PCR amplicons derived from total RNA isolated from cells of a variety of pigeon tissues. It is evident that pKir2.1 mRNA is found in all of the tissues tested.

Figures 6 and 7 demonstrate that the pKir2.1 DNA amplicons shown in Fig. 5 were translated into protein in the vestibular labyrinth, vestibular ganglion, and neck muscle. Decalcified pigeon heads were embedded in paraffin and serially sectioned. The sections were 6 μm thick. In situ immunohistochemistry using a rabbit polyclonal antibody to Kir2.1 was employed. The epitope based on hKir2.1 had 14 of 18 identical residues to the pKir2.1 sequence. Figure 6 shows different regions of the sensory epithelium with control tissue on the left and tissue showing HRP staining of the antibody reaction on the right. It can be seen that hair cells and supporting cells showed a reaction in all regions of the epithelium. This result is confirmed by patch clamp studies showing I_Kir current in both pigeon hair cells (39, 65) and
supporting cells (39). Insets in Fig. 6D are photomicrographs of single type I and type II hair cells showing immunoreaction. The arrows point at both types of cells showing reaction product.

Figure 7 shows control sections (Fig. 7, A and C) and sections with reaction product in vestibular ganglion cells and in neck muscle (Fig. 7, B and D). This result complements the results shown in Fig. 5 indicating that Kir2.1 DNA (Fig. 5) and translated protein (Fig. 7) is in the vestibular nerve and neck skeletal muscle.

Figure 8 presents intracellular recordings from oocytes expressing pKir2.1. Figure 8A shows two-electrode voltage clamp traces to a voltage protocol shown above the current traces. The current traces in Fig. 8A-a show rapidly activating, rapidly inactivating currents in response to hyperpolarizing membrane potentials. The outward current in response to depolarizing voltages is highly attenuated. Figure 8A-b shows traces indicating the reduction in current during superfusion of the oocyte by a solution containing 100 μM BaCl2. Figure 8B is a current-voltage (I/V) plot indicating a partial block of stead-state current by 5 μM BaCl2 and a strong block by 100 μM BaCl2. The block relative to the control bath (ND96) almost completely recovered following washout of the BaCl2 by the control bath. Figure 8C indicates the sensitivity of the pKir2.1 current to K+ ions. When a ND96 solution containing 80 mM potassium gluconate was substituted for the control ND96 solution, the I/V curve shifted to the right and crossed the abscissa, 75 mV more depolarized. This shift in reversal potential is indicative of K+ sensitivity and is characteristic of the Kir2.1 subfamily of inward rectifiers. Figure 8D presents a comparison of Ikir currents from a native hair cell from the utricle (Fig. 8D-a), an oocyte injected with pKir2.1 cRNA (Fig. 8D-b), and a CHO cell transfected with pKir2.1 DNA (Fig. 8D-c). When a monoexponential function was fitted to the inactivation of the currents, it became obvious that the oocyte expression system, probably because of its size (capacitance and resistance), was not a good model system to compare currents studied in this HES to those found in smaller native hair cells.

Therefore, three vectors were developed to deliver pKir2.1 DNA to the CHO cell plasmalemma. Figure 9 confirms that pKir2.1 was expressed in the cell membrane (see arrows in Fig. 9, B and C). In each case, enhanced green fluorescent protein (EGFP) was coexpressed as a fluorescent marker. Figure 9 shows confocal photomicrographs of optical slices through CHO cells transfected with two fusion proteins (Fig. 9, B and C) and a dual promoter vector (Fig. 9D) 72 h earlier. In the case of the latter vector, it can be observed that the EGFP is diffused throughout the cytoplasm and nucleus, whereas for the two fusion proteins the EGFP is localized at or near the plasmalemma, not in the nucleus, but in vesicles throughout the cytoplasm. The ionic currents resulting from patch clamping the membrane of any of the three vectors transfected into CHO cells were equivalent.
Figure 10 presents a collage of single-channel results based upon cell-attached single-channel patch clamp recordings from CHO cells expressing pKir2.1. Figure 10A presents traces for different levels of hyperpolarization. It can be seen that the trace represents a two-state response (open-closed) and that the amplitude of the openings increase with levels of hyperpolarization. The channel does not open for levels more positive than −20 mV (traces not shown). The $I/V$ curve (means ± SE, n = 7) in Fig. 10C summarizes group responses. The calculated linear slope conductance of $29 \text{pS}$ is consistent with other values (single-channel slope conductances in the twenties) found for Kir2.1 channels (46). Figure 10, B and D, shows other features of the single-channel response that are characteristic of Kir2.1 channels: long open dwell times (200–300 ms) and multiconductance levels, respectively.

**DISCUSSION**

This paper presents new data demonstrating the cloning of the ORF for the ion channel pKir2.1 expressed in pigeon vestibular hair cells. Tissue from the semicircular canal ampullae was used to obtain the amplicons, which were subsequently cloned and sequence confirmed. To ensure that hair cell cytoplasm provided the mRNA for the amplicons derived from the ampullae, cytoplasm was extracted (Fig. 3) from single hair cells following patch clamp recording of $I_{K\text{ir}}$ ionic currents from the same cells. This was necessary because ion channels that are found in vestibular hair cell membranes are also found in supporting cells that surround the hair cells and also may be found in afferent and efferent nerve terminals that abut the hair cells (Fig. 7). Previous work (45), which cloned a related ion channel in chicken auditory papillae (cIRK1), used whole regions of the papillae for RT-PCR expression and cloning. It is unclear whether the tissue, the HES (oocytes), or just sampling in that study produced the response features of the wild-type clone that were unlike members of the Kir2.1 subfamily of inward rectifiers. For example, it was necessary to produce a mutant Q125E to increase the single-channel conductance from $16 \text{pS}$ and to lower the EC$_{50}$ for Ba$^{2+}$ from 12 to 2 $\mu$M (45). In the present study, the ORF for pKir2.1 (Fig. 2) with a glutamine at location 125 (as in unmutated cIRK1) produced a single-channel conductance of $29 \text{pS}$ (Fig. 10) and an EC$_{50}$ for Ba$^{2+}$ of 3.7 $\mu$M (data not shown). These numbers correspond closely to those reported for Kir2.1 subfamily members (32, 61, 67).

Further verification of Kir2.0 subfamily membership of pKir2.1 using different techniques (genomics, electrophysiology, and immunocytochemistry) is the second novel feature of this paper. The ORF sequence for pKir2.1 (Fig. 2) shows high identity (>80%) with other Kir2.1 subfamily members in a variety of tissues and species, including primates (Table 2, Fig. 4A). Like other Kir2.1 subfamily members, there is considerable opportunity for posttranslational modulation of the ion channel by second messenger systems to ultimately control cell membrane potential (and therefore excitability), cellular K$^+$ homeostasis, and gating of the channel itself. Fourteen of the 30 identified serine motifs have a 50% or greater chance of being active phosphorylation sites. The motifs for a PKA, a PKC, and a tyrosine kinase phosphorylation site are present. Four threonine and seven tyrosine motifs have a probability of 0.5 or greater of being a phosphorylation site. Kir2.1 channels
are regulated by protein phosphorylation (9, 12, 22, 64, 66) and direct G protein activation (14, 26, 57). The inhibitory and facilitatory modulation probably fine tunes the cell’s excitability. There have been no studies of phosphorylation of Kir2.1 channels by protein kinase CK2. However, protein kinase CK2 has been shown to bind to and phosphorylate the carboxy termini of ENaC subunits (60) and \( \beta \)-subunits of a voltage-dependent Ca\(^{2+} \) channel (30).

The ORF of pKir2.1 has the signature binding motif for PIP\(_2\). PIP\(_2\) is a second messenger that regulates the activity of a number of channels (13, 20). It has been shown to bind electrostatically (28) to positive base residues of Kir channels including Kir2.1 (24, 63, 68). PIP\(_2\) depletion causes \( I_{K_{ir}} \) current rundown that can be reversed when PIP\(_2\) is restored. Recently, it has been shown (36) that a residue, R218, that is important in PIP\(_2\) binding when neutralized (R218/QW) is also associated with the channelopathy identified as Andersen syndrome (49). The presence of dysmorphic bodily features (seen in Andersen syndrome) suggests that, in addition to its role in cell excitability, K\(^+\) homeostasis and action potential repolarization, pKir2.1 might have a role in bodily feature development.

Table 2 and Fig. 4 suggest that there is a good deal of phylogenetic conservation of nucleotides carrying the message for \( I_{K_{ir}}/K_{ir} \). For example, there is 85% identity between the Kir2.1 ORFs (~1,200 nucleotides) for pigeon hair cells and human heart cells. As expected, the phylogram (Fig. 4A) shows avian epithelial cells forming a node at one end of the phylogram and primate cells forming a node at the other end with rodents in between. Figure 4B extends the phylogenetic analysis of pKir2.1 and suggests that it is indeed a homomeric Kir2.1 subunit and not a pKir2.2, pKir2.3, or pKir2.4 subunit homomer or a mixed heteromer.

Kir2.1 is generally expressed in neurons, glial cells, and brain vasculature, but it is also expressed in smooth and skeletal muscle. Figure 5 shows a similar distribution of mRNAs encoding pKir2.1 in pigeon also with transcripts found in cell types and tissues as diverse as the sensory epithelia of the ear (hair cells from semicircular canals and utricle), epithelia from the eye (lens), neurons in the peripheral nervous system (vestibular ganglion cells), brain (brain stem, optic tectum and cerebellum), skeletal muscle (neck muscle), and cells from the heart and liver.
Figure 6 shows expression of pKir2.1 in the vestibular neuroepithelium, and Fig. 7 shows expression of pKir2.1 in vestibular ganglion cell bodies and neck muscle. In the vestibular epithelium, supporting cells and both types of hair cells (type I and type II) show immunoreactivity (see inset in Fig. 6D). It has previously been shown (39) from patch clamp studies that the primary current in pigeon vestibular supporting cells is \( I_{\text{Kir}} \). \( I_{\text{Kir}} \) currents have been shown to be in type II hair cells in a number of species (4, 23, 39, 42, 58). The presence of \( I_{\text{Kir}} \) in type I vestibular hair cells is more controversial. Griguer et al. (18) recorded an inward rectifier current in type I hair cells in guinea pig. Subsequently, it was determined (54) that deactivation of a large outward rectifier current present in almost all pigeon type I hair cells (55) may have been mistakenly identified as the inward rectifier current. However, the present findings of Kir2.1 antibody immunoreactivity in type I hair cells suggest that pKir2.1 channels may be coexpressed in pigeon type I hair cells. Previous patch clamp studies (39, 65) have shown that \( I_{\text{Kir}} \) currents are regionally distributed in different zones of the pigeon vestibular epithelium. In the present study, there were no gradients of immunoreactivity in different areas of the epithelium (Fig. 6). It must be assumed that the antibody assay is too insensitive to detect the distributional variation of pKir2.1 channels in the epithelium as assayed by patch clamp recordings.

The traces in Fig. 1 point out several physiological roles for \( I_{\text{Kir}} \) channels in native pigeon hair cells. Those roles include setting the resting membrane potential and limiting membrane potential hyperpolarization, particularly when \( I_{\text{h}} \) is coexpressed. When pKir2.1 is blocked, the hair cell’s membrane potential depolarizes by \(-30 \, \text{mV} \) (moving it from near \( E_{\text{K}} = -98 \, \text{mV} \)), and \( I_{\text{h}} \) is unmasked, causing a large hyperpolarization relative to the control response traces where both \( I_{\text{Kir}} \) and \( I_{\text{h}} \) are activated. It has been suggested (16, 17) that \( I_{\text{Kir}} \) has an additional physiological role in turtle auditory hair cells; that of contributing to hair cell tuning to different sound frequencies. The size of \( I_{\text{Kir}} \) varies inversely with the resonant frequency of the turtle auditory hair cell. Interestingly, in pigeon vestibular hair cells, resonant frequency is statistically significantly lower (\(-1/2 \)) in regions of the epithelium (zone 3 of the crista and the ES zone of the utricle) where pKir2.1 is present and prominently expressed (65).

The reversal potential for Kir2.1 current shifts as \( E_{\text{K}} \) shifts. As such, outward current flows only over a limited voltage range positive to \( E_{\text{K}} \) regardless of the value of \( E_{\text{K}} \). Figure 8 illustrates this in oocytes transfected with pKir2.1. In Fig. 8C,
the reversal potential shifts based on calculations using the Nernst equation. The membrane potential should have shifted 93 mV, but it actually shifted 87 mV. This difference was probably due to uncompensated leak current. No large shift occurred when 80 mM Na\(^+\)/H\(^+\) was washed onto the cell. It has been estimated that for Kir2.1 channels, the K\(^+\)/H\(^+\) selectivity is 1,500:1 (Ref. 53). Figure 8B shows another signature feature for Kir2.1 channels: Ba\(^2+\)/H\(^+\) sensitivity. In Fig. 8, 100 \(\mu\)M Ba\(^{2+}\) almost completely blocks the channel, yet 5 \(\mu\)M Ba\(^{2+}\) only blocks the channel by ~15%. Previous work (61, 67) has shown that the EC\(_{50}\) for dose response curves for Kir2.1 channels is ~2.5 \(\mu\)M. We carefully constructed a dose response curve (data not shown) for pKir2.1 expressed in CHO cells, and the EC\(_{50}\) was 3.7 \(\mu\)M.

From the cell-attached patch clamp data presented in Fig. 10, A and C, it can be seen that when using symmetrical 150 mM K\(^+\) solutions, the pKir2.1 channel activates at ~30 mV and the inward current increases linearly over the range ~30 to ~150 mV. The mean slope conductance over this range is 29 pS. This value corresponds almost identically with other Kir2.1 channels [chick lens, 29 pS (52); and mouse macrophages, 30 pS (32)] as well as \(I_{\text{Kir}}\) channels [guinea pig ventricular cells, 27 pS (59); guinea pig alveolar cells, 31 pS (44); and rabbit osteoclasts, 31 pS (29)]. Figure 10B presents mean open and shut time for seven pigeon \(I_{\text{Kir}}\) experiments. Like mouse macrophage \(I_{\text{Kir}}\) expressed in oocytes (6), pKir2.1 single-channel recordings had single exponential open time distributions with mean open times ranging from 200–300 ms, but unlike Kir2.1 expressed in oocytes (6) (which had 4 discrete closed states), pKir2.1 patches had a single exponential closed time distribution with mean closed times of ~25 ms. The above results for pKir2.1 single-channel recording were based on two state recordings. However, it must be pointed out that like other Kir2.1 channels (52, 53) multiconductance states were frequently noticed.

The average resting membrane potential for type II hair cells in zone 3 of the pigeon crista (primarily pKir2.1 channels) is \(V_z = -68\) mV (65). The average input resistance at that voltage is ~1.5 G\(\Omega\) (8); i.e., a total conductance (\(G\)) of 667 pS. These values were obtained in solutions where \(E_K = -94\) mV.
In this paper, we report cloning pKir2.1 and measuring the single-channel conductance; γ = 29 pS. Thus 23 pKir2.1 channels open all the time should be activated. But only 72% of the conductance at rest is for potassium since Vf = −68 mV not −94 mV. Thus 17 pKir2.1 channels are needed to account for the K+ conductance. However, the single-channel open probability at Vf = −68 mV is 0.65–0.8 (data not shown). Because of the open probability, 21–26 pKir2.1 channels (84–104 homotetrameric molecules of pKir2.1) are required to account for the conductance. The other currents I1, I[K(Ca)], Ica, and Itr (coexpressed in pigeon type II vestibular hair cells) do not play a role in the above calculations since they do not activate until the membrane potential reaches at least −40 mV.

In conclusion, we have cloned a Kir2.0 subfamily channel in pigeon vestibular hair cells. We have concluded from phylogenetic analysis that it is the 2.1 subunit and not the other Kir2.0 subunits that is encoded in pigeon vestibular hair cells. We have shown that pKir2.1 has high ORF sequence identity with other members of the Kir2.1 subfamily in a variety of tissues from avians to humans. In pigeons, pKir2.1 transcripts are found in the vestibular epithelium, vestibular nerve, skel- etal neck muscle, lens of the eye, heart, liver, and brain. Hair cells and supporting cells in the vestibular epithelium, as well as cells in the vestibular ganglion and neck muscle, immuno-react with Kir2.1 antibody, demonstrating pKir2.1 ion channel protein expression. We injected and transfected pKir2.1 cRNA and DNA into oocytes and CHO cells, respectively. We carried out whole cell patch clamp electrophysiological studies and demonstrated that the K+ and Ba2+ sensitivities of the channels are the same as that reported for other Kir2.1 channels. Finally, we carried out cell-attached single-channel studies in CHO cells transfected with pKir2.1 and demonstrated that the single-channel features such as single-channel conduc- tance and subconductance states were exactly like other Kir2.1 channels. The results obtained using all of the above tech- niques converge to the conclusion that the fast inward rectifier potassium channel in pigeon vestibular hair cells belongs to the Kir2.1 subfamily of inward rectifiers.

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25. Jan LY and Jan YN. Cloned potassium channels from eukaryotes and DNA into of oocytes and CHO cells, respectively. We injected and transfected pKir2.1 cRNA and DNA into oocytes and CHO cells, respectively. We carried out whole cell patch clamp electrophysiological studies and demonstrated that the K+ and Ba2+ sensitivities of the channels are the same as that reported for other Kir2.1 channels. Finally, we carried out cell-attached single-channel studies in CHO cells transfected with pKir2.1 and demonstrated that the single-channel features such as single-channel conduc- tance and subconductance states were exactly like other Kir2.1 channels. The results obtained using all of the above tech- niques converge to the conclusion that the fast inward rectifier potassium channel in pigeon vestibular hair cells belongs to the Kir2.1 subfamily of inward rectifiers.


