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KCNQ4, a Novel Potassium Channel Expressed in Sensory Outer Hair Cells, Is Mutated in Dominant Deafness

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Summary

Potassium channels regulate electrical signaling and the ionic composition of biological fluids. Mutations in the three known genes of the KCNQ branch of the K^+ channel gene family underlie inherited cardiac arrhythmias (in some cases associated with deafness) and neonatal epilepsy. We have now cloned KCNQ4, a novel member of this branch. It maps to the DFNA2 locus for a form of nonsyndromic dominant deafness. In the cochlea, it is expressed in sensory outer hair cells. A mutation in this gene in a DFNA2 pedigree changes a residue in the KCNQ4 pore region. It abolishes the potassium currents of wild-type KCNQ4 on which it exerts a strong dominant-negative effect. Whereas mutations in KCNQ1 cause deafness by affecting endolymph secretion, the mechanism leading to KCNQ4-related hearing loss is intrinsic to outer hair cells.

Introduction

Hearing loss is the most frequent inherited sensory defect in humans. Approximately 70 million people worldwide suffer from a hearing loss exceeding 55 dB (Wilson, 1985). The proportion of people affected by hearing loss increases dramatically with age. Hearing loss can be due to environmental and genetic factors, and the progressive hearing loss of the elderly (presbyacusis) most often seems to be due to a combination of both.

Inherited deafness can be classified as nonsyndromic (isolated hearing loss) or syndromic (associated with other anomalies). Several hundred syndromes, consisting of hearing loss associated with defects in a variety of other organ systems, have been described (Gorlin et al., 1995). Nonsyndromic deafness is classified according to its mode of inheritance as DFN, DFNA, and DFNB (X-linked, autosomal dominant, and autosomal recessive, respectively). In general, autosomal recessive deafness has an early onset and is very severe. Autosomal dominant deafness, by contrast, more often develops slowly over several decades. It is hoped that genes identified in families with dominant deafness may also underlie some forms of presbyacusis.

A large number of loci for nonsyndromic deafness were identified in the last 4 years. There are at least 19 loci for autosomal dominant deafness (DFNA1 to DFNA19) and 22 loci for DFNB. Sometimes, depending on the particular mutation, the same gene can be involved in dominant or recessive deafness (Kelsell et al., 1997; Liu et al., 1997; Weil et al., 1997; Denoyelle et al., 1998; Verhoeven et al., 1998; Mustapha et al., 1999). Several genes involved in syndromic and nonsyndromic deafness have already been identified and are reviewed in Petit (1996) and Kalatzis and Petit (1998). Among others, their gene products include unconventional myosin isoforms (Weil et al., 1997; Wang et al., 1998a), connexin 26 (a gap junction protein) (Kelsell et al., 1997), and two genes encoding potassium channel subunits (*KCNQ1* and *KCNE1*) (Neyroud et al., 1997; Schulze-Bahr et al., 1997).

Ion channels play important roles in signal transduction and in the regulation of the ionic composition of intra- and extracellular fluids. Mutations in ion channels were since long suspected as possibly underlying some forms of hearing loss. In the cochlea, the transduction current through the sensory cells is carried by potassium and depends on the high concentration of that ion in the endolymph. So far, only two genes encoding K^+ channel subunits, *KCNQ1* and *KCNE1*, have been found to be mutated in syndromic hereditary deafness. The gene products of both genes, the KCNQ1 (or KvLQT1) and the minK (or IsK) proteins, respectively, form heteromeric K^+ channels (Barhanin et al., 1996; Sanguinetti et al., 1996). KCNQ1 is a typical member of the voltage-gated K^+ channel superfamily with six transmembrane domains. The minK protein has a single transmembrane span (Takumi et al., 1988) and cannot form K^+ channels on its own. However, as a β-subunit it enhances and modifies currents mediated by KCNQ1. These heteromeric channels participate in the repolarization of the heart action potential. Certain mutations in either *KCNQ1* or *KCNE1* cause a form of the autosomal dominant long QT syndrome (LQTS) (Wang et al., 1996; Splawski et al., 1997), a disease characterized by repolarization anomalies of cardiac action potentials resulting in arrhythmias and sudden death. Other mutations in either gene lead to the recessive Jervell and Lange-Nielsen (JLN) syndrome that combines LQTS with congenital deafness (Neyroud et al., 1997; Schulze-Bahr et al., 1997). In order to cause deafness, KCNQ1/minK currents must be reduced below levels that are already sufficiently low to cause cardiac arrhythmia.

KCNQ1 and *KCNE1* mutations lead to cardiac disease because they directly impair electrical signaling. The mechanism underlying the syndromal deafness in JLN is different. KCNQ1/minK channels probably mediate

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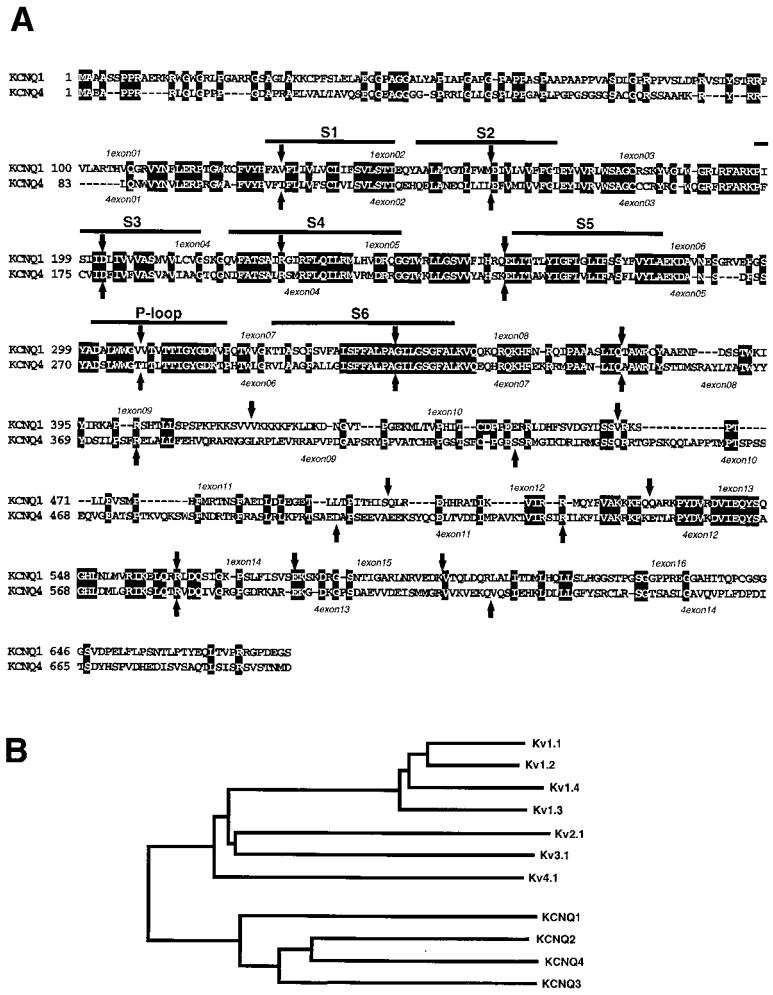

Figure 1. Sequence Comparison of KCNQ Potassium Channels

(A) Alignment of KCNQ1 (KvLQT1) and KCNQ4 K^+ channel amino acid sequences. Identical residues are marked by a black background. The transmembrane domains S1 through S6 and the pore-forming P loop are indicated by bars above the sequence. Positions of introns in both KCNQ1 and KCNQ4 are indicated by arrows, and exons are numbered for both channels. The KCNQ4 cDNA sequence has been deposited in the GenBank database under accession #AF105202. The sequences of all exons with adjacent intronic sequences are available under accession #AF105203–AF105216.

(B) Dendrogram (ClustalX, default parameters) comparing all known KCNQ K^+ channels to selected K^+ channels of the Kv class. It is evident that KCNQ channels form a distinct branch of the K^+ channel superfamily. Its individual members are less related to each other than, for example, Kv1.1 through Kv1.4.

the secretion of potassium into the endolymph that bathes the apical surface of the hair cells that convert mechanical stimulation into electrical signals. Indeed, both minK and KCNQ1 are coexpressed in the stria vascularis that secretes the endolymph (Sakagami et al., 1991; Neyroud et al., 1997), and this secretion is defective in mice deleted for the minK gene (Vetter et al., 1996).

In this work, we have cloned and characterized KCNQ4, a novel member of the KCNQ family of K^+ channels. *KCNQ4* was mapped to the DFNA2 locus, and a dominant-negative *KCNQ4* mutation that causes deafness in a DFNA2 pedigree was identified. In contrast to KCNQ1, KCNQ4 probably does not contribute to endolymph secretion but is directly important for the function of sensory outer hair cells. Further, similar to KCNQ2 that forms functional heteromers with KCNQ3 that probably underlie the M current (Wang et al., 1998b), KCNQ4 also forms heteromeric channels with KCNQ3. This work opens novel perspectives for cochlear physiology and for this interesting branch of the K^+ channel family.

Results

Cloning and Characterization of the KCNQ4 cDNA

Using a KCNQ3 K^+ channel partial cDNA as a probe, a human retina cDNA library was screened and an ~1 kb cDNA encoding a protein fragment homologous to KCNQ channels was isolated. It was distinct from the known members KCNQ1 (KvLQT1), KCNQ2, and KCNQ3. We named the novel gene *KCNQ4*. Overlapping cDNAs containing the entire open reading frame were obtained by rescreening the cDNA library and by extending the 5′ end in RACE (rapid amplification of cDNA ends) experiments.

The cDNA encodes a polypeptide of 695 amino acids with a predicted mass of 77 kDa (Figure 1A). Its overall amino acid identity to KCNQ1, KCNQ2, and KCNQ3 is 38%, 44%, and 37%, respectively. Together with these proteins it forms a distinct branch of the superfamily of voltage-gated K^+ channels (Figure 1B). Like a typical member of this gene family, it has six predicted transmembrane domains and a P loop between transmembrane domains S5 and S6. In K^+ channels, which are tetramers of identical or homologous subunits, four of these highly conserved P loops combine to form the ion-selective pore (Doyle et al., 1998). As other KCNQ channels, KCNQ4 has a long predicted cytoplasmic carboxyl terminus that accounts for about half of the protein. A conserved region present in the carboxyl termini of KCNQ1, -2, and -3 is also present in KCNQ4 (roughly represented by exon 12). Its function is currently unknown. The sequence of KCNQ4 predicts several potential

sites for phosphorylation by protein kinase C. In contrast to KCNQ1 and KCNQ2, however, it lacks an amino-terminal consensus site for cAMP-dependent phosphorylation. Phosphorylation of that site enhances currents of heteromeric KCNQ2/KCNQ3 channels (Schroeder et al., 1998). Several splice variants have been described for KCNQ1 through KCNQ3. Most of these occur in the cytoplasmic carboxyl terminus (e.g., Biervert et al., 1998). In the corresponding region of KCNQ4, we found a splice variant that lacked exon 9, leading to an in-frame deletion of 54 amino acids (residues 378 to 431). PCR experiments on adult human brain cDNA showed that this was a minority transcript (data not shown). Northern analysis of KCNQ4 expression in human tissues revealed faint bands in heart, brain, and skeletal muscle (data not shown).

Genomic Structure and Chromosomal Mapping to the DFNA2 Locus

A PAC was isolated that contains the entire KCNQ4 coding region. The genomic structure of the *KCNQ4* gene was established (Figure 1A and Experimental Procedures). The transmembrane block S1–S6 is encoded by six exons (exons 2 to 7) having the same limits as in *KCNQ2* and *KCNQ3* (Schroeder et al., 1998). In *KCNQ1*, an additional intron interrupts the sequence encoding domain S4. The exon–intron structures of *KCNQ* genes diverge most in the carboxyl termini of these proteins.

Using hybridization to human chromosomes, *KCNQ4* was mapped to 1p34. Several disease loci have been mapped to this region, including DFNA2, a locus for dominant progressive hearing loss (Coucke et al., 1994). Due to the critical role of K^+ homeostasis in auditory mechanotransduction, we considered *KCNQ4* an excellent candidate gene for DFNA2. The DFNA2 locus has been mapped between markers D1S255 and D1S193 (Coucke et al., 1994; van Camp et al., 1997). We therefore refined the localization of *KCNQ4* in comparison to published physical and genetic maps using a YAC (yeast artificial chromosome) contig of this region (Figure 2). *KCNQ4* was present on CEPH YAC clone 914c3, a result that places this gene within the DFNA2 region.

Expression of KCNQ Genes in the Inner Ear

The expression of *KCNQ4*, as well as other *KCNQ* genes, was studied by semiquantitative RT-PCR on mouse cochlear RNA. These results were compared with those obtained with vestibular and brain RNA (Figure 3A). KCNQ1, KCNQ3, and KCNQ4 messages can be detected in the cochlea, and additional PCR cycles revealed a weak KCNQ2 expression as well. At this high amplification, KCNQ1 was also detected in brain (data not shown). KCNQ1 and KCNQ4 appear to have the highest cochlear expression. KCNQ1 expression is higher in the cochlea than in brain (which was negative by Northern analysis [Wang et al., 1996]). The reverse is true for KCNQ2 and KCNQ3, both of which are broadly expressed in brain (Schroeder et al., 1998). KCNQ4 expression is significant in both of these tissues.

In situ hybridizations were performed on cochlea sections from mice at postnatal day P12 with a KCNQ4 antisense probe. Sensory outer hair cells were strongly

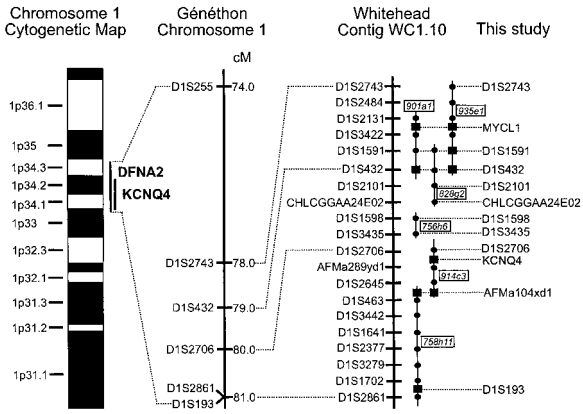

Figure 2. Genomic Mapping of the *KCNQ4* Gene

Left, cytogenetic map of a part of the short arm of human chromosome 1. The published borders of the DFNA2 region (Coucke et al., 1994; van Camp et al., 1997), as well as the localization of *KCNQ4* according to our FISH analysis, are indicated. Center, an enlargement of this region showing the corresponding Généthon map (1996), including microsatellite markers and distances in centimorgan (cM). Right, the Whitehead WC1.10 contig of that region. The bars at the right side represent YACs in that region (names in boxes) that were used here. Tested markers are shown at right. Filled circles denote markers already tested previously, while rectangles are markers newly placed on these YACs in this work. In contrast to the suggestion of van Camp et al. (1997) that was based on a single recombinant in one pedigree, our work places MYCL1 telomeric to D1S432. This agrees with the Location Data Base (LDB). Our mapping shows that *KCNQ4* maps to the unambiguous DFNA2 region.

labeled (Figures 3B and 3C). By contrast, the inner hair cells appeared negative. The stria vascularis, the site of KCNQ1 expression (Neyroud et al., 1997), was negative as well (Figure 3C). Control hybridization with a KCNQ4 sense probe (Figure 3D) revealed that the staining of outer hair cells was specific.

A KCNQ4 Pore Mutation in a Pedigree with Autosomal Dominant Deafness

These results indicated that *KCNQ4* was an excellent candidate gene for autosomal dominant deafness. We screened 45 families with autosomal dominant deafness without previous linkage analysis. In most of these families, the hearing loss had been diagnosed before adulthood, that is, before the age of onset reported for most of the DFNA forms, including DFNA2. Mutation screening was limited to exons 4 to 7, which encode the pore region and adjacent transmembrane domains. A *KCNQ4* mutation was found in a French family with profound hearing loss. Its clinical features (given in detail in Experimental Procedures) include progressive hearing loss that is more prominent with higher frequencies, tinnitus in one patient, and no indication for vestibular defects nor gross morphological changes in the inner ear. A missense mutation (G285S) was present in exon 6 in a heterozygous state (Figure 4A). It segregated with all affected members in the pedigree (Figure 4C). This mutation was not found on 150 control Caucasian chromosomes.

The G285S mutation affects the first glycine in the GYG signature sequence of K^+ channel pores (Figure 4B). This glycine is highly conserved across different

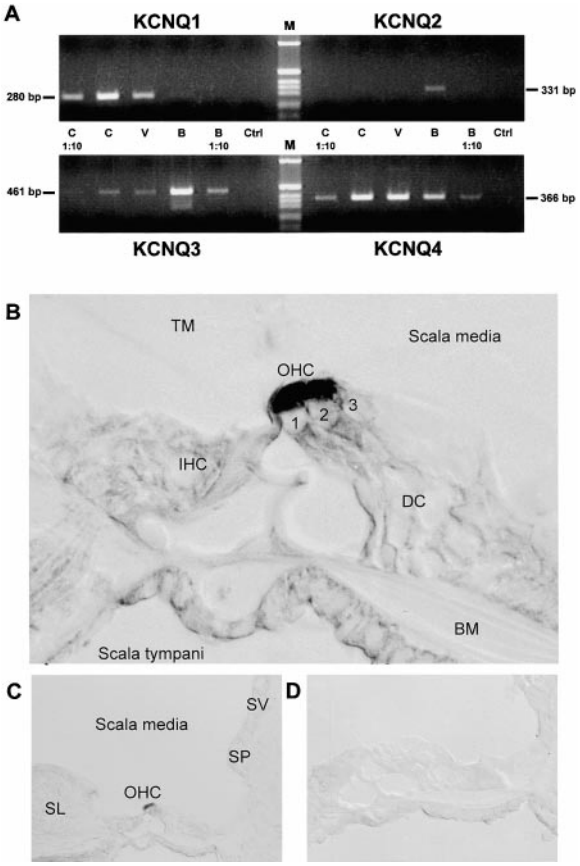

Figure 3. Expression of KCNQ4 in the Inner Ear

(A) Expression of KCNQ channels in the cochlea, vestibule, and brain as revealed by RT-PCR. c, cochlear RNA; v, vestibular RNA; b, brain RNA. If 1/10 of the amount of cDNA was employed for amplification, this is indicated by 1:10. As preparation of cochlear and vestibular RNA is difficult, there may be a small cross contamination between these preparations.

(B and C) In situ hybridization of the mouse cochlea (at postnatal day P12) using a mouse KCNQ4 antisense probe or, as a control, a sense probe (D). KCNQ4 message is present in the three outer hair cells (OHC), labeled 1 to 3, but is not detected in the inner hair cell (IHC) nor in the stria vascularis (SV). DC, Deiters cells; BM, basilar membrane; SP, spiral prominence; SL, spiral limbus; TM, tectorial membrane. (B) is a higher magnification of (C). The predominantly apical staining of OHCs is typical for these cells and is probably related to the basal localization of the nucleus.

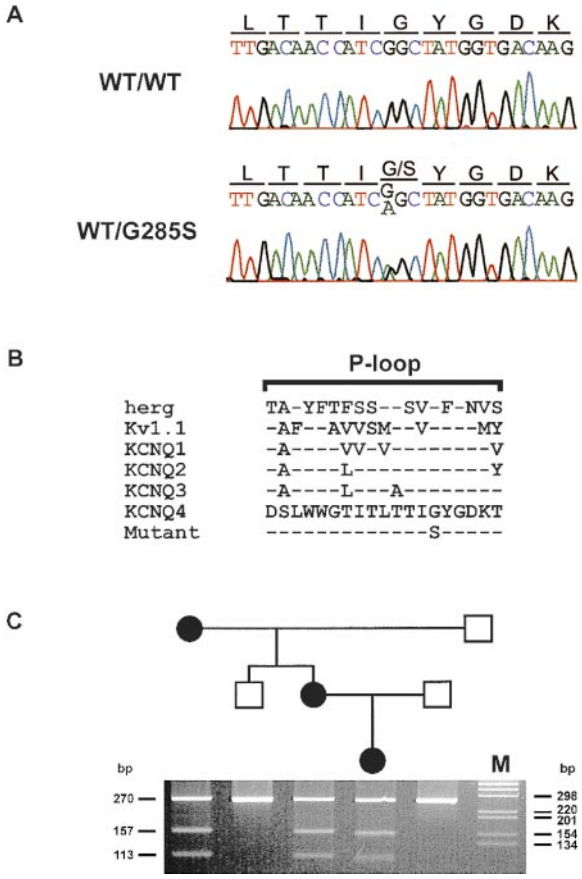

Figure 4. Mutation Analysis of *KCNQ4* in a DFNA2 Pedigree

(A) Sequence analysis of the pore region of KCNQ4 in an unaffected (WT/WT) and an affected member (WT/G285S) of the pedigree. Direct printouts from the ABI377-automated sequencer are shown. They reveal a G-to-A transition on one allele of the patient, leading to the G285S missense mutation. The mutation leads to a novel AluI restriction site (AGCT).

(B) Sequence comparison of the pore-forming P loop region between all published KCNQ channels and two more distantly related K^+ channels (herg and Kv1.1). Amino acids identical to the KCNQ4 residues are indicated by dashes. The G285S mutation identified in the pedigree (line at bottom) changes the first glycine of the highly conserved GYG motif to a serine. These three residues line the narrowest portion of the K^+ channel pore.

(C) Segregation of the G285S mutation in the DFNA2 pedigree. Exon 6 of KCNQ4 was amplified by PCR from genomic DNA from all members of the pedigree we had access to (no DNA was available from one grandparent). This yielded PCR products of 270 bp that were digested with AluI, which is diagnostic for the G285S mutation and results in fragments of 157 and 113 bp. Since the mutation is present in a heterozygous state, WT bands are present for each individual. The mutation cosegregates with deafness.

classes of K^+ channels in all species. The crystal structure of the *Streptomyces lividans* K^+ channel reveals that these three amino acids line the narrowest part of the pore (Doyle et al., 1998). Mutations in these amino acids disrupt the selectivity filter and in most cases abolish channel function. Interestingly, an identical change in amino acids at the equivalent position was found in the *KCNQ1* gene of a patient with the dominant LQTS (Russell et al., 1996). It disrupted channel activity and exerted a dominant-negative effect on coexpressed WT KCNQ1 channels (Wollnik et al., 1997). These mutations also have dominant-negative effects when inserted into KCNQ2 and KCNQ3 (Schroeder et al., 1998). This is strong evidence that the progressive hearing loss in this family is due to the KCNQ4 G285S mutation.

Functional Expression of KCNQ4 Potassium Channels

KCNQ4 was expressed in *Xenopus* oocytes and its activity was investigated by two-electrode voltage clamping. Similar to KCNQ1, KCNQ2, and KCNQ3, KCNQ4 also yielded currents that activated upon depolarization (Figure 5A). Compared to those other KCNQ channels, current activation was slower and occurred with a time constant in the order of 600 ms at +40 mV (KCNQ2/KCNQ3 channels have a corresponding time constant

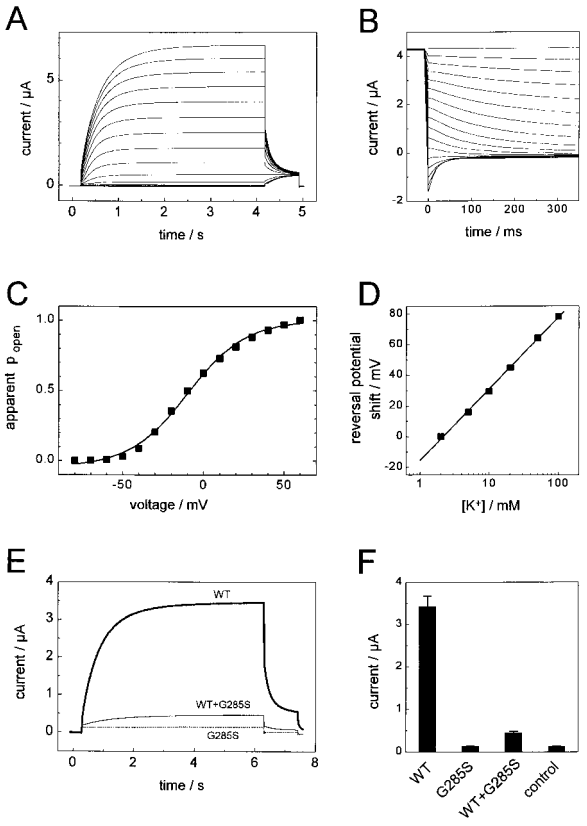

Figure 5. Electrophysiological Properties of KCNQ4 Currents

(A) Two-electrode voltage-clamp current traces from a *Xenopus* oocyte injected with KCNQ4 cRNA. From a holding potential of −60 mV, cells were clamped for 4 s to voltages between −80 to +60 mV in 10 mV steps, followed by a constant pulse to −30 mV.

(B) Inactivation behavior of KCNQ4 at different voltages. After an activating voltage pulse (3.5 s at +40 mV), the cell was clamped to voltages between +40 to −120 mV in 10 mV steps.

(C) Apparent open probability (p_{open}) as a function of voltage determined from tail current analysis of currents as in (A). Half-maximal p_{open} is achieved at −10.0 ± 1.2 mV, and the apparent gating charge is 1.4 ± 0.1, as obtained by fitting a Boltzmann function to the data (n = 14 from two oocyte batches, ± SEM).

(D) Shift of the reversal potential with the extracellular K^+ concentration. Total monovalent cation concentration was 100 mM, and K^+ replaced Na^+. The reversal potential shift of 46.7 ± 0.9 mV per decade indicates a K^+-selective channel (n = 18 from three oocyte batches, ± SEM). Substitution of external K^+ with other cations gave the following permeability ratios: P_K/P_{Na} = 52.3 ± 4.4, P_K/P_{Cs} = 7.8 ± 0.7, and P_K/P_{Rb} = 0.94 ± 0.03 (permeability sequence: $Rb^+ \approx K^+ > Cs^+ > Na^+$, n = 15 from three oocyte batches, ± SEM).

(E) Current traces of WT KCNQ4 (thick solid line), a 1:1 coinjection of WT KCNQ4 and $KCNQ4_{G285S}$ mutant (thin solid line), and $KCNQ4_{G285S}$ mutant (dotted line). $KCNQ4_{G285S}$ currents were indistinguishable from water-injected control oocytes. From a holding potential of −60 mV, the cells were clamped for 6 s to +40 mV, followed by a −30 mV step.

(F) Mean currents after 4 s at +40 mV, averaged from several experiments as in (E) (n = 20 to 35, four oocyte batches, ± SEM).

of ~300 ms). This time constant was very sensitive to temperature. Deactivation of currents at physiological resting potentials (~−70 mV) was considerably faster (Figure 5B). Similar to KCNQ2 (Biervert et al., 1998), currents often showed some inward rectification at positive potentials. When oocytes were depolarized to +60 mV for 10 s or more, an apparent slow inactivation was observed (data not shown) that resembled the one described for KCNQ3 (Yang et al., 1998). Currents began to activate at about −40 mV, with half-maximal activation at −10 mV (Figure 5C). The channel is selective for K^+ (Figure 5D). It has a $K^+ \approx Rb^+ > Cs^+ > Na^+$ permeability sequence. KCNQ4 currents were inhibited by more than 80% by 5 mM Ba^{2+} (data not shown).

We next examined the effect of the G285S mutation found in the affected family (Figures 5E and 5F). The mutant channel did not yield any detectable currents when expressed in *Xenopus* oocytes. $KCNQ4_{G285S}$ was then injected at a 1:1 ratio with WT KCNQ4 to mimic the situation in a heterozygous DFNA2 patient. This reduced currents by about 90%, indicating a strong dominant-negative effect of the mutant. The channels present in coinjected oocytes still showed a strong preference of potassium over sodium or calcium (data not shown). This implies that the deafness is due to a quantitative loss of KCNQ4 K^+ currents rather than to an influx of Na^+ or Ca^{2+}.

KCNQ1 assembles with minK (IsK) to form channels that yield larger currents and activate much slower (Barhanin et al., 1996; Sanguinetti et al., 1996). We tested by coexpression whether minK affects KCNQ4 as well. At concentrations (1 ng minK cRNA per oocyte) leading to marked changes in KCNQ1 currents in parallel experiments, there was no significant change in KCNQ4 currents (data not shown).

Different KCNQ subunits can form heteromeric channels. Coexpression of KCNQ2 with KCNQ3, but not with KCNQ1, gave currents that were about 10-fold larger than those from homomeric channels (Schroeder et al., 1998; Wang et al., 1998b; Yang et al., 1998). Since KCNQ1 and KCNQ3 (and to a lesser degree, KCNQ2) are also expressed in the cochlea (Figure 3A), we investigated whether these proteins interact functionally. Oocytes coinjected (at the same total cRNA concentration) with KCNQ1 and KCNQ4 cRNAs yielded currents that seemed not different from a linear superposition of currents from the respective homomeric channels (Figure 6A), and the same was true for oocytes coexpressing KCNQ2 and KCNQ4 (Figure 6B). In addition, a dominant-negative KCNQ1 mutant (Wollnik et al., 1997) did not suppress KCNQ4 currents (Figure 6A), and this also holds for the equivalent KCNQ2 mutant (Schroeder et al., 1998) (Figure 6B).

By contrast, coexpression of KCNQ3 with KCNQ4 yielded currents that were significantly larger than could be explained by a superposition of currents from the respective homomeric channels (Figures 6C and 6D). Further, KCNQ4 currents were markedly suppressed by coexpressing a dominant-negative KCNQ3 mutant (Schroeder et al., 1998) (Figure 6C). Currents from coinjected oocytes activated faster than KCNQ4 currents (Figure 6D), and there was an ~10 mV shift of the open probability toward negative voltages (Figure 6E). Compared to KCNQ2/KCNQ3 channels, which may underlie the M current (Wang et al., 1998b), KCNQ3/KCNQ4 heteromers open at slightly more positive voltages. To compare KCNQ3/KCNQ4 channels to M channels, we used the typical voltage protocol employed for these channels (Wang et al., 1998b) and found currents superficially

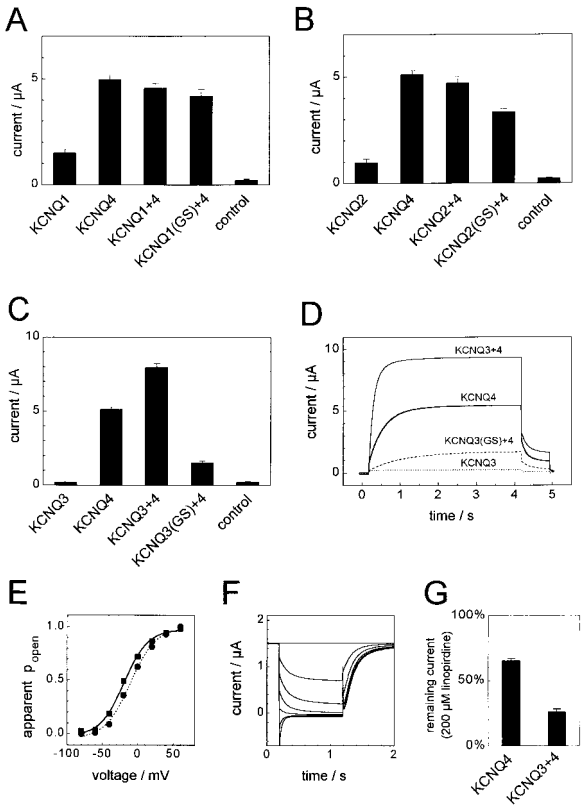

Figure 6. Functional Interaction of KCNQ4 with Other KCNQ Subunits

Coexpression of KCNQ4 with KCNQ1 (A), KCNQ2 (B), and KCNQ3 (C) and derived dominant-negative mutants ($KCNQ1_{G219S}$, $KCNQ2_{G279S}$, $KCNQ3_{G318S}$) (n = 10 to 31, three oocyte batches, ± SEM).

(D) Representative currents from experiments as in (C) showing altered activation kinetics for the coinjection of KCNQ4 with KCNQ3 or $KCNQ3_{G318S}$, respectively. From a holding potential at −60 mV, the voltage was clamped for 4 s at +40 mV, followed by a step to −30 mV. Time constants and amplitudes obtained from two-exponential fits were KCNQ4: τ_1 = 360 ms, A_1 = −4.9 µA, τ_2 = 1700 ms, A_2 = −0.34 µA; KCNQ3 + KCNQ4: τ_1 = 120 ms, A_1 = −6.3 µA, τ_2 = 560 ms, A_2 = −1.3 µA.

(E) Apparent p_{open} as a function of voltage for currents from oocytes coinjected with KCNQ4 and KCNQ3 cRNA, determined from tail current analysis (squares, solid curve). Half-maximal p_{open} is achieved at $V_{0.5}$ = −19.1 ± 2.0 mV, and the apparent gating charge is 1.5 ± 0.2 (n = 23 from three oocyte batches, ± SEM), as obtained from a fit of a Boltzmann function. The p_{open} curve for KCNQ4 is shown for reference (circles, dashed curve).

(F) Current traces recorded from an oocyte coinjected with KCNQ3 and KCNQ4 cRNA using a typical M current voltage protocol. From a holding potential at −30 mV, the cell was progressively hyperpolarized for 1 s to voltages between −30 and −90 mV in −10 mV steps.

(G) Differential effects of 200 µM linopirdine on KCNQ4 (n = 10, ± SEM) and KCNQ3 + KCNQ4 (n = 6, ± SEM). Currents were measured at +40 mV, and percentage of current remaining with linopirdine is shown. Steady-state inhibition was reached after ~1 min for KCNQ4 currents and after ~3 min for KCNQ3 + KCNQ4 currents.

resembling M currents (Figure 6F). Linopirdine, a potent and rather specific inhibitor for M currents, nearly completely inhibits KCNQ2/KCNQ3 channels at a concentration of 200 µM (Wang et al., 1998b). This concentration of linopirdine inhibited KCNQ4 by about 30%, while a significantly larger inhibition (~75%) was observed with KCNQ3/KCNQ4 coexpression (Figure 6G).

Discussion

In this work, we have identified the gene for DFNA2, a locus involved in autosomal dominant deafness. It encodes KCNQ4, a novel potassium channel. It is expressed in several tissues, including the cochlea, where it is present in outer hair cells. The mutant channel identified in a DFNA2 family exerts a dominant-negative effect on WT KCNQ4. Interestingly, the pathomechanism leading to deafness is different with mutations in *KCNQ4* or *KCNQ1*.

KCNQ Channels in Genetic Disease

It is remarkable that mutations in every known *KCNQ* gene lead to human disease: mutations in *KCNQ1* (KvLQT1) cause the autosomal dominant LQTS (Wang et al., 1996) and, when present on both alleles, the JLN syndrome (Neyroud et al., 1997), whose symptoms include deafness in addition to cardiac arrhythmias. Mutations in either *KCNQ2* or *KCNQ3*, which form heteromers that probably represent the M channel (Wang et al., 1998b), cause benign familial neonatal convulsions (BFNC) (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). The present work adds KCNQ4 and the associated autosomal dominant deafness to that list.

After KCNQ1, KCNQ4 is now the second KCNQ channel whose loss of function leads to deafness. However, there are important differences. The hearing loss caused by *KCNQ1* mutations is syndromic, severe, and congenital, and both alleles of the gene are mutated. By contrast, the deafness associated with *KCNQ4* is nonsyndromic, progresses slowly, and is dominant. The reduction of currents associated with dominant-negative *KCNQ1* mutations causes severe cardiac arrhythmias but does not affect normal cochlear function (Wollnik et al., 1997). By contrast, a dominant-negative *KCNQ4* mutation suffices to cause progressive hearing loss. Surprisingly, the mutation found here ($KCNQ4_{G285S}$) is exactly equivalent to a *KCNQ1* mutation found in dominant LQTS (Russell et al., 1996) that had a similar dominant-negative effect (Wollnik et al., 1997). Thus, in the ear, a moderate loss of KCNQ1 function is better tolerated than a similar loss of KCNQ4. The expression levels of KCNQ2 and KCNQ3 channels are even closer to a critical threshold in the brain. A slight current reduction due to mutations lacking a dominant-negative effect on one allele suffices to cause neonatal epilepsy (Schroeder et al., 1998). Thus, the safety margins separating normal current levels from the disease-causing threshold differ widely between different KCNQ channels and organs.

Heteromers Formed by KCNQ Subunits

The KCNQ channels described so far function physiologically as heteromers. KCNQ1 associates with minK, and KCNQ2 and KCNQ3 form heteromeric channels that may underlie the M current (Wang et al., 1998b), an important determinant of neuronal excitability that is regulated by several neurotransmitters (Marrion, 1997). Therefore, a relevant question is whether KCNQ4 forms heteromeric channels with any of these proteins.

We addressed this issue irrespective of their colocalization in vivo by coexpressing these different potassium channel subunits in *Xenopus* oocytes. There were no obvious effects when KCNQ4 was coexpressed with minK at concentrations that markedly affect KCNQ1 currents. Further, expression of these proteins overlaps neither in brain nor in the cochlea (but may do so in heart). minK mRNA could not be found in brain (Chouabe et al., 1997). In the cochlea, KCNQ4 is expressed in outer hair cells and minK in the stria vascularis (Sakagami et al., 1991).

We could neither detect a functional interaction of KCNQ4 with KCNQ1 or KCNQ2. However, it is difficult to rule out such an interaction by coexpressing the WT channels. Currents of a hypothetical heteromer may not differ enough from a superposition of currents mediated by the respective homomultimers. We therefore resorted to coexpressing dominant-negative mutants. Neither KCNQ1 nor KCNQ2 mutants suppressed KCNQ4 currents, arguing against the formation of heteromers. Similar to minK, KCNQ1 is expressed specifically in the stria vascularis (Neyroud et al., 1997) and does not overlap with KCNQ4. Our RT-PCR experiments suggest that KCNQ2 expression in the cochlea is low. Together, these experiments indicate that KCNQ4 does not form heteromeric channels with KCNQ1 or KCNQ2 in the cochlea.

By contrast, KCNQ4 interacts with KCNQ3. Currents from homomeric KCNQ3 are very small (Schroeder et al., 1998; Wang et al., 1998b). Coexpression of KCNQ3 with KCNQ4 markedly increased current amplitudes, but this increase was less than the 10-fold stimulation observed with KCNQ2/KCNQ3 coexpression (Schroeder et al., 1998; Wang et al., 1998b; Yang et al., 1998). Significantly, heteromeric KCNQ3/KCNQ4 currents activated faster and at more negative voltages than KCNQ4 and displayed a different drug sensitivity. Further, a dominant-negative KCNQ3 mutant strongly suppressed KCNQ4 currents. Since KCNQ3 is also expressed in the cochlea (Figure 3A), the formation of KCNQ3/KCNQ4 channels in outer hair cells is a distinct possibility that needs to be confirmed by localizing KCNQ3 to these cells. The formation of cochlear KCNQ3/KCNQ4 channels would not contradict the involvements of KCNQ3 in epilepsy and of KCNQ4 in deafness. All mutations identified so far in BFNC lack a dominant-negative effect (Schroeder et al., 1998). Therefore, they are not expected to reduce currents of the cochlear heteromer to the levels found with the present KCNQ4 mutation. On the other hand, KCNQ4 expression in brain may be low. Thus, it may lack a significant effect on the KCNQ2/KCNQ3 channels that are affected in BFNC.

KCNQ2/KCNQ3 heteromeric channels were recently shown to have properties of the physiologically important M current (Wang et al., 1998b). We have shown here that KCNQ3/KCNQ4 heteromers also share some characteristics with M channels. It will be interesting to see whether these heteromers mediate variants of M currents in certain regions of the nervous system.

Pathophysiology of Deafness due to KCNQ Mutations: Endolymph Secretion versus Direct Hair Cell Defects

The receptor cells in the organ of Corti that transduce mechanical stimuli to electrical signals are the sensory hair cells (for a recent review on cochlear physiology, see Nobili et al., 1998). The synaptic output of inner hair cells creates the main electric signal that mediates hearing. The function of outer hair cells is mainly to tune the cochlea by converting their receptor potential into a mechanical force. The apical membranes of both inner and outer hair cells are bathed in the endolymph that fills the scala media. In contrast to normal extracellular saline, it has a high potassium concentration of roughly 150 mM and a positive potential (+80 mV with respect to the normal extracellular space). Mechanical stimulation of the stereocilia of hair cells leads to an influx of K^+ driven by the large electrical voltage ($\sim$−150 mV) across their apical membranes.

The potassium taken up by hair cells leaves these cells via their basolateral membrane. This most likely involves K^+ channels. K^+ is thought to be recycled to the endolymph via a cellular system connected by gap junctions (Kikuchi et al., 1995; Spicer and Schulte, 1996, 1998). In this model, K^+ is taken up by the Deiters cells that contact the base of outer hair cells. It then diffuses intracellularly through gap junctions to the stria vascularis. There it is finally secreted into the endolymph by the marginal cells of the stria vascularis.

Physiological and immunocytochemical studies have implicated KCNQ1/minK channels in this secretion. In a mouse model with a disrupted minK gene (Vetter et al., 1996), the scala media collapses shortly after birth. Within a few days, the hair cells degenerate. Similar morphological features were described in patients with JLN syndrome (Friedmann et al., 1966). Thus, there is very strong evidence that the profound, early deafness in that disease is due to a defect in endolymph production.

The fact that the short circuit current across the stria vascularis is nearly abolished in minK knockout mice argues against the hypothesis that KCNQ4 may be an important parallel pathway for endolymph secretion in this epithelium. Further, we could not detect functional interactions of KCNQ4 with KCNQ1 or minK. This excludes the formation of a common channel involved in endolymph secretion. Finally, in contrast to KCNQ1 and minK, KCNQ4 is not expressed in the stria vascularis, but in outer hair cells.

What might be the function of KCNQ4 in outer hair cells? At this point, we can only speculate. It seems unlikely that it is involved in the primary signal transduction at the apical membrane, as the strongly negative voltage across this membrane prevents it from being open (by contrast, the voltage across the apical membrane of marginal cells of the stria vascularis is in the −10 mV range, allowing K^+ secretion via apical KCNQ1/minK channels). KCNQ4 will more likely contribute to the basolateral K^+ conductance that is important both for the modulation of electrical excitation and for the removal of intracellular K^+ taken up apically from the endolymph. Currents that activate slowly with depolarization have been described in outer hair cells (Housley and Ashmore, 1992; Mammano and Ashmore, 1996). However, their pharmacology (e.g., sensitivity to 4-aminopyridine) does not fit with KCNQ4 or KCNQ3/KCNQ4 currents (T. F. and T. J. J., unpublished observation). Nonetheless, the hypothesis that KCNQ4 is involved in regulating the excitability of outer hair cells is attractive. It is indirectly supported by the finding that KCNQ2 and

Table 1. Solution Contents (Concentrations in mM)

ND98	ND100	KD100	Rb100	Cs100
98 NaCl 2 KCl	100 NaCl	100 KCl	100 RbCl	100 CsCl
0.2 $CaCl_2$	0.2 $CaCl_2$	0.2 $CaCl_2$	0.2 $CaCl_2$	0.2 $CaCl_2$
2.8 $MgCl_2$	2.8 $MgCl_2$	2.8 $MgCl_2$	2.8 $MgCl_2$	2.8 $MgCl_2$

Buffer solution was 5 mM HEPES (pH 7.4).

KCNQ3 may constitute the M channel that regulates neuronal excitability (Wang et al., 1998b).

A complementary view is that KCNQ4 is important for the basolateral removal of K^+ from hair cells. Possibly, a chronic K^+ overload leads to a slow degeneration of these cells. This might explain the slowly progressive hearing loss in DFNA2 patients. Importantly, mutations in connexin 26, which are expected to disrupt the recycling of K^+ to the endolymph, also lead to deafness (Kelsell et al., 1997; Denoyelle et al., 1998). There is no morphological information yet on a possible degeneration of hair cells in patients with either DFNA2 or mutations in connexin 26.

In the family we studied, as well as in previously described DFNA2-affected families (Coucke et al., 1994), some affected individuals suffered from tinnitus. Interestingly, some forms of this disorder were linked to a dysfunction of outer hair cells (Kakehata and Santos-Sacchi, 1996). Ion channels are good targets for drugs, and K^+ channel openers have already been developed. KCNQ4 may be an interesting target for the prevention of progressive hearing loss and possibly for the treatment of tinnitus. The establishment of transgenic mouse models will be necessary to fully elucidate the pathophysiology of *KCNQ4*/DFNA2 deafness. These mice may also be a valuable model for the frequent condition of presbyacusis that develops slowly over decades.

Experimental Procedures

Cloning of KCNQ4 cDNA

A human retina cDNA λ phage library (Clontech, #HL1132a) was screened with a partial KCNQ3 cDNA clone. A cDNA encoding a fragment of a novel KCNQ homolog subsequently named KCNQ4 was isolated. Rescreening extended the sequence toward the 3′ end. The 5′ end was cloned by 5′ RACE using a Marathon kit (Clontech) with human skeletal muscle cDNA. A complete cDNA was assembled and cloned into the NcoI and XhoI sites of the oocyte expression vector PTLN (Lorenz et al., 1996). This provides an optimal Kozak sequence at the initiator AUG and *Xenopus* β-globin untranslated sequences to boost expression in oocytes. Mutations were introduced by recombinant PCR using Pfu polymerase. PCR-derived fragments were fully sequenced.

RT-PCR Analysis of Mouse KCNQ mRNA Expression

Approximately 2 μg of mouse total brain RNA and mouse cochlear and vestibular RNA were reverse transcribed using the SuperScript II (GIBCO-BRL) reverse transcriptase. The cDNA was amplified for 30 cycles (96°C for 30 s, 61°C for 30 s, and 68°C for 45 s) using a 2400 Thermocycler System (Perkin Elmer). Each 50 μl reaction contained 2.5 U polymerase (Expand Long Template PCR System, Boehringer Mannheim) and 5% DMSO. KCNQ1 primers were based on the mouse cDNA sequence (GenBank #U70068): MK1a 5′-aaggc tggatcagtccattgg-3′ and MK1r 5′-aggtgggcaggctgttgctgg-3′ (280 bp). As no mouse KCNQ2 sequence was available, we chose sequences conserved between human (Y15065) and rat (AF087453) KCNQ2: MK2a 5′-gccacggcacctcccccgtgg-3′ and MK2r 5′-ccctctgcaatg tagggcctgac-3′ (331 bp). KCNQ3 primers were derived from a mouse EST (AA386747): MK3a 5′-ccaaggaatgaaccatatgtagcc-3′ and MK3r 5′-cagaagagtcaagatgggcaggac-3′ (461 bp). Mouse KCNQ4 primers were MK4a 5′-agtacctgatggagcgccctctcg-3′ and MK4r 5′-tcatccaccgtaagctcacactgg-3′ (366 bp). Amplification products were verified by direct sequencing.

In Situ Hybridization of Mouse Cochlea

A mouse KCNQ4 cDNA corresponding to bp 618 to 1602 of the human KCNQ4 ORF was cloned into pBluescript. Sense and antisense probes were transcribed using T3 and T7 RNA polymerases after appropriate linearization. After DNase digestion, the probes were ethanol precipitated twice with 0.4 M LiCl. They were labeled with digoxigenin-11-UTP as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Mouse inner ears were fixed for 1 hr at 4°C in 4% paraformaldehyde in PBS. After three rinses in PBS, they were immersed in 20% sucrose overnight at 4°C. Cryostat sections (10–14 μm) were postfixed and rinsed in PBS. Following prehybridization at room temperature for ≥3 hr, they were hybridized overnight at 58°C in a humid chamber. Sections were then washed and incubated with sheep antidigoxigenin antibody coupled to alkaline phosphatase. Staining by NBT/BCIP (Boehringer Mannheim) was done for 2 hr at 37°C and overnight at room temperature. Sections were then mounted in Aquatex (Merck, USA).

Genomic Structure and Amplification of KCNQ4 Exons from Genomic DNA

The genomic structure was established by a PCR approach from genomic DNA. Sequences of exons and adjacent introns were deposited in GenBank (accession numbers AF105203–AF105216). Individual KCNQ4 exons and adjacent short intronic sequences were amplified by PCR from human genomic DNA using intronic oligonucleotide primers. The sequence of these primers and the corresponding PCR protocols can be obtained from the authors. To screen unlinked pedigrees with autosomal dominant deafness, we amplified only exons 4 through 7. After amplification and agarose gel purification, PCR products were directly sequenced using the amplification primers and an ABI377-automated DNA sequencer.

Chromosomal Localization of *KCNQ4*

A PAC containing the coding sequence of KCNQ4 was isolated using intronic KCNQ4 oligonucleotide primers and PCR. It was used to localize *KCNQ4* to 1p34 using FISH (Genome Systems). *KCNQ4* was then mapped on the Whitehead Contig WC1.10 using several of the intronic primers given above and published STS markers by PCR amplification from individual YAC clones.

DFNA2-Affected Family

The family with autosomal dominant, progressive deafness (Figure 4C) is of French origin. In the first generation, deafness was detected in early childhood, in the second generation around puberty, and in the third generation in early childhood. The third generation individual has been complaining about tinnitus since she was about 3 years old. The deafness is more severe in the third generation than in the second, which is again more severe than in the first. In all three individuals, the hearing loss is more severe on high frequencies. The hearing loss is between 50 and 90 dB at 500 Hz and between 90 and 120 dB at 2 and 4 kHz. The second generation individual started walking when about 10 months old, and all affected individuals did not complain about balance problems. Thus, there is no indication for a vestibular involvement. There were no hints for a morphogenetic defect on CT scans.

Expression in *Xenopus laevis* Oocytes and Voltage-Clamp Studies

After linearization of the KCNQ4-containing PTLN vector with HpaI, capped cRNA was transcribed in vitro using the mMessage mMachine kit (Ambion). Usually 5–15 ng of cRNA was injected into *Xenopus* oocytes previously isolated by manual defolliculation and short collagenase treatment. In coexpression experiments, cRNAs were generally injected at a 1:1 ratio. Oocytes were kept at 17°C in modified Barth's solution (90 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, 100 U penicillin—100

μg streptomycin/ml [pH 7.6]). Two-electrode voltage-clamp measurements were performed at room temperature 2–4 days after injection using a Turbotec 05 amplifier (npi instruments) and pClamp 5.5 software (Axon Instruments). Currents were usually recorded in ND98 solution (see Table 1). Solutions for Na^+/K^+ replacement experiments were prepared from appropriate mixtures of solution KD100 and ND100. Linopirdine (RBI, Natick, MA) was prepared as a 100 mM stock solution in DMSO and added to a final concentration of 200 μM to ND98. Reversal potentials were determined from tail currents after a 2 s depolarizing pulse to +60 mV and corrected for liquid junction potentials. The permeability ratios were calculated according to $P_K/P_X = \exp(-F \cdot V_{rev}/R \cdot T)$. To determine the voltage dependence of apparent open probability, oocytes were clamped for 4 s to values between −80 mV to +50 mV in 10 mV steps, followed by a constant −30 mV test pulse. Tail currents extrapolated to t = 0 were obtained from monoexponential fits, normalized to the value at 0 mV, and used to determine apparent p_{open}. Data analysis used pClamp6 and Microcal Origin 5.0.

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GenBank Accession Numbers

The KCNQ4 cDNA sequence has been deposited in the GenBank database under accession number AF105202. Exons and intronic sequences have been deposited under accession numbers AF105203 through AF105216.