The Function and Molecular Identity of Inward Rectifier Channels in Vestibular Hair Cells of the Mouse Inner Ear

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**ABSTRACT**

Inner ear hair cells respond to mechanical stimuli with graded receptor potentials. These graded responses are modulated by a host of voltage-dependent currents that flow across the basolateral membrane. Here we examine the molecular identity and the function of a class of voltage-dependent ion channels that carries the potassium-selective inward rectifier current known as I_{K1}. I_{K1} has been identified in vestibular hair cells of various species, but its molecular composition and functional contributions remain obscure. We used quantitative RT-PCR to show that the inward rectifier gene, Kir2.1, is highly expressed in mouse utricle between E15 and adulthood. We confirmed Kir2.1 protein expression in hair cells by immunolocalization. To examine the molecular composition of I_{K1}, we recorded voltage-dependent currents from type II hair cells in response to 50-msec steps from $-124$ to $-54$ in 10 mV increments. Wild-type cells had rapidly activating inward currents with reversal potentials close to the $K^+$ equilibrium potential and a whole-cell conductance of $4.8 \pm 1.5$ nS (n=46). In utricle hair cells from Kir2.1^{-/-} mice, I_{K1} was absent at all stages examined. To identify the functional contribution of Kir2.1 we recorded membrane responses in current-clamp mode. Hair cells from Kir2.1^{-/-} mice had significantly (p<0.001) more depolarized resting potentials and larger, slower membrane responses than those of wild-type cells. These data suggest that Kir2.1 is required for I_{K1} in type II utricle hair cells and contributes to hyperpolarized resting potentials and fast, small amplitude receptor potentials in response to current inputs such as those evoked by hair bundle deflections.

**Keywords:** Kir2.1, KCNJ2, hair cell, utricle, vestibular, I_{K1}.
INTRODUCTION

In the vestibular system head movements are converted into sensory signals by mechanotransduction channels located at the apex of sensory hair bundles. The sensory signals then propagate as graded receptor potentials from the apex to the base of the hair cell. Small changes in the electrical properties of the basolateral membrane can modify the hair cell receptor potential and, consequently, neurotransmitter release at the afferent synapse. These changes include the developmental acquisition of various ion channels required for proper modulation and transmission of the sensory signal, which continues until hair cells reach functional maturity around P8 (Rüsch et al. 1998) as well as modulation by various efferent mechanisms. To extend an understanding of the functional development and the molecular components required for hair cell signaling we focus here on the Kir2 family of potassium inward rectifier channels and their contributions to mammalian vestibular hair cell function.

Kir2 channels play a physiological role in a variety of cells: they can affect resting potential, action potential firing rates, neurotransmitter release, insulin release, cell volume and blood flow (Doupnik et al. 1995; Isomoto et al. 1997; Nichols and Lopatin 1997; Bichet et al. 2003). Kir2 channels are K⁺-selective and are active around the resting potential. They pass significantly larger inward currents at membrane potentials negative to the potassium reversal potential \( E_K = -82 \text{ mV} \), than outward currents at positive potentials (Lu et al. 2004). However, unlike voltage-gated channels, Kir2 channels lack the voltage-sensing structure, S4, specific to those channels (Kurachi et al. 2003) and thus are not gated by membrane voltage. Rather, their voltage-dependence or rectification is derived from blockade of K⁺ efflux by intracellular Mg²⁺ (Matsuda et al.
1987) or polyamines (Lopatin et al. 1994) at depolarized potentials. These biophysical properties provide a negative feedback mechanism that acts to repolarize the membrane potential following small deviations that would otherwise drive the cell away from resting potential.

The Kir2 ion channel family includes four subunits in mouse and two additional subunits, Kir2.5 and 2.6, which are present in the carp and human genomes, respectively (Hassinen et al. 2008; Ryan et al. 2010). Most Kir2 channels form functional homomeric tetramers, while some assemble into heteromeric tetramers (Preisig-Müller et al. 2002; Schram et al. 2002). All Kir2 channels have a descending P-loop that includes the canonical GYG K⁺-selectivity sequence. Mutations in the GYG sequence render Kir2 channels nonfunctional and lead to disorders such as Andersen Tawil Syndrome (Plaster et al. 2001).

Expression of Kir2 channels has been reported in mouse auditory hair cells (Marcotti et al. 2003; Ruan et al. 2008) and avian vestibular hair cells (Navaratnam et al. 1995; Correia et al. 2004), but their physiological correlates and contributions have not been elucidated, particularly in mammals. In inner ear hair cells, $I_{K1}$ contributes to sensory signaling by maintaining resting potential at more negative values, lowering input resistance (Holt and Eatock 1995; Rüsch et al. 1998; Brichta et al. 2002; Géléoc et al. 2004) and enhancing receptor potentials by deactivation of their negative feedback mechanism at depolarized membrane potentials (Goodman and Art 1996). Previously, Géléoc et al. (2004) showed a correlation between the acquisition of $I_{K1}$ in mouse utricle in early development and more negative resting potentials.
Here we used quantitative PCR, a fluorescence activated cell sorted database and immunolocalization to examine the expression of Kir2 channels in the mouse vestibular system. Data were collected at different time points during development from E15 through adulthood to identify temporal correlation between gene expression and physiological expression of $I_{K_1}$. We used the whole-cell, tight-seal recording technique to characterize the biophysical properties of $I_{K_1}$ in the postnatal mouse utricle. We show that $I_{K_1}$ can be blocked by BaCl$_2$, a known blocker of Kir2 channels. In addition, we examined basolateral conductances in wild-type hair cells, hair cells transfected with a dominant-negative Kir2.1 construct and hair cells excised from Kir2.1$^{-/-}$ mice. We found that cells that lack functional Kir2.1 do not express $I_{K_1}$, have more depolarized resting potentials and slower but larger amplitude responses to hyperpolarizing current injections. These data indicate that Kir2.1 is essential for $I_{K_1}$ and plays a significant role in vestibular hair cell signaling.

MATERIALS AND METHODS

Tissue Preparation. Utricle epithelia were excised from mice between embryonic day (E) 15 to postnatal day (P) 386 according to a protocol approved by the Animal Care Committee at the University of Virginia (#3123) and at Children’s Hospital Boston (#1959). Briefly, mice were euthanized by rapid decapitation. The temporal bone was excised and placed in MEM with Glutamax (Invitrogen, Carlsbad, CA) containing 10 mM HEPES (Sigma, St. Louis, MO) and 0.05 mg/mL Ampicillin (pH 7.4). The utricle sensory epithelium was carefully removed and placed under two thin glass fibers on a glass coverslip to stabilize and flatten the tissue. We used tissue from mice of three
different genotypes, all obtained from The Jackson Laboratory, (Bar Harbor, ME). FVB/NJ wild-type mice served as a control, while Kir2.1<sup>+/−</sup> mice were used to breed heterozygous and homozygous mice lacking the Kir2.1 gene (Kir2.1<sup>−/−</sup>). Kir2.1<sup>−/−</sup> pups did not survive 12 hours past birth and all postnatal experiments were done on vestibular tissue cultured for the indicated number of days. To confirm that our culture conditions did not alter patterns of ion channel gene expression we compared data from acutely excised wild-type tissue with data from wild-type tissue maintained in culture up to 20 days and found no electrophysiological differences (data not shown).

**Quantitative-PCR.** Utricles were acutely dissected from pre- and postnatal wild-type mice at six different ages: E15 (n=20), E18 (n=15), P0 (n=24), P7 (n=14), P25 (n=30) and P180 (n=14). RNA was isolated using an RNAqueous-micro kit (cat# 1931, Ambion, Austin, TX). RNA was purified using an RNA purification kit (DNA-free RNA kit; cat# R1013, Zymo Research, Irvine, CA). To eliminate genomic DNA and reverse transcribe isolated RNA into cDNA we used the QuantiTect Reverse Transcription kit (Qiagen Inc., Valencia, CA). RNA concentration was determined on a spectrophotometer (Nandodrop, ND1000, Thermo Fisher Scientific, Pittsburgh, PA). For quality assurance, samples from isolated RNA were analyzed for purity with a Bioanalyzer (Agilent Technologies, SantaClara, CA) and then reverse transcribed into cDNA and later analyzed by quantitative PCR. Quantitative PCR primers were designed with a melting temperature of 54°C for Kir2.1. The Kir2.1 qPCR primer sequences were:

CACAGCTTCTCAAATCTAGGATCA and CTATTTCTGAACGATAGTGATGG.

For qPCR IQ-Sybr-Green Supermix (Invitrogen #11761-100, Carlsbad, CA) was used.
We tested expression levels on the housekeeping gene, ribosomal 29S, with the following primers that had a melting temperature of 62°C: GGAGTCACCCACGGAAGTTCGG and GGAAGCAGCTGGCGGCACATG. To confirm primer specificity we generated a plasmid that carried the Kir2.1 amplicon (130 base pairs). The size was confirmed by agarose gel electrophoresis and the gel-purified product was sequenced to confirm Kir2.1 identity. To calculate expression ratios we used the delta-delta CT method, normalizing cycle thresholds first to the housekeeping gene, 29S and then to the E15 sample. The final analysis was performed using Origin 7.1 (OriginLab, Northampton, MA).

**Generation of adenoviral vectors.** The coding sequence for murine Kir2.1 in the pCDNA1/Amp vector was previously cloned and kindly provided by Dr. Lily Y. Jan (Kubo et al. 1993). Mutation from GYG to SYG in the pore loop region using RT-PCR was confirmed by sequencing the mutated gene including the pore loop region using the following primers Kir2.1MutXhoIForw - CTCGAGACTGTTTTCTAAAGCAGAAA (TM 55°C) and Kir2.1MutEcoRVRev -GATATCTTTCTGAAACCTTTGGCT (TM 56°C). The mutated gene, Kir2.1-G144S, was subcloned into the pAdTrack-CMV-GFP shuttle plasmid (He et al. 1998). The plasmid was linearized with the restriction endonuclease Pme I, and transformed into BJ5183 competent cells (Agilent Technologies, Lexington, MA) with the adenoviral backbone plasmid, pAdEΔpol (Hodges et al. 2000). To confirm successful homologous recombination, kanamycin-resistant recombinants were digested with restriction endonuclease Pac I, and analyzed after running the product on agarose gels. We transfected C7 cells, a cell line used for adenovirus-packaging (Amalfitano et al. 1998) with the linearized homologous
recombinant plasmids. Following five rounds of serial passage, the crude lysate was filtered and purified with an AdenoX viral purification kit (BD Biosciences, Sparks, MD) to yield 2ml Ad-CMV-GFP-CMV-Kir2.1-G144S, at a titer of $2.2 \times 10^8$ viral particles/ml, which was aliquoted in 100 μl vials and stored at −80°C. Ad-GFP-hKir2.1 and pAd-VgRXR at titer of $4.3 \times 10^{11}$ viral particles/ml were generated as previously described (Holt et al. 1999).

**Immunohistochemistry.** Sensory epithelia were harvested at P0 from wild-type and Kir2.1−/− mice (The Jackson Laboratory, Bar Harbor, ME) and from adult mice then placed in Glyo Fixx (Thermo Scientific, Rockford, IL) overnight at 4 ºC. After permeabilization with 0.1% Triton X-100S (Sigma Aldrich, St. Louis, MO), utricles were incubated with a Kir2.1 rabbit polyclonal antibody targeting the C-terminus (Prestige antibodies, Sigma Aldrich) at a concentration of 1:600 in 1% PBT at 4°C overnight. After incubation with 488 -Alexa Fluor donkey anti-rabbit secondary antibodies and 647 - phalloidin (Invitrogen, Carlsbad, CA) at 1:200 dilution in 1% PBT, sensory epithelia were mounted on coverslips using SlowFade Gold antifade reagent (Invitrogen, Carlsbad, CA). Adult vestibular tissue (Fig. 3D) was prepared with an alternate procedure. Mice were perfused transcardially with 4% paraformaldehyde, 1% picric acid, 1% acrolein, 5% sucrose in 0.1 M PB. Sections were cut on a freezing sliding microtome, digested with 2% Triton X-100 for 30 min, and incubated for 2 days in a primary antibody cocktail of the same rabbit anti-Kir2.1 (1:200) and goat anti-calretinin (1:400), then rinsed and transferred to a secondary antibody cocktail of donkey anti-rabbit Alexa 594 and donkey anti-goat Alexa 488 (both diluted 1:200). Calretinin was used to immunolabel
extrastriolar type II hair cells and calyx afferents in the striolar region (Desai et al., 2005).

All tissue was viewed through a 63x objective on a Zeiss 510 confocal laser-scanning microscope (Oberkochen, Germany). Images were analyzed using the Zeiss LSM Image Browser.

Electrophysiology. Organotypic cultures were generated from utricles, from wild-type, Kir2.1+/− and Kir2.1−/− mice ranging from age P0 to P386, P0 to P383 and P0 to P0 + 20 days in vitro, respectively. To remove the otolithic membrane, utricles were treated with protease XXIV (Sigma) added at 0.1 mg/ml for 20 min. and mounted on coverslips as described above. Epithelia were cultured in MEM with Glutamax (Invitrogen, Carlsbad, CA) containing 10 mM HEPES (Sigma) and 0.05 mg/mL Ampicillin (pH 7.4), and 2% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA). Current or voltage was recorded in standard artificial perilymph solution containing (in mM) 144 NaCl, 0.7 NaH₂PO₄, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 5.6 D-glucose, and 10 HEPES. Vitamins (1:50; Cat #11120) and amino acids (1:100; Cat #11130) were added from concentrates (Invitrogen). Final adjustment to pH 7.4 and ~320 mOsmol/kg was done with NaOH. BaCl₂ (Sigma) was added to bath solution at 100 μM concentration. Hair cells were observed from the apical surface using an upright Axioskop FS microscope (Zeiss, Oberkochen, Germany) equipped with a 63X water immersion objective with differential interference contrast optics. Recording pipettes (3-5 MΩ), pulled from R6 capillary glass (King Precision Glass, Claremont, CA), were filled with intracellular solution containing (in mM): 135 KCl, 5 EGTA, 5 HEPES, 5 K₂ATP, and 0.1 CaCl₂, adjusted to pH 7.4 and ~290 mOsmol/kg with KOH. Current or voltage were recorded in
the whole-cell, voltage-clamp configuration at room temperature (22–24°C) with an Axopatch 200B (Molecular Devices, Palo Alto, CA), filtered at 1 kHz with a low pass Bessel filter, digitized at ≥20 kHz with a 12-bit acquisition board (Digidata 1322) and pClamp 8.2 (Molecular Devices). Data was stored on disk for offline analysis using OriginPro 7.1 (OriginLab, Northampton, MA). Analysis of Variance (ANOVA) tests were applied to compare multiple means and results are shown as means ± S.D. Activation curves were fitted with a Boltzmann equation as follows:

$$G(V_m) = G_{min} + \frac{G_{max} - G_{min}}{1 + \exp \left[ \frac{(V_m - V_{1/2})}{S} \right]}$$

$G_{max}$ and $G_{min}$ are the maximum and minimum conductances, respectively. $V_{1/2}$ is the membrane potential at which half of the conductance is activated, and $S$ is the slope of the curve at the midpoint.

Type I and type II cells were identified based on presence or absence, respectively, of the type I flask-shaped morphology which was well preserved in the intact sensory epithelium. As a second indication we used a voltage protocol designed to highlight the type I specific current $I_{K,L}$ which is evident as early as E18 (Géléoc et al. 2004). Cells that expressed $I_{K,L}$ were excluded from the analysis.

RESULTS

Inward rectifier currents in utricle hair cells

Consistent with previous studies (Rüsch et al. 1998; Géléoc et al. 2004), we observed strong inward rectifier currents throughout early development in mouse utricle type II hair cells. We used the whole-cell, tight-seal technique in voltage-clamp mode to
record fast inward rectifier currents that were evoked by a protocol that included
hyperpolarizing voltage steps from a holding potential of −64 mV to potentials between
−124 and −54 mV in increments of 10 mV. **Figure 1A** and **1C** show representative
currents recorded from utricle type II hair cells at P2 and P26. These currents belong to
the I_{K1} class of inward rectifiers as opposed to the slow inward rectifiers that belong to
the I_{h} class (Rüsch et al. 1998; Géléoc et al. 2004; Horwitz et al. 2011). We did not
record from type I hair cells because they express the low voltage-activated outward
current, I_{K,L}, which is active at voltages similar to I_{K1} making it difficult to distinguish the
latter. I_{K,L} confers more hyperpolarized resting potentials to type I hair cells. However,
Holt et al. (2007) concluded that loss of I_{K,L} in type I hair cells did not depolarize the
cells’ resting potential to the extent expected because in the absence of I_{K,L}, persistent I_{K1}
may compensate.

**Figure 1B** and **1D** show mean steady-state I(V) curves from type II cells at the
same ages shown in **Figure 1A** and **1C**, respectively. Typical of I_{K1} in other studies, the
I(V) curves reveal small outward currents positive to −40 mV and large inward currents
negative to the potassium equilibrium potential (E_K=−82 mV). To confirm that the
currents we recorded were K^+-selective, we measured their reversal potential. The mean
instantaneous I(V) curve recorded at P26 had a reversal potential of −81.8 mV as shown
in **Figure 1E-F**. The mean reversal potential for all wild-type cells was −83.1 ± 2.8 mV
(n=36), close to the K^+ equilibrium potential. To extend previous studies (Géléoc et al.
2004; Rüsch et al. 1998), which examined I_{K1} up to P3 in mouse utricle type II hair cells,
here we describe I_{K1} throughout development and into adulthood up to P386.
Voltage dependence of $I_{K1}$

We plotted the activation curve for $I_{K1}$ recorded from a representative wild-type hair cell at P9 as shown in Figure 2A. The data revealed voltage dependence similar to that described for $I_{K1}$ in a previous study by Rüsch et al. (1998). We fitted the data from wild-type cells with a first-order Boltzmann function. The Boltzmann fits revealed a mean of $V_{1/2} = -83.5 \pm 4.0$ mV, a mean slope of $8.8 \pm 1.8$ mV (n=8). The mean slope conductance was $4.8 \pm 1.5$ nS (n=46). To examine developmental changes we plotted the conductance as a function of postnatal age (Fig. 2B) but found no further increase in the maximal conductance up to one year of age. Similarly, we plotted the activation curve parameters, $V_{1/2}$ and slope, as function of age and found they were stable up to 6 months, the latest stage tested (Fig. 2C).

$I_{K1}$ activated within a few milliseconds with single exponential kinetics and was almost fully activated at $-124$ mV consistent with previous characterizations (Holt and Eatock 1995; Rüsch et al. 1998). To quantify the activation kinetics we fit traces evoked by steps to $-104$ mV with a single exponential function (Fig. 2D). The mean time constant of activation was $0.9 \pm 0.2$ msec (n = 20). Time constants from individual cells plotted as a function of age revealed little change through development and into adulthood (Fig. 2D). As such, the currents were similar in their voltage range of activation, activation kinetics and K$^+$ selectivity at all ages examined and were similar to those previously described as $I_{K1}$ in prenatal and postnatal mouse utricle epithelia up to P3 (Géléoc et al. 2004; Rüsch et al. 1998). The data were also consistent with previous reports from acutely excised saccular hair cells from the leopard frog (Holt and Eatock 1995). The similar biophysical properties for $I_{K1}$ in vestibular hair cells of various
species and ages suggests the currents may be carried by channels with a similar molecular identity.

**Expression of Kir2 mRNA and protein**

To narrow in on the potassium channel genes that might underlie $I_{K1}$ in mouse vestibular hair cells, we began with a quantitative RT-PCR screen for Kir2.1 mRNA since Kir2.1 expression was reported previously in avian vestibular hair cells (Navaratnam et al. 1995; Correia et al. 2004). We extracted mRNA from acutely excised sensory epithelia from pre- and postnatal mice ranging from E15 to P180 and used validated, specific primers designed for linear amplification of Kir2.1 mRNA (Fig. 3A). We found Kir2.1 was highly expressed at all developmental ages tested and that there was a rise in Kir2.1 expression that paralleled the developmental acquisition of $I_{K1}$ in mouse utricle type II hair cells which begins as early as E15 (Géléoc et al. 2004). The increase in Kir2.1 mRNA expression during the first postnatal week also coincided with the continued maturation of the vestibular system in mice (Rüsch et al. 1998). Furthermore, the total number of mature hair cells continues to increase until P16 (Kirkegaard and Nyengaard 2005; Li et al. 2008) which may contribute to the higher Kir2.1 mRNA expression level measured at P25 compared to P7.

As a second test for Kir2 gene expression we screened a database (https://shield.hms.harvard.edu) derived from RNA sequencing of a fluorescence-activated cell sort (FACS). Vestibular hair cells that expressed green fluorescent protein (GFP) driven by the hair cell promoter, Pou4f3 were dissociated and FACS sorted. GFP-negative, non-hair cells were also collected. The experiment was repeated at
developmental stages between E16 and P16 and the entire transcriptome for both GFP-positive hair cells and GFP-negative, non-hair cells was sequenced, quantified and mapped against the NCBI build 37/mm9 mouse genome assembly. The number of mRNA transcript reads for Kir2.1 - Kir2.4 is plotted as a function of development (Fig. 3B). The mRNA sequencing data show high Kir2.1 expression with a pattern that parallels both the qPCR data and the developmental acquisition of I_{K1}. Importantly, the data also show little or no expression of Kir2.2, Kir2.3 or Kir2.4 and very low level Kir2.1 expression in non-hair cells.

To confirm Kir2.1 protein expression in hair cells, we examined immunolocalization throughout utricles of organotypic cultures excised at P0 and incubated several days in vitro (Fig. 3C). Hair bundles were counterstained with AlexaFluor 647–phaloidin (red) to illuminate actin and distinguish them from basolateral membranes. The immunohistochemistry data revealed that Kir2.1 localizes (green) to the basolateral membranes of type I and type II hair cells, but not hair bundles. We then confirmed the specificity of the Kir2.1 antibody in tissue excised from Kir2.1^{-/-} mice that have a complete deletion of the open reading frame (Zaritsky et al. 2000; 2001). Utricles excised from Kir2.1^{-/-} mice at the same age and stained using an identical protocol and identical image acquisition parameters revealed robust actin staining but no Kir2.1 protein expression in hair cells or elsewhere in the sensory epithelium (Fig. 3C). We also confirmed Kir2.1 protein expression in utricles harvested from young adult wild-type mice. In this case the tissue was sectioned and counterstained with calretinin which labels 70-80% of extrastriolar type II hair cells (Desai et al. 2005). The image reveals
robust Kir2.1 expression in the basolateral membranes of type I hair cells and in type II
hair cells identified by colocalization with calretinin (Fig. 3D).

**Dominant-Negative suppression of I_{K1}**

To examine the correlation between Kir2 channels and I_{K1}, we transfected
organotypic cultures generated from wild-type mouse utricle dissected at P0 with a
dominant-negative construct that carried a point mutation in the Kir2.1 channel gene that
causes Andersen Tawil Syndrome (ATS), a hereditary cardiac disorder (Plaster et al.
2001; Lu et al. 2006; Tristani-Firouzi and Etheridge 2010). The point mutation (G144S)
occurs in the Kir2.1 channel pore motif GYG and leads to irregular spiking activity and
atrial fibrillation. To investigate the identity and function of I_{K1} in hair cells we used the
dominant-negative mutation and generated an adenoviral construct, Ad-mKir2.1-G144S.
The bicistronic construct carried a CMV promoter to drive expression of GFP as a
transfection marker and a second CMV promoter to drive expression of mKir2.1-G144S
(Fig. 4A). Wild-type utricle epithelia were exposed to Ad-mKir2.1-G144S at titers of 2.2
X 10^8 viral particles/ml for 4 h and maintained in culture for up to 10 days. We recorded
from control GFP-negative cells and transfected GFP-positive cells which presumably
also expressed the dominant-negative construct. The GFP-negative cells showed robust
I_{K1} (Fig. 4B), while the GFP-positive cells (Fig. 4D) lacked I_{K1} entirely, but retained I_h
(Fig. 4C), evident as the small slowly activating currents at the end of the step. The mean
I(V) curve taken near the end of the step from six wild-type cells transfected the
dominant-negative construct revealed little or no residual I_{K1} (Fig. 4E). Since Kir2.1 can
coassemble with all members of the Kir2 family to exert dominant-negative inhibition,
这些数据表明，Kir2家族对IK1的贡献在于前庭毛细胞，但并不指明具体是哪一种Kir2成员参与。

Kir2.1⁻/⁻ 毛细胞缺乏IK₁

为了检查哪种Kir2亚单位对于IK₁是必要的，我们记录了Kir2.1⁻/⁻小鼠的数据。我们从Kir2.1⁻/⁻小鼠中切取囊状体，并在电压钳模式下记录II型毛细胞来分析生理性的IK₁表达。在所有检视的Kir2.1⁻/⁻小鼠细胞（n=76）中，IK₁在所有阶段（从P0到20天）均完全缺失（图5E和F）。从野生型和杂合子细胞（图5A-D）中记录的代表电流显示，IK₁具有相似的平均电流。进一步地，IK₁在早出生后阶段和成年期间在野生型和Kir2.1杂合子细胞之间相似。这些数据强烈表明Kir2.1是IK₁的一个分子组成部分。接下来，我们想知道在Kir2.1⁻/⁻小鼠中残留的电流（<−25 pA）是否是由其他Kir家族亚单位造成的。大多数Kir通道形成功能性的同四聚体，而有些则形成异四聚体。先前的研究报告了每个Kir2通道亚单位在异源系统中可与其他Kir2亚单位共组装（Preisig-Müller et al. 2002；Schram et al. 2002）。为了调查其他Kir基因的潜在贡献，我们从Kir2.1⁻/⁻小鼠中切取囊状体，并在添加100 μM BaCl₂到外部溶液后记录电流（图6A）和添加前（图6C）。从两个数据集得到的稳态I(V)曲线没有显示任何差异，这表明BaCl₂没有抑制残余的IK₁电流（图6B和D）。这些结果确认...
that none of the other Kir subunits contributed to $I_{K1}$ in Kir2.1$^{-/-}$ mice. In other words, in
the absence of the Kir2.1 gene in utricle type II hair cells, $I_{K1}$ was completely abolished.

Because our results from Kir2.1$^{-/-}$ utricle hair cells showed that Kir2.1 channels
are necessary to evoke $I_{K1}$ in these cells, we wanted to test whether, conversely, Kir2.1
expression is sufficient to generate $I_{K1}$. For this experiment we used an adenoviral
construct that carried the wild-type coding sequence of the human Kir2.1 gene and
transfected HEK cells, which do not express an endogenous $I_{K1}$. We used an inducible
expression system, which required transfection with two vectors, Ad-VgRXR and Ad-
GFP-human Kir2.1, the latter containing the wild-type Kir2.1 adenoviral construct (Fig. 7A)
as described previously (Holt et al. 1999). Ad-VgRXR, which had a titer of $4.3 \times 10^{11}$ viral particles/ml, contained the gene for the ecdysone receptor controlled by a CMV
promoter and the gene for the retinoid X receptor controlled by an RSV promoter. Ad-
GFP::hKir2.1 contained the human Kir2.1 gene fused to the GFP, under the control of the
inducible ecdysone promoter. We transfected HEK cells with both constructs for 4 h,
added 4 $\mu$M of the ecdysone analogue muristerone A after 24 h to activate the ecdysone
receptor, and incubated overnight. Muristerone A was reported to enhance gene
expression driven by this promoter greater than 30-fold (Johns et al. 1999). Therefore,
we anticipated that cells infected with both adenoviral vectors and treated with
muristerone A would express the GFP::hKir2.1 fusion protein. We observed membrane
restricted GFP expression as early as 24 h after transfection. HEK cells that were GFP-
negative did not have $I_{K1}$ (data not shown), while data recorded from GFP-positive HEK
cells revealed robust $I_{K1}$ (Fig. 7A) with properties similar to $I_{K1}$ in wild-type hair cells
(Fig. 7B). The maximal conductance was $8.4 \pm 2.6$ nS, with a $V_{1/2}$ of $-86 \pm 2.5$ mV and
slope factor of 5.9 ± 1.6 mV (n=3). The time constant of activation evoked by a step to −104 mV was 1.6 ± 0.3 msec.

We then transfected wild-type human Kir2.1 into organotypic cultures generated from mouse Kir2.1−/− utricles extracted at P0. The tissue was exposed to the vector for 24 hours and maintained in culture for two days. In contrast to GFP-negative Kir2.1−/− cells (Fig. 7C), transfected, GFP-positive cells (Fig. 7F) from Kir2.1−/− mice had robust IK1 (Fig. 7D). Steady-state I(V) curves from Kir2.1−/− cells transfected with the human Kir2.1 displayed pronounced inward rectification, characteristic of IK1 (Fig. 7E). The maximal conductance was 21.7 ± 11.8 nS, with a V½ of −84 ± 3.2 mV and slope factor of 6.4 ± 2.0 mV. The time constant of activation evoked by a step to −104 mV was 1 ± 0.3 msec. Other than the maximal conductance—the result of overexpression of Kir2.1—there were no significant differences in the biophysical properties of exogenous Kir2.1 currents expressed in HEK cells and Kir2.1−/− hair cells and those of endogenous IK1 in type II hair cells. These data demonstrate that Kir2.1 alone is sufficient to produce robust inward rectifier currents with all the properties of the native hair cell IK1.

The mean whole-cell inward rectifier conductances measured under the experimental conditions described in the previous sections are summarized in Figure 8A. Taken together the data show that Kir2.1 is both necessary and sufficient to produce IK1 in type II hair cells of the mouse utricle.

**Functional contribution of Kir2.1**

Since the preceding data demonstrated that Kir2.1 is required for IK1, we were able to use the various IK1 blockers—BaCl₂, Kir2.1 gene deletion, Kir2.1-G144S—and
IK\textsubscript{1} rescue to examine the functional contributions of Kir2.1 to vestibular hair cell signaling. Because Kir2.1 is potassium-selective and active at the hair cell resting potential, we hypothesized that Kir2.1 may play a role in setting the membrane resting potential and regulating membrane excitability in vestibular hair cells. We predicted that the positive slope of the steady-state I(V) relationship should act through negative feedback to shift the resting membrane potential toward the K\textsuperscript{+} equilibrium potential. For small membrane potential deviations around the K\textsuperscript{+} equilibrium potential, Kir2.1 may serve to stabilize the membrane response driving the cell toward the K\textsuperscript{+} equilibrium potential. However, for larger depolarizations, we predicted rapid Kir2.1 deactivation, which would allow the cell to respond more freely without the stabilizing influences of Kir2.1. In other words, depolarizing inputs sufficient to overcome the negative feedback mechanism of Kir2.1 would tend to drive membrane potentials more positively which would enhance Ca\textsuperscript{2+} influx (Rodriguez-Contreras et al. 2001; Bao et al. 2003) and neurotransmitter release.

To test these hypotheses we designed current-clamp protocols with small current injections that ranged from $-40$ pA to 30 pA in 10 pA increments and recorded the voltage responses of type II vestibular hair cells. We chose this range of current injections because it permitted hyperpolarized voltage responses in wild-type cells that remained positive to $-90$ mV, the most negative K\textsuperscript{+} equilibrium potential, given physiologically relevant K\textsuperscript{+} concentrations. In addition, 10-20% of the hair cells’ transduction conductance is active at rest (Hudspeth 1989). Thus, the hyperpolarizing current steps were designed to mimic the physiological current amplitudes ($\leq 50$ pA) evoked by negative hair bundle deflections and transduction channel closure as well as
small hyperpolarizing currents that might result from efferent feedback onto type I and
type II hair cells (Holt et al. 2006). With zero current injection we found that Kir2.1 contributed to more hyperpolarized resting potentials with a mean resting potential of \(-59 \pm 5.1 \text{ mV (n = 43; Fig. 8B).}\) Data recorded from Kir2.1\(^{-/-}\) cells showed significantly more depolarized resting potentials by about 10 mV on average, relative to wild-type cells. Regardless of experimental manipulation, inhibition of Kir2.1 activity led to depolarized resting potentials while preservation or restoration of Kir2.1 activity conferred more negative potentials (Fig. 8B). To explore the correlation between the amplitude of the inward rectifier conductance and the resting membrane potential, we generated a scatter plot (Fig. 8C) of resting potential data as a function of inward rectifier conductance for each cell shown in Figure 8A-B. The data were fit with a linear regression that had a slope of -1.2 mV/nS (r = 0.66), which suggests that for every 0.83 nS of inward rectifier conductance, the hair cell resting potential hyperpolarizes by 1 mV.

Figure 9 shows representative membrane responses under various conditions. In wild-type hair cells, hair cells from Kir2.1\(^{+/-}\) mice, and in cells from Kir2.1\(^{-/-}\) mice exposed to Ad-hKir2.1, we recorded fast, small amplitude membrane responses that followed the onset of the current step (Fig. 9A, B and F). In other words, cells that expressed either endogenous or exogenous Kir2.1 had similar membrane responses. In contrast, Kir2.1\(^{-/-}\) hair cells, wild-type hair cells exposed to BaCl\(_2\), and cells exposed to Ad-Kir2.1-G144S had slow, large amplitude voltage responses (Fig. 9C, D and E). To quantify the data, we plotted V(I) curves taken from the peak membrane responses as a function of the current injected from cells of various conditions. We saw a significant difference in the membrane potential responses, particularly over the voltage range
Kir2.1 was active (Fig. 10A-C). Furthermore, wild-type cells exposed to BaCl₂, cells that lacked Kir2.1, and cells transfected with mutant Kir2.1 displayed more hyperpolarized membrane potential responses to current injections than cells that expressed Kir2.1 currents (Fig. 10A-C).

We quantified the speed of the membrane response to −40 pA current injections (Fig. 10D) by fitting the change in membrane voltage with a single exponential equation. The time constant of the exponential fits was taken as the membrane time constant. Cells that lacked Kir2.1 had significantly slower responses (48 ± 22 msec, n=12) than cells that expressed Kir2.1 currents (11 ± 4 msec, n=10; Fig. 10D). The data confirmed that Kir2.1 currents help to speed the membrane potential response to small physiologically relevant current injections.

**DISCUSSION**

**Kir2.1 is the molecular correlate of Iₖ1**

While previous studies have described Iₖ1 in vestibular hair cells of goldfish (Sugihara and Furakawa 1996), frogs (Holt and Eatock 1995) turtles (Goodman and Art 1996; Brichta et al. 2002), pigeons (Masetto and Correia 1997) and mice (Rüsch et al. 1998; Géléoc et al. 2004) the molecular correlate has not been identified. Navaratnam et al. (1995) identified Kir2.1 mRNA expressed in the apical half of the chick cochlear sensory epithelium and Correia et al. (2004) identified Kir2.1 mRNA and cloned a Kir2.1 channel from pigeon vestibular hair cells. Based on their results they hypothesized that Kir2.1 may contribute to Iₖ1 in chick and pigeon hair cells, respectively. While these studies did not test for expression of the other three members of the Kir2 channel
subfamilies, Correia et al. (2004) suggested, based on phylogenetic analysis, that pigeon Kir2.1 does not combine with other Kir2 family subunits. However, previous data showed that Kir2.1 can combine into heteromers with other Kir2 channel subunits in other systems (Preisig-Müller et al. 2002; Schram et al. 2002; Panama et al. 2010).

In the present study we showed that of the four Kir2 channel subfamilies described in the mouse genome Kir2.1 mRNA was most highly expressed in mouse vestibular epithelia. In addition, we found a strong correlation between the temporal expression pattern of Kir2.1 mRNA and the physiological maturation of $I_{K1}$ in the mouse vestibular system during embryonic stages between E15 and E18 (Géléoc et al., 2004) and during the first postnatal week (Rüsch et al. 1998). None of the other Kir2 family subunits displayed substantial expression at the developmental onset of $I_{K1}$ or at any time point we examined. Further, Kirkegaard and Nyengaard (2005) found that hair cell numbers in mouse vestibular epithelia increase until P16. A larger number of hair cells expressing Kir2.1 is consistent with our data that show an increase in Kir2.1 mRNA expression between P7 and P25.

Another result in support of Kir2.1 playing a major role in vestibular hair cells stemmed from our immunohistochemical data in which we localized Kir2.1 protein to the basolateral membranes of utricle hair cells in wild-type mice but not in Kir2.1$^{-/-}$ mice. Kir2.1 was present in the basolateral membranes at both neonatal and adult stages. We also found that hair cells transfected with exogenous Kir2.1 that carried a dominant-negative mutation in the selectivity filter (G144S) lacked $I_{K1}$, which confirmed the involvement of Kir2 family members. The strongest evidence in support of our hypothesis that $I_{K1}$ is composed of Kir2.1 channels is based on our electrophysiological
data from Kir2.1−/− mice. We showed that I_{K1} was present in wild-type and heterozygous
hair cells that expressed Kir2.1, while it was completely abolished in hair cells that
lacked Kir2.1 (Fig. 8). These data suggest that Kir2.1 channels are necessary for I_{K1} in
mouse utricle hair cells.

To investigate the sufficiency of Kir2.1 we used an adenoviral vector that
expressed the wild-type gene to transfect HEK cells and hair cells of the cultured utricles
excised from Kir2.1−/− mice. In both transfected cell types we restored I_{K1} with all the
properties of the endogenous currents recorded from wild-type hair cells. Based on these
data we conclude that Kir2.1 is both necessary and sufficient to form the ion channels
that carry I_{K1} in mouse vestibular hair cells.

Furthermore, we provide evidence that I_{K1} can be detected in mouse vestibular
hair cells not only in early development as previous studies showed (Géléoc et al. 2004;
Rüsch et al. 1998), but into adulthood, past one year of age (Fig. 2B). In contrast to
cochlear hair cells where I_{K1} is transiently expressed (Marcotti et al. 1999), I_{K1} expression
in adult mouse utricle hair cells suggests that it plays a significant role in the mature
vestibular system.

Function of Kir2.1 in vestibular hair cells

Based on our electrophysiological data and the biophysical characteristics of I_{K1}
we hypothesized that Kir2.1 channels provide several functional contributions to
vestibular hair cell signaling. Previous studies in mice showed that I_{K1} is active at rest
and has a reversal potential close to E_K (−82 mV; Rüsch et al. 1998; Géléoc et al. 2004).
We corroborated these data with our results from wild-type utricle hair cells, which had a
mean reversal potential of $-83.1 \text{ mV} \pm 2.8 \text{ mV}$ (n=36). In addition, Géléoc et al. (2004) found a temporal correlation in utricle hair cells between the acquisition of $I_{K1}$ at E15 and the cells’ resting potential which became increasingly more negative between E15 and E19. Based on these results, we predicted that Kir2.1 channels would contribute to sensory hair cell signaling by a) keeping the resting potential more hyperpolarized, thus reducing cell excitability, b) decreasing the amplitude of receptor potential responses and c) increasing the speed of receptor potentials due to lower input resistances and faster membrane time constants. We found that cells in which Kir2.1 channels were blocked with BaCl$_2$ and cells that lacked functional Kir2.1 channels, resting potentials were significantly more depolarized, by 8 to 11 mV, respectively (Fig. 8B). Further, when we measured membrane receptor potentials in response to current injections that ranged from $-40 \text{ pA}$ to $30 \text{ pA}$, cells that expressed Kir2.1 displayed fast, small amplitude responses within a physiologically relevant range, positive to $-90 \text{ mV}$ (Fig. 9A, D, F and Fig. 10). However, in cells that lacked functional Kir2.1 channels, responses to hyperpolarizing steps of $-20$ to $-40 \text{ pA}$ responses were slower (Fig. 9B, C and E) and amplitudes were larger, mostly exceeding physiological values. The slow responses were due to the increased membrane time constants, in turn the result of higher membrane resistance in the cells that lacked Kir2.1 channels open at rest. The large amplitude responses in cells that lacked Kir2.1 channels were also the result of higher membrane resistance at rest. Thus, for a given input current the larger membrane resistance predicted larger, slower voltage responses, which was the case in all cells that lacked functional Kir2.1 channels. If we consider the contribution of Kir2.1 to signal processing in the frequency domain, we predict that Kir2.1 may extend the response range to higher frequencies. In wild-type
type II hair cells we measured mean membrane time constants of ~10 msec which is predicted to yield a low-pass corner frequency of ~16 Hz ($1/2\pi\tau$). Cells that lacked Kir2.1 had mean time constants of ~50 msec or corner frequencies of ~3 Hz. As these are both within the physiologic range of rodent head movements (up to 16 Hz, Hullar et al. 2005), we suggest that Kir2.1 expression may also contribute to tuning of the type II hair cell response to physiologically relevant stimulus frequencies.

The large cell-to-cell variability in the amplitude of Kir2.1 expression may allow type II hair cells a mechanism to regulate their resting, K$^+$-selective, membrane resistance and therefore, their resting potential, the speed and amplitude of their response to small current input and perhaps their frequency response *in vivo*. In heterologous cells, single-channel conductance of mouse Kir2.1 ranged between 21 and 30 pS (Kubo et al. 1993; Takahashi et al. 1994; Panama et al. 2010), while Kir2.1 isolated from avian inner ear hair cells displayed single channel conductances between 13 and 29 pS (Navaratnam et al. 1995; Correia et al. 2004; Zampini et al. 2008). Our results showed an average whole-cell Kir2.1 conductance of 4.8 nS. Considering the single-channel conductance data from previous mouse Kir2.1 studies this suggests that vestibular type II hair cells express 160 to 229 functional channels / cell on average. If we consider our most extreme measures of Kir2.1 whole-cell conductance we estimate a range of Kir2.1 channel expression between about 80 and 400 channels / cell. The exact number of Kir2.1 channels expressed in a given hair cell may be the result of position within the epithelium, number and type of synaptic contacts as well as other signaling requirements of the cell. For example, if hair cells utilize Kir2.1 channel expression as a mechanism to regulate their
resting potential, we estimate that for every ~33 Kir2.1 channels or 132 protein monomers expressed the cell can hyperpolarize its resting membrane potential by 1 mV.

Other factors may regulate the activity and properties of Kir2.1 in hair cells including intracellular polyamines and Mg\(^{2+}\). Kir2 channels have distinct binding sites along the lining of the channel pore that allow Mg\(^{2+}\) and polyamines, such as spermine and spermidine to interact and block K\(^+\) ion flux (Matsuda et al. 1987; Lopatin et al. 1994). Panama and Lopatin (2006) showed in heterologous cells that the magnitude of the block depends on polyamine concentration. Kir2 family members can also be inactivated by several kinases, such as src kinases and PKC, while others, like PKA, increase Kir2 current (Zitron et al. 2004, 2008). Several Kir2 channel subunits can be activated by alkalization due to the pH sensitivity of histine residues in the linker region of the outer transmembrane helix and in the pore-forming loop (Coulter et al. 1995; Zhu et al. 1999; Qu et al. 2000; Hughes et al. 2000). And lastly, previous studies showed that Kir2.1 interacts strongly with PIP\(_2\) (phosphatidylinositol 4,5-bisphosphate; Du et al. 2004). These various signaling pathways may serve to further modulate the activity of Kir2.1 in hair cells allowing the cells additional mechanisms to regulate their resting potentials and responses to small current inputs.

In summary, we have demonstrated Kir2.1 is both necessary and sufficient to form the channels that carry I\(_{K1}\) in mouse vestibular type II hair cells. Our data show that Kir2.1 channels contribute to vestibular hair cell signaling by conferring more hyperpolarized resting potentials; by shaping hair cell receptor potentials in response to small depolarizing and hyperpolarizing stimuli, such as those that occur due to mechanotransduction or efferent input; and by decreasing membrane resistance allowing
for faster responses. We also show that Kir2.1 is expressed not just in early development, but throughout adulthood during which its functional contributions persist. Further studies in mice with conditional deletion of Kir2.1 restricted to the vestibular system may shed light on the significance of $I_{K1}$ activity for normal vestibular function in mice and humans. Lastly, since we have demonstrated that the dominant-negative G144S mutation in Kir2.1, which causes Andersen-Tawil Syndrome in humans, also causes vestibular hair cell dysfunction, we suggest that patients with Andersen-Tawil Syndrome may also suffer from vestibular abnormalities.

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**FIGURE LEGENDS**

Figure 1. I_K1 in mouse utricle hair cells. **A**, Representative currents from a P2 type II hair cell reveal voltage-dependent K⁺ inward rectifier currents of the I_K1 variety. Currents were evoked by the protocol shown at the bottom of panel C which included voltage steps in 10 mV increments between −54 and −124 mV from a holding potential of −64 mV. **B**, Mean steady-state I(V) curve derived from current families, such as the one shown in panel A, for five P2 hair cells. **C**, A representative family of currents from a P26 type II hair cell. The scale bar shown in panel A also applies to panel C. **D**, Mean steady-state I(V) curve derived from current families of six P26-P29 hair cells. **E**, A family of I_K1 traces evoked by the protocol (below) designed to facilitate estimation of the I_K1 reversal potential. From the holding potential of −64 mV the cell was hyperpolarized to −114 mV to fully activate I_K1 then stepped to a series of voltages between −124 and −54 mV. **F**, Mean instantaneous I(V) generated from nine P24-P31 hair cells. Currents were sampled at the time point indicated by the arrow in panel E. Reversal potential was taken as the zero-current potential of the instantaneous I(V) relation.
**Figure 2.** Activation range and developmental expression of $I_{K1}$.  

**A,** A representative activation curve recorded from a P9 wild-type type II hair cell. The data were fit with a Boltzmann equation with a $V_{1/2}$ of $-84$ mV, a slope of 5.9 mV, and a maximal conductance of 5.2 nS.  

**B,** Maximal inward rectifier conductances from type II hair cells plotted as function of age. The data were plotted on a log scale to help spread the data along the x-axis.  

**C,** Slope (S) and $V_{Half}$ values derived from eight representative Boltzmann fits plotted as a function of postnatal age.  

**D,** Activation kinetics at $-104$ mV fitted with a single exponential (red trace). Below, the time constants (tau) are plotted as a function of postnatal age for 20 representative cells.

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**Figure 3.** Expression of Kir2 subunits in mouse utricle.  

**A,** Quantitative RT-PCR was used to estimate mRNA expression of Kir2.1 at various time points. The number of wild-type mouse utricles used to derive the template mRNA was: E15=20, E18=15, P0=24, P7=14, P25=30, P180=14. Error bars represent standard error of the mean for three replicates.  

**B,** Normalized number of mRNA reads plotted as a function of age for hair cells and non-hair cells. Transcript counts are plotted for Kir2.1 – Kir2.4. Two replicates are shown for the E16 sample. Data provided courtesy of Drs. Zheng-Yi Chen and David Corey (https://shield.hms.harvard.edu).  

**C,** Confocal images of utricles at P0 from wild-type (left) and Kir2.1<sup>−/−</sup> mice (right). Projections through a series of focal planes through the epithelium show that Kir2.1 (green) is expressed in the basolateral membrane of hair cells, but not in stereocilia (red). Scale bar = 10 µm.  

**D,** Confocal image of a cross-section through an extrastriolar region of a wild-type P25 mouse utricle. The tissue was immunolabeled for Kir2.1 (green) and calretinin (red). Type I (I) and type
II (II) hair cells are indicated in the figure. Scale bar = 10 µm. Image provided courtesy of Dr. Anna Lysakowski and Mr. Steven Price.

Figure 4. Adenoviral expression of dominant-negative Kir2.1 in wild-type utricle hair cells. A, Adenoviral vector map for Ad-mKir2.1-G144S. The vector includes the coding sequence for Kir2.1 carrying a point mutation (G144S) in the conical GYG selectivity filter. Promoter sequences are shown in blue, GFP in green, and the coding sequences for Kir2.1 in red. B, Representative currents recorded from a wild-type cell at P7 (GFP-negative). The scale bar in panel C also applies to panel B. The stimulus protocol for recordings in B and C shown at the bottom of panel B. C, Data from a GFP-positive cell transfected with dominant-negative Kir2.1 (G144S) construct. The data were recorded from the same tissue as the data shown in panel B (+3 days in culture). D, Image of the GFP-positive cell recorded from in panel C. E, Mean I(V) curve sampled near the end of the voltage steps, derived from six GFP-positive cells transfected with Ad-Kir2.1-G144S.

Figure 5. Inward rectifier currents recorded from Kir2.1 knockout mice. A and C, Representative currents recorded from wild-type and heterozygous cells at P2. B, Mean steady-state I(V) relation taken from 10 P0-P1 wild-type cells. D, Mean steady-state I(V) relation taken from six P2 cells from Kir2.1⁺⁻ mice. E, A family of representative currents recorded from a utricle type II hair cell excised from a Kir2.1⁺⁻ mouse at P0 and maintained in culture for two days. The stimulus protocol and scale bars shown below panel E also apply to panels A and C. F, Mean steady-state I(V) relation taken eight P0 hair cells from Kir2.1⁺⁻ mice.
Figure 6. Current families recorded in the presence of BaCl₂.  

A, Current traces recorded from a Kir2.1⁻/⁻ II hair cell at P1 prior to application of Kir2.1⁻/⁻.  The scale bar applies to panels A and C.  

B, Mean steady-state I(V) relation taken from the five Kir2.1⁻/⁻ cells prior to bath application of BaCl₂.  

C, Currents recorded from the same cell shown in panel A after bath application of 100 µM BaCl₂, a pan-Kir2 blocker.  The voltage protocol at the bottom applies to panels A and C.  

D, Mean steady-state I(V) relation taken from the same five cells of panel B after application of 100 µM BaCl₂.

Figure 7. Adenoviral expression of wild-type hKir2.1 in HEK cells and Kir2.1⁻/⁻ hair cells.  

A, Representative traces recorded from a GFP-positive HEK cell transfected with hKir2.1.  The scale bar applies to panels A-D.  

B, Representative traces recorded from a wild-type type II hair cell excised at P0 and maintained in culture for three days.  

C, Traces recorded from a GFP-negative Kir2.1⁻/⁻ cell.  The tissue was excised at P0 exposed to Ad-hKir2.1 for 24 hours and maintained in culture for three days.  The voltage protocol applies to panels A-C.  

D, A representative family of currents recorded from a GFP-positive cell from the same tissue as described for panel C.  

E, Mean I(V) relationship sampled near the end of the voltage steps derived from eight GFP-positive Kir2.1⁻/⁻ cells transfected with hKir2.1.  

F, Confocal image of a type II hair cell transfected with hKir2.1::GFP counterstained with rhodamine-phalloidin.  Scale bar = 5 µm.

Figure 8. Summary of conductance and resting potential data.  

A, Mean maximal inward rectifier conductance measured from utricle type II hair cells under the
experimental conditions labeled at the bottom of the X-axis: wild-type (WT), Kir2.1 heterozygotes (-Het), Kir2.1 homozygotes (-KO), wild-type + 100 µM BaCl₂ (+BaCl₂), wild-type transfected with Ad-mKir2.1-G144S (+G144S) and Kir2.1 homozygotes transfected with wild-type hKir2.1 (+hKir2.1). Error bars indicate standard deviation. Asterisks mark significant difference (p<0.001) relative to wild-type cells. The number of samples for each bar is indicated on the graph. B, Mean resting potentials for the same experimental conditions indicated in panel A. Error bars indicate standard deviation. Asterisks mark significant difference (p<0.001) relative to wild-type cells. The number of samples for each bar is indicated on the graph. C, Scatter plot of resting potential as a function of inward rectifier conductance derived from the data shown in panels A and B. All data under all conditions for which we had both measurements from the same cell are plotted (n = 103 cells). The data were fit with a linear regression (red line that had a slope of −1.2 mV/nS, a Y-intercept of −51 mV and a correlation coefficient of 0.66.

**Figure 9.** Membrane responses recorded in current-clamp mode under various experimental conditions. Membrane potentials were evoked in type II hair cells by current steps that ranged from −40 to 30 pA in 10 pA. The voltage protocol shown at the bottom of panel E and the scale bars shown in panel A apply to all panels. A, Representative responses recorded from wild-type cells at P2. B, Data recorded from Kir2.1+/− heterozygous cell at P1. C, Membrane potentials recorded from a Kir2.1−/− homozygous cell at P0 + seven days in culture. D, Wild-type cell (P7) exposed to 100 µM BaCl₂. E, Data from a GFP-positive, wild-type cell transfected with Ad-Kir2.1-G144S for 24 hours at P0 and maintained in culture for 12 days. F, Data from a GFP-
positive Kir2.1\textasciitilde heterozygous hair cell excised at P0, transfected with wild-type Ad-hKir2.1 for 24 hours and maintained in culture for three days.

**Figure 10.** Mean voltage-current relationships and membrane time constants measured under various experimental conditions.  
A, Mean V(I) curves extracted from current-clamp responses to current steps between −40 to 30 pA. Responses from 10 cells were measured before (green) and after (black) application of 100 µM BaCl\textsubscript{2}. Error bars show S.E.M. The dotted lines represent, −90 mV, the negative limit of the physiological range of membrane potentials.  
B, Mean V(I) relations for 10 Kir2.1\textasciitilde cells (green) and 10 Kir2.1\textasciitilde cells (black).  
C, Mean V(I) relations for four GFP-positive wild-type cells exposed to Ad-Kir2.1-G144S (black) and eight GFP-positive Kir2.1\textasciitilde cells exposed to Ad-hKir2.1 (green).  
D, Mean membrane time constants taken from exponential fits to voltage responses evoked by −40 pA current steps. The various experimental conditions are labeled at the bottom of the X-axis: wild-type (+/+), Kir2.1 heterozygotes (+/-), Kir2.1 homozygotes (-/-), wild-type + 100 µM BaCl\textsubscript{2} (BaCl\textsubscript{2}), wild-type transfected with Ad-Kir2.1-G144S (G144S) and Kir2.1 homozygotes transfected with wild-type hKir2.1 (hKir2.1). The number of cells for each group is indicated on the graph. Error bars represent S.D. Asterisks mark significant difference (p<0.001) relative to wild-type cells.