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RESEARCH PAPER

Restoration of ion channel function in deafness-causing KCNQ4 mutants by synthetic channel openers

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BACKGROUND AND PURPOSE

DFNA2 is a frequent hereditary hearing disorder caused by loss-of-function mutations in the voltage-gated potassium channel KCNQ4 (Kv7.4). KCNQ4 mediates the predominant K^+ conductance, $I_{K,n}$, of auditory outer hair cells (OHCs), and loss of KCNQ4 function leads to degeneration of OHCs resulting in progressive hearing loss. Here we explore the possible recovery of channel activity of mutant KCNQ4 induced by synthetic KCNQ channel openers.

EXPERIMENTAL APPROACH

Whole cell patch clamp recordings were performed on CHO cells transiently expressing KCNQ4 wild-type (wt) and DFNA2-relevant mutants, and from acutely isolated OHCs.

KEY RESULTS

Various known KCNQ channel openers robustly enhanced KCNQ4 currents. The strongest potentiation was observed with a combination of zinc pyrithione plus retigabine. A similar albeit less pronounced current enhancement was observed with native $I_{K,n}$ currents in rat OHCs. DFNA2 mutations located in the channel's pore region abolished channel function and these mutant channels were completely unresponsive to channel openers. However, the function of a DFNA2 mutation located in the proximal C-terminus was restored by the combined application of both openers. Co-expression of wt and KCNQ4 pore mutants suppressed currents to barely detectable levels. In this dominant-negative situation, channel openers essentially restored currents back to wt levels, most probably through strong activation of only the small fraction of homomeric wt channels.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that by stabilizing the KCNQ4-mediated conductance in OHCs, chemical channel openers can protect against OHC degeneration and progression of hearing loss in DFNA2.

Abbreviations

BFNC, benign familial neonatal convulsion; DFNA2, deafness autosomal dominant locus 2; OHC, outer hair cell; wt, wild-type; ZnP, zinc pyrithione; ZnP/Ret, zinc pyrithione plus retigabine

Introduction

Loss-of-function mutations in the voltage-gated potassium channel KCNQ4 (Kv7.4) cause a non-syndromic, progressive form of hereditary deafness, deafness autosomal dominant locus 2 (DFNA2) (Kubisch *et al.*, 1999; Beisel *et al.*, 2000; Kharkovets *et al.*, 2000; 2006). DFNA2 is characterized by symmetric hearing loss that is progressive at all frequencies (Jentsch, 2000; Nie, 2008). Hearing impairment is moderate at young ages, but most affected individuals develop severe-to-profound high-frequency hearing loss by the age of 70 (Smith and Hildebrand, 2008). KCNQ4 is strongly expressed in sensory outer hair cells (OHCs) in the organ of Corti, where it mediates the predominant K^+ conductance, $I_{K,n}$ (Mammano

and Ashmore, 1996; Kubisch *et al.*, 1999; Marcotti and Kros, 1999; Kharkovets *et al.*, 2006; Holt *et al.*, 2007). KCNQ4 has an essential role for OHC function, as pharmacological inhibition, genetic ablation and loss of function by dominant-negative mutations lead to progressive loss of OHCs and subsequent hearing loss in animal models, recapitulating the human DFNA2 phenotype (Kubisch *et al.*, 1999; Nouvian *et al.*, 2003; Kharkovets *et al.*, 2006). KCNQ4 is also expressed in inner hair cells (Oliver *et al.*, 2003) and in neurons in the central auditory system (Kharkovets *et al.*, 2000; Beisel *et al.*, 2005), but the physiological relevance in these cells is not known. To date, eight distinct KCNQ4 point mutations generating non-functional channel subunits, two mutations producing truncated protein (Coucke *et al.*, 1999; Kamada *et al.*, 2006) and a six-amino acid deletion in the linker between the fourth and fifth transmembrane segment (Baek *et al.*, 2011) have been identified in DFNA2-affected humans. Most point mutations were located within the channel's pore region, where mutations inhibit channel function presumably by disrupting ion permeation (Coucke *et al.*, 1999; Kubisch *et al.*, 1999; Talebizadeh *et al.*, 1999; Van Hauwe *et al.*, 2000; Akita *et al.*, 2001; Van Camp *et al.*, 2002; Topsakal *et al.*, 2005) or by reducing channel surface expression (Mencia *et al.*, 2008; Kim *et al.*, 2011). A mutation in a putative splicing site in the third transmembrane region was hypothesized to produce a non-functional KCNQ4 isoform specifically in OHCs (Su *et al.*, 2007; Kim *et al.*, 2011), and a mutation in the proximal C-terminus renders channels non-functional (Coucke *et al.*, 1999; Kim *et al.*, 2011), possibly by disrupting channel gating. Voltage-gated potassium channels are homo- or heteromeric assemblies of four pore-forming subunits. Therefore, mutations in one single subunit can disrupt channel function of the tetramer in a dominant-negative manner (Biervert *et al.*, 1998; Charlier *et al.*, 1998; Schroeder *et al.*, 1998; Singh *et al.*, 1998). Accordingly, DFNA2-causing mutations are thought to reduce native $I_{K,n}$ via dominant-negative suppression of channel function in individuals heterozygous for the mutation (Kubisch *et al.*, 1999; Holt *et al.*, 2007). A variety of potassium channel openers has been identified over the last years that strongly and specifically potentiate currents through KCNQ channels (Wulff *et al.*, 2009). Some of these KCNQ openers are already in use or in clinical trials for the treatment of various neurological disorders (Wulff *et al.*, 2009). Interestingly, the KCNQ openers, retigabine and zinc pyrithione (ZnP), were recently shown to restore the channel function of epileptogenic KCNQ2-mutants (Xiong *et al.*, 2007; 2008). These channel subunits mediate the neuronal M-currents (Wang *et al.*, 1998; Shapiro *et al.*, 2000) and loss-of-function mutations cause a specific form of neonatal epilepsy [benign familial neonatal convulsion (BFNC)] (Biervert *et al.*, 1998; Charlier *et al.*, 1998; Schroeder *et al.*, 1998; Singh *et al.*, 1998). In analogy, if KCNQ openers restored channel function in DFNA2-relevant KCNQ4 mutants, these agonists may have therapeutic potential to protect against OHC degeneration and hearing loss.

Here we investigated the effects of synthetic KCNQ openers on native $I_{K,n}$ and heterologously expressed KCNQ4 and found enhancement of both currents. Channel function of the DFNA2-causing mutation that is located close to the sixth transmembrane segment, G321S, was partially restored by a combination of zinc-pyrithione and retigabine (ZnP/Ret). Moreover these KCNQ openers rescued KCNQ4 from dominant-negative inhibition by DFNA2-mutations. Channel activity was recovered substantially to wild-type (wt) levels by activation of residual homomeric wt channels. Taken together, our data suggest that channel activators have the potential to stabilize the essential KCNQ4-mediated conductance in OHCs in DFNA2.

Methods

Cell culture, transfection and mutagenesis

CHO cells were maintained in MEM Alpha Medium (Invitrogen GmbH, Darmstadt, Germany) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (both Invitrogen). For experiments cells were plated on glass cover slips (Carl Roth, Karlsruhe, Germany) and transfected with jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). Experiments on transfected cells were performed 24 h to 48 h post-transfection. The expression vectors used were pEGFP-C1-KCNQ4 and pRFP-C1-KCNQ4 (NM_004700.2), and pBK-CMV-KCNQ3 (NM_004519.2). The expressed channels are referred to as KCNQ4 wt and KCNQ3 wt (Kv7.4 and Kv7.3, respectively) according to the *Guide to Receptors and Channels* (Alexander *et al.*, 2011). Based on pEGFP-C1-KCNQ4 six DFNA2-relevant point mutations were generated by site-directed mutagenesis using the QuickChangeII XL Site-Directed mutagenesis kit (Stratagene, Santa Clara, CA, USA), and mutagenesis was verified by sequencing (SEQLAB-Sequence Laboratories Göttingen GmbH, Göttingen, Germany). KCNQ4 wt and mutations were co-expressed to examine the action of channel openers in a setting corresponding to heterozygote DNFA2 carriers. In these experiments wt and mutant plasmids were transfected at various ratios as indicated while keeping the total amount of DNA constant. To verify co-expression of wt and mutated protein, wt KCNQ4-mRFP was co-expressed with the GFP-fused mutant. The expected fraction of homomeric wt channels was calculated for each proportion assuming random assembly of channel tetramers.

Tandem concatamers consisting of KCNQ4 wt subunits or of one wt and one mutant subunit (W276S, G285S or G321S) were generated by fusing the subunits N-terminus to C-terminus. Molecular cloning of the concatamers was based on KCNQ4 in the pBK-CMV-rev vector. An *Age*I cutting site was introduced 3′ of the KCNQ4 insert and the stop codon was removed by mutagenesis. The resulting construct was digested with *Age*I and *Nhe*I, yielding a 2367 bp fragment encoding KCNQ4 that was subsequently subcloned into the KCNQ4-pEGFP-C1 plasmid to create the wt concatamer plasmid. For concatamers containing one of the mutations (W276S, G285S orG321S) we used the same cloning strategy with source vectors that carried one of the mutations. Mutagenesis and cloning were verified by sequencing.

Organ of Corti preparation

All experiments utilizing animal tissue were performed according to German law and to institutional guidelines at the Philipps-University Marburg. Organs of Corti of Wistar rats (12–20 days after birth) were acutely isolated as previ-

ously reported (Oliver *et al.*, 2000). Briefly, animals were anaesthetized using Isofluran (Baxter Deutschland GmbH, Unterschleißheim, Germany) and killed by decapitation. The cochlea was dissected, separated from modiolus, stria vascularis and and tectorial membrane and then was continuously perfused with standard extracellular solution containing (mM) 144 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 10 HEPES and 5.6 D-glucose, pH 7.4 (with NaOH), 305–310 mOsm·kg^{-1}. Experiments were performed within 3 h after the preparation at room temperature (22–25°C).

Electrophysiological recordings

Patch clamp recordings were performed in the whole cell configuration with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA). Data were sampled with an ITC-18 interface (HEKA Elektronik, Lambrecht, Germany) controlled by PatchMaster software (HEKA) via a Macintosh PowerPC (Apple Inc, Cupertino, CA, USA). Some experiments were performed using an EPC10-USB patch clamp amplifier (HEKA). Currents were low-pass filtered at 2 kHz and sampled at 5 kHz, and whole cell capacitance was compensated in whole-cell configuration. Patch pipettes were pulled from borosilicate glass (Sutter Instrument Company, Novato, CA, USA) to an open pipette resistance of 1.5–3 MΩ after backfilling with intracellular solution containing (mM) 135 KCl, 2.4 $CaCl_2$ (0.1 μM free Ca^{2+}), 3.5 $MgCl_2$, 5 HEPES, 5 EGTA, 2.5 Na_2ATP, pH 7.3 (with KOH), 290–295 mOsm·kg^{-1}. Series resistance (R_s) typically was below 8 MΩ and R_s compensation (80–90%) was applied throughout the recordings. Experiments were performed on OHCs of the third row of apical cochlear turns, and recordings were only included in analysis if the membrane potential was more hyperpolarized than –69 mV. Access to the basolateral membrane of OHCs was achieved by gently removing surrounding tissue with a suction pipette. In CHO cells robust expression of wt and/or mutant KCNQ4 was verified by GFP and/or RFP fluorescence, and only one CHO cell was investigated per cover slip. All experiments were performed at room temperature (22–25°C). Membrane potentials shown are not corrected for liquid junction potentials (approximately 4 mV).

Chemicals

Retigabine {[*N*-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester]}, flupirtine {*N*-[2-amino-6-((4-fluorophenyl)methyl)amino)-3-pyridinyl]carbamic acid}, BMS-204352 {maxipost; [(*S*)-3-(5-chloro-2-methoxyphenyl)-3-fluoro-6-(trifluoromethyl)-1,3-dihydro-2*H*indole-2-one]} (all kindly provided by Neurosearch, Ballerup, Denmark), ZnP (Sigma-Aldrich, Munich, Germany) and XE991 [10,10-*bis* (4-pyridinylmethyl)-9(10*H*)-anthracenone dihydrochloride] (Tocris Bioscience, Bristol, UK) were applied from the extracellular side via a glass capillary at the concentrations indicated in results.

Data analysis and statistics

Data were analysed with PatchMaster (HEKA) and IGOR Pro (Wavemetrics, Lake Oswego, OR, USA). Steady-state KCNQ4 currents were quantified as the outward current 1200 ms after a voltage pulse (as indicated) from the holding potential (–60 mV). Data are presented normalized to membrane capacitance as the measure for membrane area, yielding current densities (pA/pF). Membrane capacitance was determined with the C_M compensation circuitry of the patch clamp amplifier. Drug-induced changes in current amplitudes were calculated by normalizing to the current amplitude at the beginning of each experiment prior to application of the respective channel opener ($I/I_{control}$). Concentration-response relations were derived from normalized KCNQ4 steady-state currents at 0 mV at various drug concentrations. Responses were fitted with a Hill equation with $I/I_b = I_b + (I_{max} - I_b)/(1 + (EC_{50}/[S])^{n_H})$, where I is the normalized current, I_b and I_{max} denote the minimal and maximal currents at low and high drug concentrations, EC_{50} is the concentration at the half maximal effect, [*S*] is the drug concentration and n_H is the Hill coefficient. Voltage-dependence of channel activation was derived from tail current amplitudes using the voltage protocols given in the figures. Current-voltage curves were derived from tail currents and were fitted with a two-state Boltzmann function with $I = I_{min} + (I_{max}\text{-}I_{min})/\{1 + \exp[(V\text{-}V_h)/s]\}$, where I is current, *V* is the membrane voltage, V_h is the voltage at half maximal activation, and s describes the steepness of the curve. Results are shown as conductance-voltage curves, obtained by normalizing to (I_{max}-I_{min}), obtained from fits to the data of the individual experiments. To account for biophysical differences between heterologous KCNQ4 and native $I_{K,n}$ (i.e. hyperpolarized voltages of activation for $I_{K,n}$), different voltage protocols were used for OHCs and CHO cells (Kubisch *et al.*, 1999; Marcotti and Kros, 1999; Oliver *et al.*, 2003). Thus, $I_{K,n}$ current amplitudes were measured as the tail currents at –120 mV following pre-pulses to voltages between –160 and +40 mV (increments of 10 mV), and heterologous KCNQ4 tail currents were recorded at 0 mV from pre-pulses between –100 and +50 mV.

Statistical analysis was performed with (paired) *t*-test and Dunnett test for multi-comparison, and significance was assigned at $P \leq 0.05$ (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Data are presented as mean ± SEM, with *n* representing the number of independent experiments (individual cells).

Results

Potentiation of heterologous KCNQ4 by channel openers

The effects of channel openers on heterologously expressed KCNQ channels have been investigated previously. However, the efficacy of current potentiation differs between channel openers and depends on the KCNQ isoform (Schroder *et al.*, 2001; Tatulian *et al.*, 2001; Xiong *et al.*, 2007). To identify the most potent activator of KCNQ4, we systematically compared the potencies of openers that have been described previously, namely flupirtine, retigabine, BMS-204352 and ZnP.

At concentrations of 10 μM, all openers except flupirtine strongly increased steady-state KCNQ4 currents (at 0 mV) 4- to 12-fold (Figure 1A,B; Supporting Information Figure S1). In these experiments the rank order of current potentiation was: flupirtine < retigabine < BMS-204352 < ZnP < ZnP/Ret. Despite different degrees of current augmentation, heterologous KCNQ4 showed comparable sensitivities for flupirtine

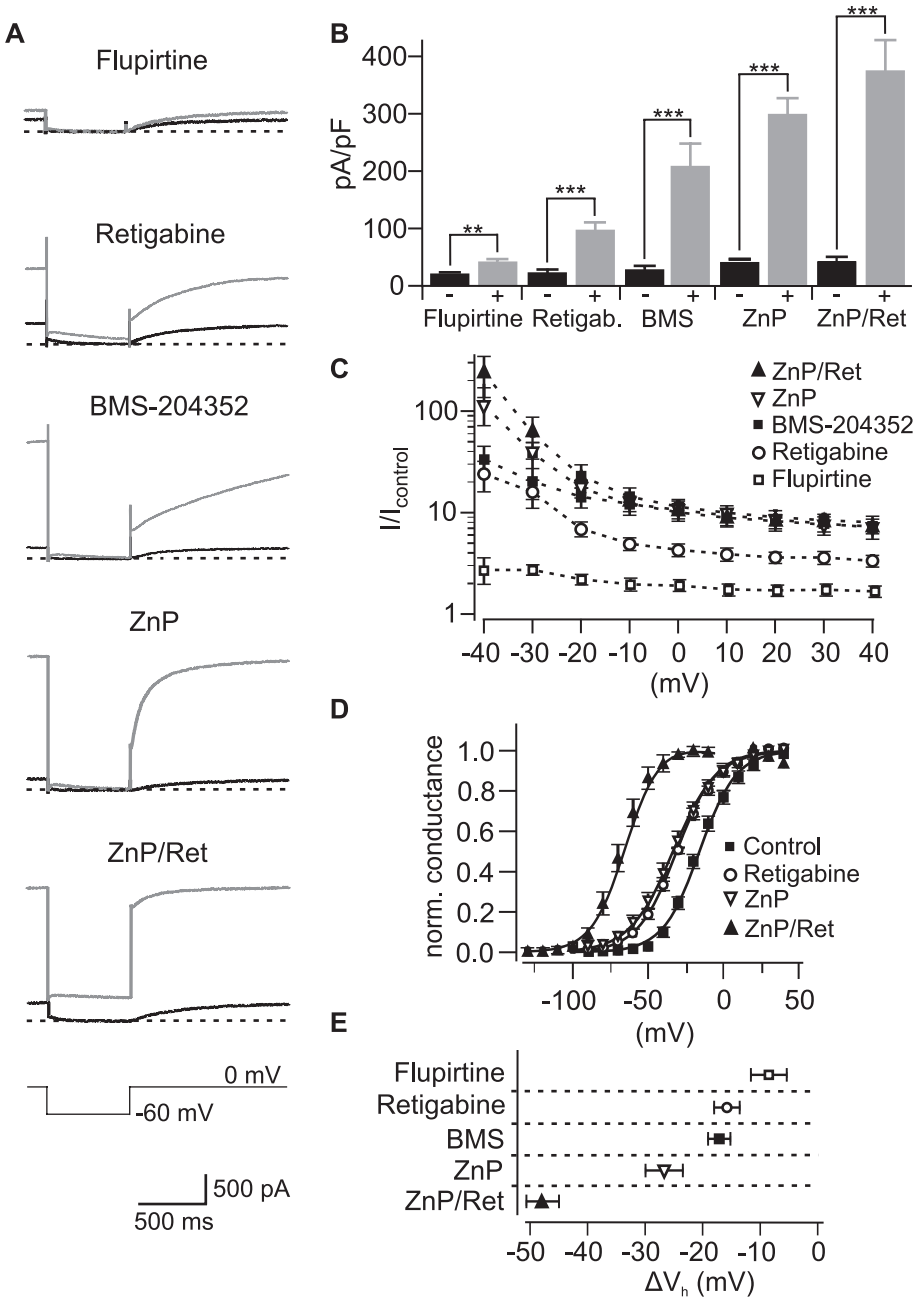

Figure 1

Potentiation of heterologous KCNQ4 currents by channel openers. (A) Representative recordings from CHO cells transiently transfected with wt KCNQ4 under control conditions and after application of flupirtine, retigabine, BMS-204352, zinc pyrithione (ZnP) or a combination of zinc pyrithione and retigabine (ZnP/Ret) (10 μM each, voltage command as indicated). Dashed lines indicate zero currents, and scale bar applies to all recordings. (B) Summary of steady-state currents at 0 mV normalized to plasma membrane area (membrane capacitance), obtained from recordings as presented in (A). Concentration of all openers was 10 μM. (C) KCNQ4 current potentiation by KCNQ openers was voltage-dependent. Steady-state currents at different holding potentials were normalized to currents obtained before the application of KCNQ channel openers. KCNQ4 current increase was more pronounced at hyperpolarized voltages, and saturated at approximately 10 mV for all openers. (D) Voltage- dependence of KCNQ4 currents with and without KCNQ channel openers. Conductance-voltage relations were derived from tail current recordings as shown in Supporting Information Figure S2 and normalized to maximum currents derived from Boltzmann fits to the data. Continuous lines indicate a Boltzmann fit to the averaged data. Fits to current-voltage relation of individual measurements yielded V_h –21.8 ± 2.0 mV for controls, –31.7 ± 2.7 mV with retigabine, –33.4 ± 2.9 mV with ZnP, and –64.0 ± 2.5 mV with ZnP/Ret (all openers at 10 μM). Current-voltage relationships for BMS-204352 and flupirtine are shown in Supporting Information Figure S1. (E) Summary of the shifts of the half-maximal voltage of activation (ΔV_h) induced by application of KCNQ openers, calculated from recordings as in (D). Flupirtine, retigabine and BMS-204352 exhibited modest shifts (approximately –10 to –20 mV), ZnP (–26.7 ± 3.3 mV) and ZnP/Ret (–47.8 ± 2.9 mV) extremely shifted voltages of activation to hyperpolarized potentials. (Numbers of cells for all panels were 11 flupirtine, 11 retigabine, 12 BMS-204352, 11 ZnP and 8 ZnP/Ret)

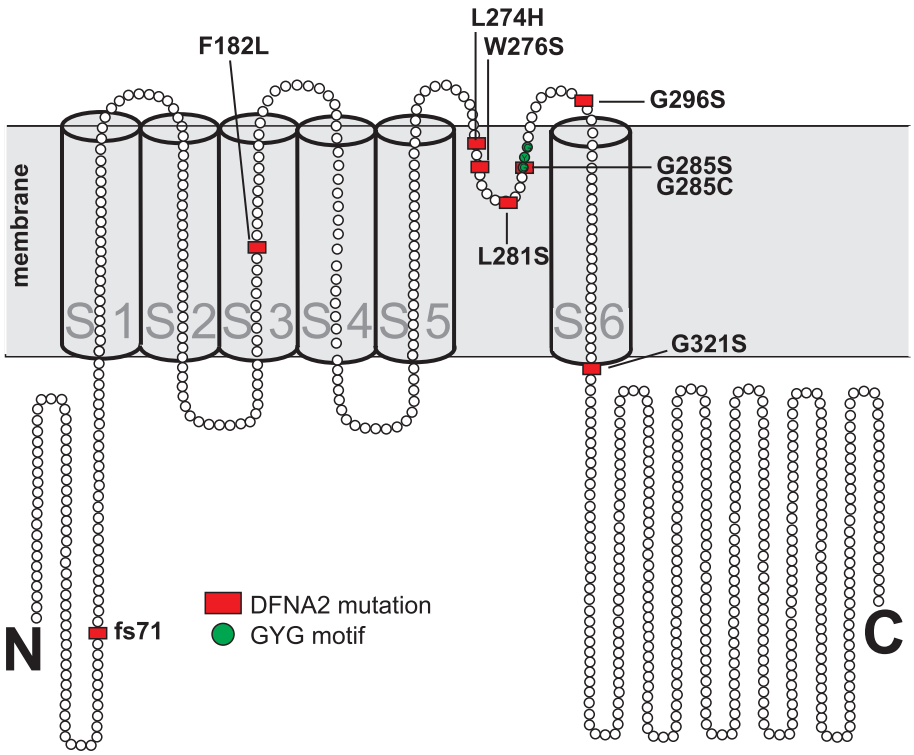

Figure 2

Topology of DFNA2-relevant mutations in the KCNQ4 channel protein. Point mutations described as causative for DFNA2 are presented in red and the K^+ channel pore motif glycine–tyrosine–glycine (GYG) is depicted in green.

(EC_{50} = 2.9 μM), retigabine (EC_{50} = 3.7 μM), BMS-204352 (EC_{50} = 3.5 μM), and for ZnP (EC_{50} = 6.8 μM) (Supporting Information Figure S1). Thus, 10 μM produced saturating or nearly saturating effects for all openers and this concentration was therefore used in the further studies. Figure 1C shows that current potentiation was stronger at hyperpolarized voltages, with highest current gain observed with ZnP/Ret. The voltage-dependence of current enhancement resulted from a shift of the voltage-dependence of activation to hyperpolarized potentials for all openers, as reported previously (Figure 1D) (Schroder *et al.*, 2001; Tatulian *et al.*, 2001; Xiong *et al.*, 2007). Taken together these results show that combined application of ZnP/Ret produced the strongest amplification of KCNQ4 currents. By enhancing steady-state conductance at 0 mV by a factor of approximately 11 (Figure 1B,C), and by shifting the voltage of half-maximal activation from –21.8 ± 2.0 mV to –64.0 ± 2.5 mV (Figure 1D,E), ZnP/Ret-mediated current potentiation amounted to more than 200-fold at –40 mV (Figure 1C). These results are consistent with a previous report, showing strong and additive enhancement of KCNQ currents by ZnP and retigabine via distinct molecular interaction sites in the channel protein (Xiong *et al.*, 2008). In fact, the shift in voltage sensitivity by ZnP/Ret produced currents that closely resembled the native $I_{K,n}$ currents from OHCs, which are characterized by their unique negative activation range (Supporting Information Figure S2) (Mammano and Ashmore, 1996; Marcotti and Kros, 1999; Oliver *et al.*, 2003).

KCNQ4 mutations in the pore region cannot be restored by ZnP/Ret

We next explored whether channel openers might be able to rescue channel function of the various KCNQ4 loss-of-function mutants that cause DFNA2 (Figure 2). To this end, we used the combination of ZnP and retigabine that exhibited the highest efficacy in potentiation of the wt channel.

Figure 3 summarizes the effects of ZnP/Ret (10 μM both) on KCNQ4 channels carrying mutations within or close to the pore. None of the mutant channels produced detectable currents when expressed in CHO cells, consistent with previous reports (Kubisch *et al.*, 1999; Holt *et al.*, 2007; Baek *et al.*, 2011; Kim *et al.*, 2011). Application of the channel opener combination did not uncover detectable currents for W276S, L281S and G285S (Figure 3A–E, G). In cells transfected with the G296S mutant subunits ZnP/Ret produced small outward currents (approximately 1 pA/pF at 0 mV) that were clearly identified as KCNQ-mediated currents, since they were abolished by the KCNQ antagonist XE991 (10 μM; Figure 3D,G). However, equivalent currents were also found in non-transfected cells (Figure 3E, G) and thus most likely arise from a minuscule expression of endogenous channels that is uncovered by the KCNQ openers. Apparently, expression of the pore mutants (W276S, L281S and G285S) suppressed endogenous currents by the dominant-negative effect described previously (Kubisch *et al.*, 1999; Holt *et al.*, 2007). In contrast to these mutations, channel dysfunction of G296S

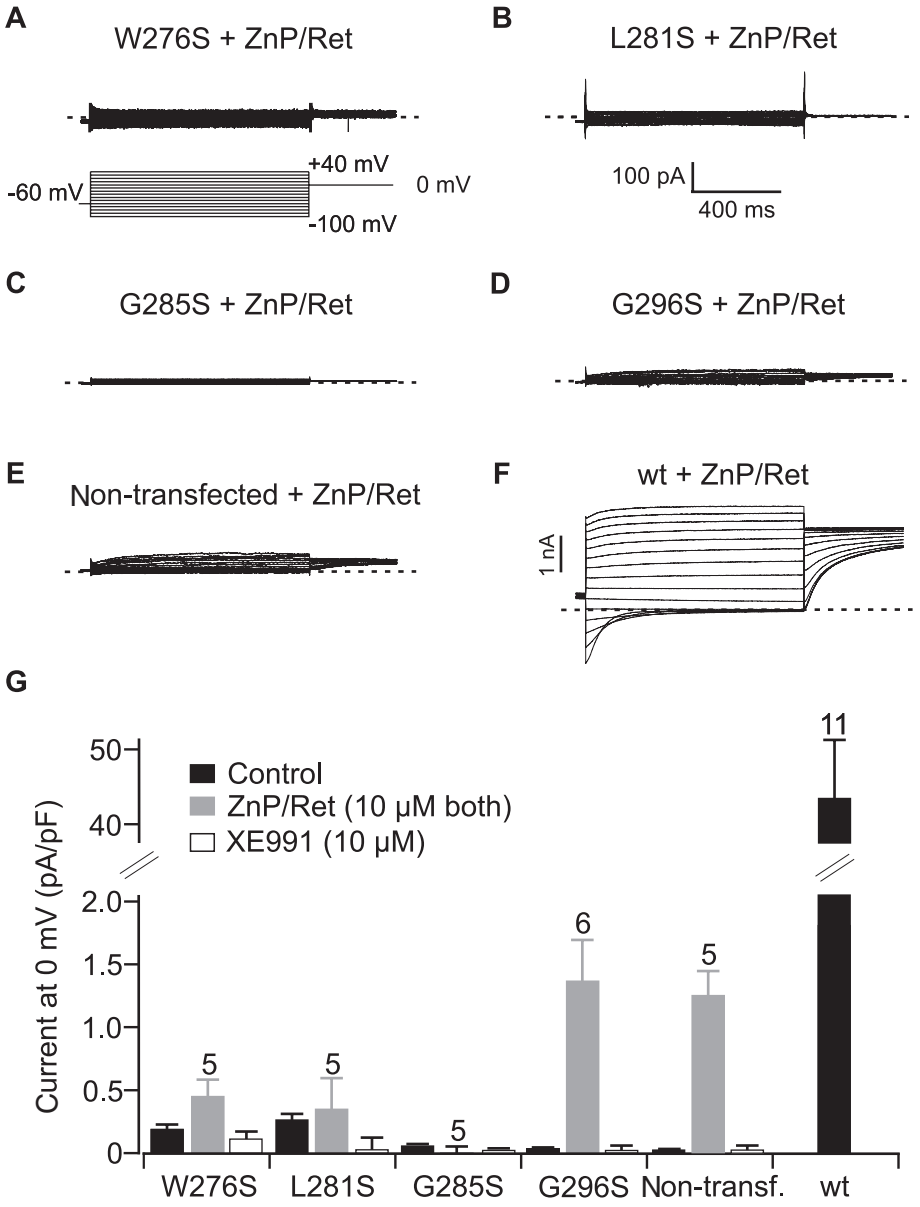

Figure 3

KCNQ4 mutations in the pore region cannot be rescued by ZnP/Ret. (A–E) Representative currents measured from cells expressing the DFNA2 mutations W276S (A), L281S (B), G285S (C) and G296S (D), and from non-transfected CHO cells (E) in the presence of ZnP/Ret (10 µM each). Scale bars apply to (A–E). Voltage protocol as indicated in (A). (F) wt KCNQ4 currents in the presence of ZnP/Ret. Voltage protocol as indicated in (A). Note the different current scaling. (G) Summarized steady-state current densities at 0 mV obtained from experiments as in (A–F), under control conditions, with ZnP/Ret and during subsequent application of the KCNQ antagonist XE991. None of the mutations produced substantial currents either in the absence or presence of ZnP/Ret. Application of the openers revealed small currents in non-transfected cells and in cells transfected with G296S that most likely arose from endogenous expression of KCNQ-like channels in CHO cells (see text). Note the compressed axis scaling for wt currents.

appeared not to interfere with the function of the endogenous channels, in line with a recent report of defective membrane targeting of this mutation (Mencia *et al.*, 2008). It should be noted that the currents recorded from non-transfected CHO cells or cells transfected with G296S were extremely small when compared with wt KCNQ4 currents (Figure 3D–G).

Taken together, these experiments show that homomeric KCNQ4 channels composed of subunits carrying a mutation in or close to the pore region cannot be rescued by application of the most potent KCNQ openers tested in this study, ZnP/Ret.

Partial restoration of channel function of a mutation in the proximal C-terminus

Two DFNA2 mutations have been identified within or close to transmembrane regions distinct from the pore structure (F182L and G321S, respectively; see Figure 2) (Coucke *et al.*,

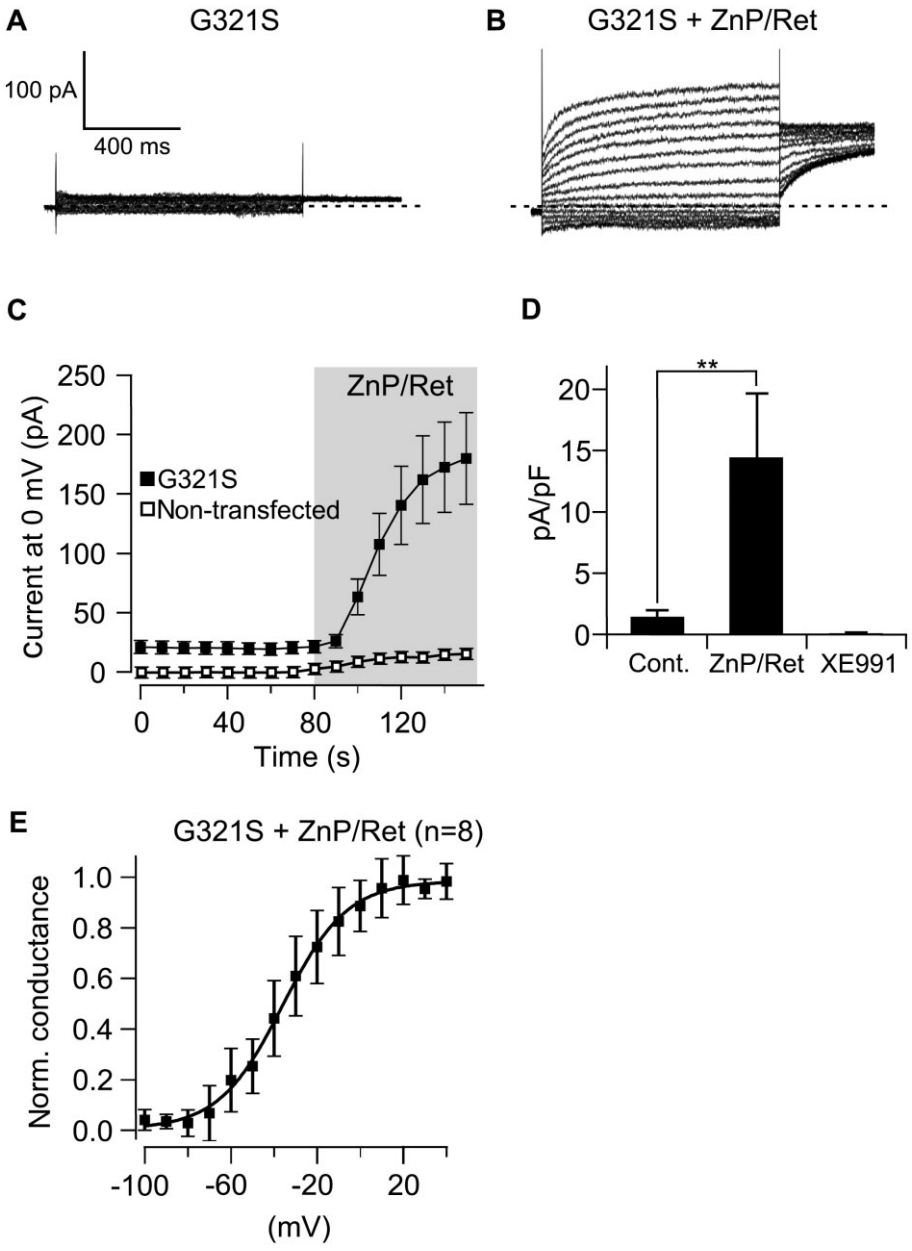

Figure 4

Partial rescue of a KCNQ4 mutation in the proximal C-terminus by ZnP/Ret. (A, B) Representative recordings from the same CHO cell transfected with KCNQ4-G321S under control conditions (A) and in the presence of ZnP/Ret (10 μM both) (B). Voltage protocol as in Figure 3. (C) Time course of current potentiation by application of ZnP/Ret in cells transfected with KCNQ4-G321S (n = 14) and in non-transfected CHO cells (n = 5). Steady-state currents were measured at 0 mV. (D) Average current densities obtained from the experiments shown in (C). Potentiated currents were fully blocked by XE991 (10 μM, n = 14). (E) Voltage-dependence of G321S-mediated currents in the presence of ZnP/Ret. Conductance-voltage relations were derived from tail current recordings as shown in (B) and derived from Boltzmann fits to the data (see Figure 1 and Supporting Information Figure S2). Continuous line indicates a Boltzmann fit to the averaged data. Fits to current-voltage relation of individual measurements yielded V_h = –36.0 ± 4.3 mV and k = –14.8 ± 1.2 mV (n = 8). Currents without channel openers were too small to determine voltage-dependence.

1999; Su *et al.*, 2007). We reasoned that these mutant channels may be more promising targets for functional restoration by channel openers.

The G312S mutation is located at the interface of S6 and the cytoplasmic C-terminus, which we refer to as the proximal C-terminus (see Figure 2) (Coucke *et al.*, 1999). Consistent with a recent report (Kim *et al.*, 2011), homomeric KCNQ4-G321S did not produce clearly detectable voltage-dependent currents under control conditions (Figure 4A,C). However, a standing outward current at 0 mV was apparent that was significantly larger than in non-transfected cells (1.5 ± 0.6 pA/pF, n = 14; Figure 4A,C and D). Application of

ZnP/Ret (10 μM both) elicited substantial voltage-activated outward currents of 14.4 ± 5.1 pA/pF (at 0 mV; n = 14; Figure 4B–E). These currents were significantly larger than the endogenous KCNQ-like currents induced by ZnP/Ret in non-transfected CHO cells (Figure 4C) and were blocked by XE991 (Figure 4D), unequivocally identifying them as carried by KCNQ4-G321S. These G321S-mediated currents activated at somewhat more hyperpolarized voltages than KCNQ4 wt currents under control conditions (V_h = –36.0 ± 4.3 mV, slope = –14.8 ± 1.2 mV, n = 8, $P \leq 0.01$; Figure 4E). ZnP/Ret potentiated G321S-mediated currents 15- to 20-fold, yielding a conductance that was about 40% of wt currents in the absence of channel openers (also see Figure 3G). Yet, the ZnP/Ret-augmented G321S conductance was sufficient to hyperpolarize CHO cells from a membrane potential of –25.4 ± 0.7 mV to –50.3 ± 4.0 mV (n = 8; $P \leq 0.001$), which is indistinguishable from the membrane potential of cells expressing wt KCNQ4 (–48.5 ± 2.3 mV, n = 12; P = 0.50). Taken together, the current amplitudes, voltage-dependence, and subsequent hyperpolarization of CHO cells demonstrate the partial restoration of G321S channel function by ZnP/Ret.

We also examined F182L, a DFNA2 mutation located in the third transmembrane domain of KCNQ4 (Su *et al.*, 2007). In line with recent findings (Kim *et al.*, 2011), current amplitudes and voltage-dependence of this mutation were indistinguishable from wt KCNQ4. Similar to wt, application of ZnP/Ret (10 μM) shifted the voltage range of activation to hyperpolarized voltages and potentiated F182L currents (Supporting Information Figure S3). Apparently, in the case of the F182L mutant impaired channel conductance is not the cause for deafness. We therefore did not investigate this mutation further.

ZnP/Ret rescues KCNQ4 from dominant-negative suppression by DFNA2 mutations

DFNA2 is a dominantly inherited disease, i.e. a single mutant KCNQ4 allele is sufficient to severely affect function of the channel. On the molecular level, the dominant nature of these loss-of-function mutations can be explained by the tetrameric structure of the channels. Thus, channel function may be disrupted by a single mutant subunit per tetrameric channel complex. If wt and mutant subunits are synthesized at the same level and assemble in a stochastic manner, this leaves only one functional (i.e. homomeric wt) channel out of 16 (6.25%), yielding a strong dominant-negative effect of the mutation. Indeed, stoichimetric co-expression of KCNQ4 with DFNA2 mutants resulted in strong suppression of channel activity (Kubisch *et al.*, 1999; Holt *et al.*, 2007), supporting this model. Moreover, in a mouse model heterozygous for the DFNA2 mutation (G285S) the native KCNQ4 equivalent in OHCs, $I_{K,n}$, was strongly diminished in a manner compatible with this dominant-negative action (Kharkovets *et al.*, 2006). We therefore tested for effects of channel openers on KCNQ4 currents suppressed by dominant-negative inhibition. To this end, wt KCNQ4 was co-expressed with subunits carrying mutations in the selectivity filter motif, GYG (G285S), near the channel pore (W276S), or in the proximal C-terminus (G321S) (Figure 2).

As reported previously (Kubisch *et al.*, 1999; Holt *et al.*, 2007; Baek *et al.*, 2011; Kim *et al.*, 2011), co-expression of mutant subunits with wt KCNQ4 strongly reduced current amplitudes compared with wt currents alone (Figure 5A–C). Supporting Information Figure S4 confirms that this current reduction was not simply due to the lower amount of wt DNA used for transfection in these experiments, as wt current amplitudes were unaffected by decreasing the amount of DNA amount to the same level used in co-transfection experiments. Thus, the current reduction resulted from a dominant-negative interaction of the mutant channel subunits with the wt subunits. Co-expression of wt KCNQ4 with the pore mutants (1:1) reduced current densities at 0 mV to 2.4 ± 0.3% (wt/G285S; 1.0 ± 0.1 pA/pF, n = 6) and 5.7 ± 1.7% (wt/W276S; 2.3 ± 0.5 pA/pF, n = 8) of wt currents (obtained from the same batches of cells, Supporting Information Figure S4) (Figure 5A–C, lower panel). This is roughly compatible with the 6% (1/16) of functional homomeric wt channels expected to result from the assembly of co-synthesized wt and mutant subunits, assuming that co-assembly into tetrameric channels is stochastic and that a single mutated subunit is sufficient to abolish ion conductance of a channel (Kubisch *et al.*, 1999; Holt *et al.*, 2007). As shown in Figure 5A and B, application of ZnP/Ret (10 μM each) strongly potentiated these residual currents to 18.9 ± 6.8 pA/pF (wt/G285S; n = 6) and 28.5 ± 4.7 pA/pF (wt/W276S; n = 8). These potentiated current amplitudes were statistically indistinguishable from wt control currents in the absence of channel openers (43.5 ± 7.8 pA/pF, n = 5, Supporting Information Figure S4 for the same batch of cells), indicating full compensation for dominant-negative suppression of channel function by ZnP/Ret. Similar to the pore mutants, co-expression of the G321S mutant suppressed wt KCNQ4 currents (to 5.3 ± 2.1 pA/pF at 0 mV, 12.2 ± 2.7% of wt; n = 7; Figure 5C, top and lower panel). Interestingly, the remaining current was larger than the 6.3% of wt currents expected from disrupted channel function by a single mutant subunit. ZnP/Ret robustly increased these currents to 75.2 ± 16.5 pA/pF (n = 7; Figure 5, C lower panel), which was even larger than wt control currents in the absence of channel openers. In addition to the strong enhancement of maximum current amplitude, restored currents from cells co-expressing mutant and wt channels exhibited negatively shifted activation ranges, similar to ZnP/Ret-potentiated wt currents (Figure 5D,E).

How do openers rescue current amplitudes from dominant-negative suppression?

The channel population under conditions of dominant-negative suppression by DFNA2 mutants consists of homomeric mutant channels, homomeric wt channels and heteromeric channels containing both wt and mutant subunits. As shown above, ZnP/Ret strongly activates the homomeric wt channels and potentiates homomeric G321S channels but has no effect on homomeric pore mutant channels (G285S and W276S; Figures 2 and 3). However, at this point it is not clear if the current potentiation, in the dominant-negative situation may also comprise the restoration of activity of otherwise dysfunctional heteromeric wt/mutant channels.

To address this issue we first consider the degree of current enhancement. ZnP/Ret increased currents from cells co-expressing wt and pore mutant channels by factors of 17.3 ± 4.5 (n = 6; wt/G285S) and 15.1 ± 2.8 (n = 8;

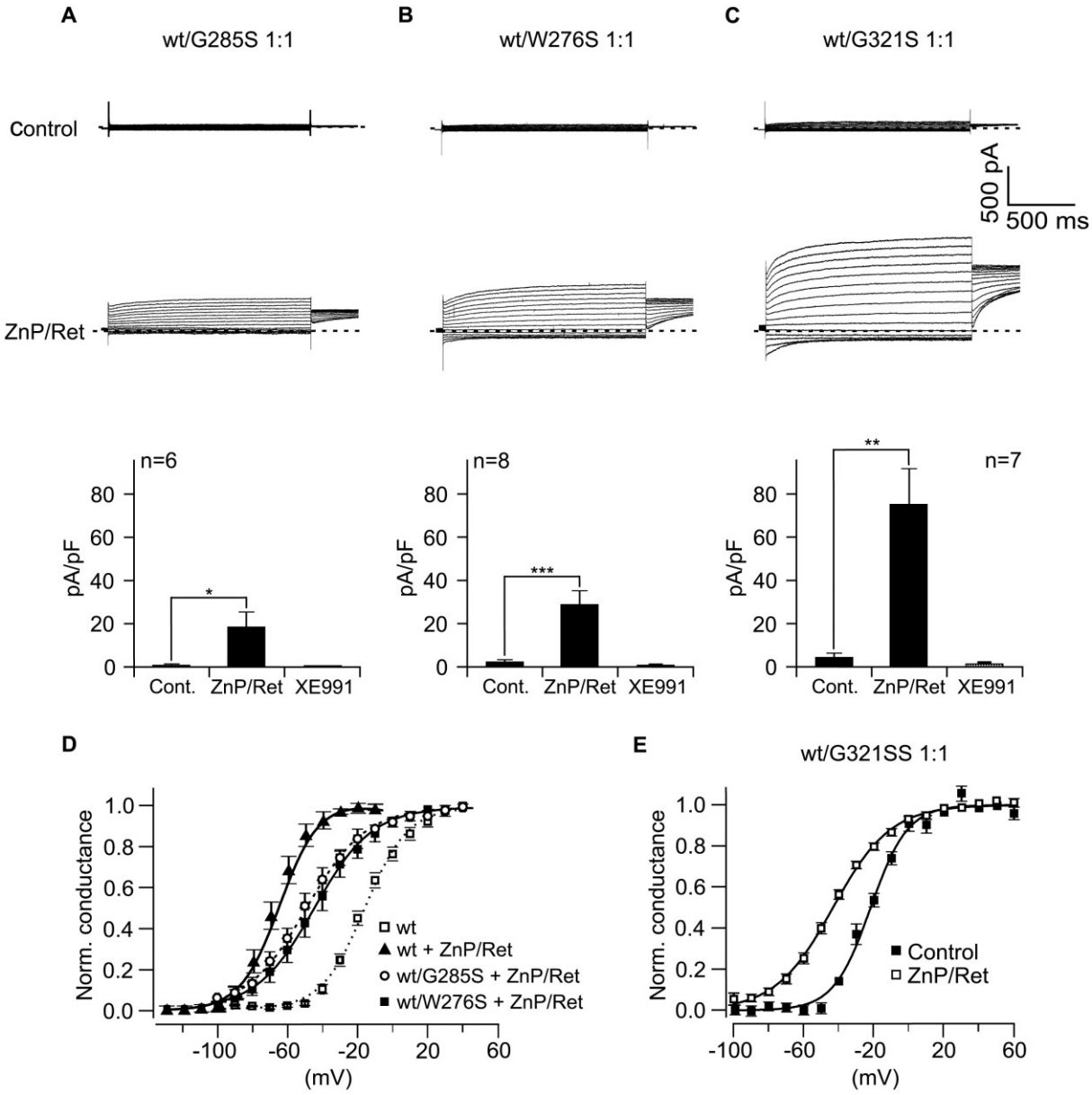

Figure 5

ZnP/Ret rescues KCNQ4 currents from dominant-negative inhibition. (A–C) Representative recordings from cells co-transfected with wt KCNQ4 and the DFNA2 mutants indicated. Current amplitudes were strongly suppressed by co-expression of the mutant channel subunits (upper panels). Application of ZnP/Ret (10 μM both) strongly increased currents for all three mutants (middle panel; same cells). Voltage protocol as presented in Figure 3. Lower panels summarize current densities (at 0 mV) before and during application of ZnP/Ret and after application of XE991 (10 μM). (D) Voltage-dependence of ZnP/Ret-induced currents in cells co-transfected with KCNQ4 wt and G285S or W276S, obtained from experiments as in (A and B): V_h = –49.1 ± 4.0 mV, n = 6 (wt/G285S); V_h = –46.8 ± 3.4 mV, n = 8 (wt/W276S). Under control conditions, currents were too small to reliably determine current-voltage relations. Conductance-voltage curves from wt KCNQ4 without and with ZnP/Ret (V_h = –64.0 ± 2.5 mV) are shown for comparison. (E) Conductance-voltage relations were determined from cells co-expressing wt KCNQ4 and G321S as in (C) before (V_h = –22.2 ± 1.2 mV) and during application of ZnP/Ret (V_h = –43.5 ± 1.2, n = 7).

wt/W276S). This is similar to the 11-fold increase in current densities obtained with wt channels (11.4 ± 2.0-fold; n = 11; see Figure 1). Therefore, the restored current may be explained solely by potentiation of the residual homomeric wt channels without the need to invoke a functional restoration of channels containing one or more mutant subunits.

However, since the above values of current enhancement may be spurious due to the minute residual current amplitudes in the absence of openers, we aimed at a more robust and quantitative examination of the degree of current enhancement. To this end, we titrated the relation between homomeric and heteromeric channel tetramers by

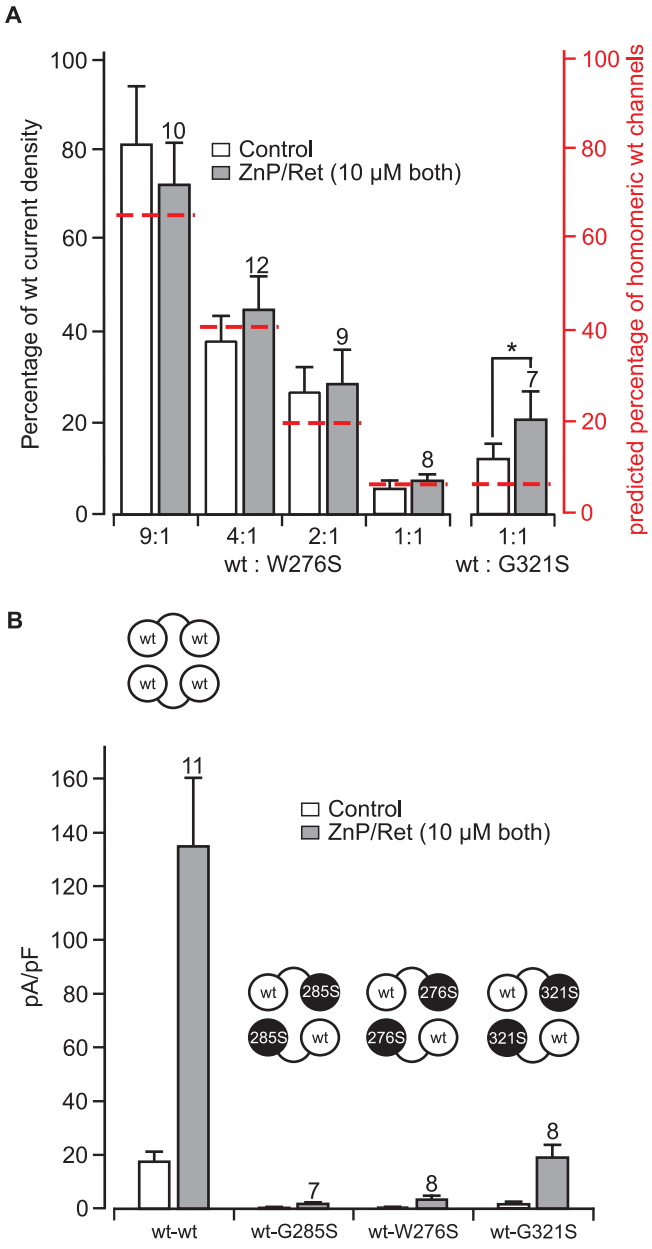

Figure 6

For pore mutants, extrication from dominant-negative inhibition is mediated by activation of residual wt channels. (A) Co-expression of KCNQ4 wt with mutant subunits reduced whole cell currents by dominant-negative inhibition. Currents were recorded from CHO cells co-transfected at the indicated ratios of wt to mutant plasmid. Current densities recorded under control conditions are presented normalized to wt control current densities recorded from control cells transfected with wt plasmid only. Currents measured with ZnP/Ret are normalized to wt current densities in the presence of the same openers. For each subunit co-expression ratio of wt and the pore mutant W276S, the level of control and potentiated currents matched the predicted fraction of residual homomeric wt channels (red dashed lines), indicating that only homomeric wt channels contributed to the currents under all conditions. In contrast, for co-expression of wt subunits with G321S (1:1), current levels normalized to pure wt currents exceeded the predicted fraction of homomeric wt channels (calculated fractions: 9:1 65.6%; 4:1 41.0%; 2:1 19.9%; 1:1 6.3%). (B) Current densities of KCNQ4 channels assembled from tandem concatamers in the absence and presence of KCNQ channel openers. Insets indicate the subunit stoichiometry of channels assembled from the respective concatameric constructs.

systematically varying the ratio of wt and W276S plasmids transfected into the cells.

As shown in Figure 6A, variation of the wt-to-mutant ratio from 9:1 to 1:1 resulted in an incremental suppression of whole-cell currents. For each plasmid ratio, the remaining current under control conditions (normalized to the current from cells expressing only wt homomers; Figure 6A, open columns) matched well with the predicted fraction of homomeric wt channels (Figure 6A, red axis), assuming random assembly of channel subunits and a linear relation between concentration of plasmid and synthesis of the respective subunit (9:1 81.4 ± 12.6%; 4:1 38.1 ± 5.4%; 2:1 26.9 ± 5.4%; 1:1 5.7 ± 1.7%). This finding strongly supports the previous notion that only homomeric wt channels are functional under these conditions, i.e. a single mutant subunit close to the pore region is sufficient to render the tetrameric channel non-functional (Kubisch *et al.*, 1999; Holt *et al.*, 2007). Similarly, the ZnP/Ret-potentiated current also agreed closely with the predicted fraction of homomeric wt channels for each subunit ratio (Figure 6A, grey columns) (9:1 72.1 ± 9.3%; 4:1 45.5 ± 6.6%; 2:1 29.2 ± 7.0; 1:1 7.6 ± 1.3).

These results suggest that in the dominant-negative situation both control and opener-enhanced currents are mediated exclusively by the residual homomeric wt channels, since the fraction of currents did not increase upon application of the channel opener. Consequently, all channels containing at least one pore mutant subunit are non-conducting and cannot be rescued by the channel openers. The rescue of dominant-negative suppression by pore mutants therefore occurs through exclusive activation of the small fraction of homomeric wt channels.

Notably, the proximal C-terminal mutant, G321S, showed a different behaviour. In cells co-expressing wt and G321S (1:1), currents in the presence and absence of ZnP/Ret were larger than the predicted fraction of homomeric wt channels (Figure 6A). This suggests that in the case of G321S, channels containing mutant subunits may be functional under control conditions and furthermore can be activated by ZnP/Ret. These findings are fully consistent with the current recovered in homomeric G321S channels (Figure 4) (control 1:1 12.2 ± 2.7%; ZnP/Ret 21.0 ± 3.2).

Finally, we directly tested the sensitivity of heteromeric wt : mutant channels to the channel openers. Homogeneous populations of heteromeric channels were achieved by generation of concatamer of a wt and a mutant subunit, which were expected to assemble into tetramers containing two wt and two mutant subunits (Figure 6B). Channels assembled from wt : wt concatamer exhibited currents indistinguishable from monomeric wt channels, and were potentiated by ZnP/Ret to the same degree (10.3 ± 2.4, n = 11; Figure 6B) as wt KCNQ4 assembled from monomeric subunits. Expression of wt:W276S or wt:G285S concatamer did not produce detectable currents. Moreover, application of ZnP/Ret did not produce currents beyond those seen in non-transfected cells, indicating that these channels containing pore mutant sub-

units cannot be activated by the channel agonists. In contrast, wt:G321S concatamer showed small currents under control conditions, and application of ZnP/Ret uncovered robust KCNQ currents. Thus heteromeric channels containing G321S subunits are not fully dysfunctional and can be restored, at least partially, by KCNQ channel openers (Figure 6B).

In conclusion, these data show that KCNQ channel openers can rescue the whole-cell KCNQ4 conductance from suppression by dominant-negative mutant subunits. For pore mutants, the recovery exclusively relies on the activation of residual homomeric wt channels. For the proximal C-terminal mutation, potentiation probably involves channels of all wt : mutant proportions and accordingly is stronger.

Chemical KCNQ openers potentiate native $I_{K,n}$ in OHCs

In DFNA2, OHC degeneration and deafness in heterozygots results from the substantial suppression of the native KCNQ4-mediated current, $I_{K,n}$ by a dominant-negative action of the mutant subunits (Kubisch *et al.*, 1999; Kharkovets *et al.*, 2000; 2006; Holt *et al.*, 2007). Our findings suggest that KCNQ channel openers, by removing this suppression, are a promising strategy for the stabilization of $I_{K,n}$ in DFNA2. Since native $I_{K,n}$ and recombinant KCNQ4 show marked biophysical differences (Mammano and Ashmore, 1996; Kubisch *et al.*, 1999; Marcotti and Kros, 1999; Kharkovets *et al.*, 2006) (see Supporting Information Figure S2), we next examined the effects of KCNQ openers on $I_{K,n}$.

Figure 7 summarizes the potentiation of native $I_{K,n}$ by retigabine (10 and 100 μM), BMS-204352, ZnP and ZnP/Ret (all applied at 10 μM). We quantified tail currents at –120 mV from voltage-steps ranging from –160 to –20 mV before and during application of KCNQ openers to measure pure $I_{K,n}$ currents not contaminated by other OHC currents that activate at more positive potentials (Figure 7A-D) (Mammano and Ashmore, 1996; Kubisch *et al.*, 1999; Marcotti and Kros, 1999; Oliver *et al.*, 2003), as demonstrated recently (Leitner *et al.*, 2011). Under control conditions $I_{K,n}$ currents were activated at around –110 mV with V_h of –75.7 ± 1.3 mV (n = 26; Figure 7A,E). Application of chemical openers robustly augmented current amplitudes and shifted voltage-dependence to hyperpolarized potentials (Figure 7C). Figure 7D shows relative current increase upon application of chemical KCNQ openers. Retigabine and ZnP/Ret (all 10 μM) robustly increased $I_{K,n}$ currents 1.4-fold (at –60 mV) to 2.2-fold (at –110 mV) with no relevant differences at physiological membrane potentials between the substances tested. Both substances quite specifically augmented $I_{K,n}$ without involvement of other OHC potassium currents, since XE991-insensitive currents were not affected by the openers, as we recently reported (Leitner *et al.*, 2011). Interestingly, BMS-204352 was barely effective, especially at membrane potentials around –70 mV, despite its high efficacy at potentiating KCNQ4-mediated currents in CHO cells. Since BMS-204352 failed to potentiate $I_{K,n}$ and thus is unlikely to be useful in the case of DFNA2, we did not further investigate the effects of this substance on native OHCs.

As for heterologous KCNQ4 channels, KCNQ openers shifted voltage-dependence of $I_{K,n}$ to hyperpolarized potentials (Figure 7E,F). These shifts were comparable for retigabine at 10 and 100 μM to V_h of –86.0 ± 3.3 mV (n = 9) and –92.1 ± 2.0 mV (n = 8), respectively, and for ZnP/Ret (both 10 μM) to –92.5 ± 3.29 mV (n = 11). In contrast, ZnP and BMS-204352 produced rather modest shifts of V_h to –79.7 ± 4.4 mV (n = 6) and –82.0 ± 3.5 mV (n = 8), respectively. These data show that 10 μM retigabine is a saturating concentration, yielding comparable sensitivities for this chemical opener for $I_{K,n}$ as for heterologous KCNQ4. Additionally, the recordings suggest equal sensitivity of $I_{K,n}$ for ZnP and retigabine, without additional current potentiation by the combination of the two substances (see also Figure 1) (Xiong *et al.*, 2008). This is also represented by the average shifts of V_h by the openers: Application of retigabine resulted in V_h shifts of –14.0 ± 1.7 mV (n = 8) and –15.4 ± 0.9 mV (n = 8) at 10 and 100 μM, respectively. Similarly, ZnP/Ret shifted V_h by –17.2 ± 1.9 mV (n = 11) (Figure 7F), which was not higher than the effect of retigabine alone. This is in strong contrast to heterologous KCNQ4, and suggests retigabine has equivalent effects on OHCs to ZnP/Ret. Thus, retigabine may be used to stabilize the KCNQ conductance in OHCs, similar to ZnP/Ret for heterologous KCNQ4.

Discussion

Here we showed for the first time that synthetic channel openers can functionally rescue KCNQ4-mediated currents from deafness-causing mutations. While homomeric pore mutant channels remained non-functional even in the combined presence of ZnP/Ret, these openers were able to activate channels carrying the DFNA2-related mutation, G321S, located in the proximal C-terminus. Moreover, ZnP/Ret rescued KCNQ4 currents from dominant-negative suppression by co-expressed mutant channel subunits irrespective of the specific mutant, that is, both with pore mutants and the mutant located in the proximal C-terminus. For the pore mutants the restored function resulted exclusively from potentiation of the small population of channels assembled from wt channel subunits. Current enhancement in the case of G321S also involved activation of channels containing mutant subunits.

Implications of the rescue of DFNA2 mutants

In DFNA2, patients suffer from slowly progressing hearing loss that amounts to severe to profound deafness (Kharkovets *et al.*, 2006; Smith and Hildebrand, 2008). As KCNQ4 mediates the predominant K^+ conductance of OHCs, $I_{K,n}$, DFNA2 most likely results from OHC dysfunction. Indeed, a mouse model for KCNQ4 shows progressive OHC degeneration and hearing loss (Kharkovets *et al.*, 2006). KCNQ4 is also expressed in inner hair cells, where it mediates an $I_{K,n}$-like conductance (Kharkovets *et al.*, 2000; Oliver *et al.*, 2003). Yet, this inner hair cell current seems to be less critical, since IHC function was not impaired in the KCNQ4 knock-out mouse model (Kharkovets *et al.*, 2006). Apart from sensory hair cells, KCNQ4 is also expressed in various neurons along the central auditory pathway (Kharkovets *et al.*, 2000; Beisel *et al.*, 2005); however, it is currently not known whether neuronal defects contribute to the pathology of DFNA2. Given the importance of KCNQ4 for the maintenance of hearing, the restoration of

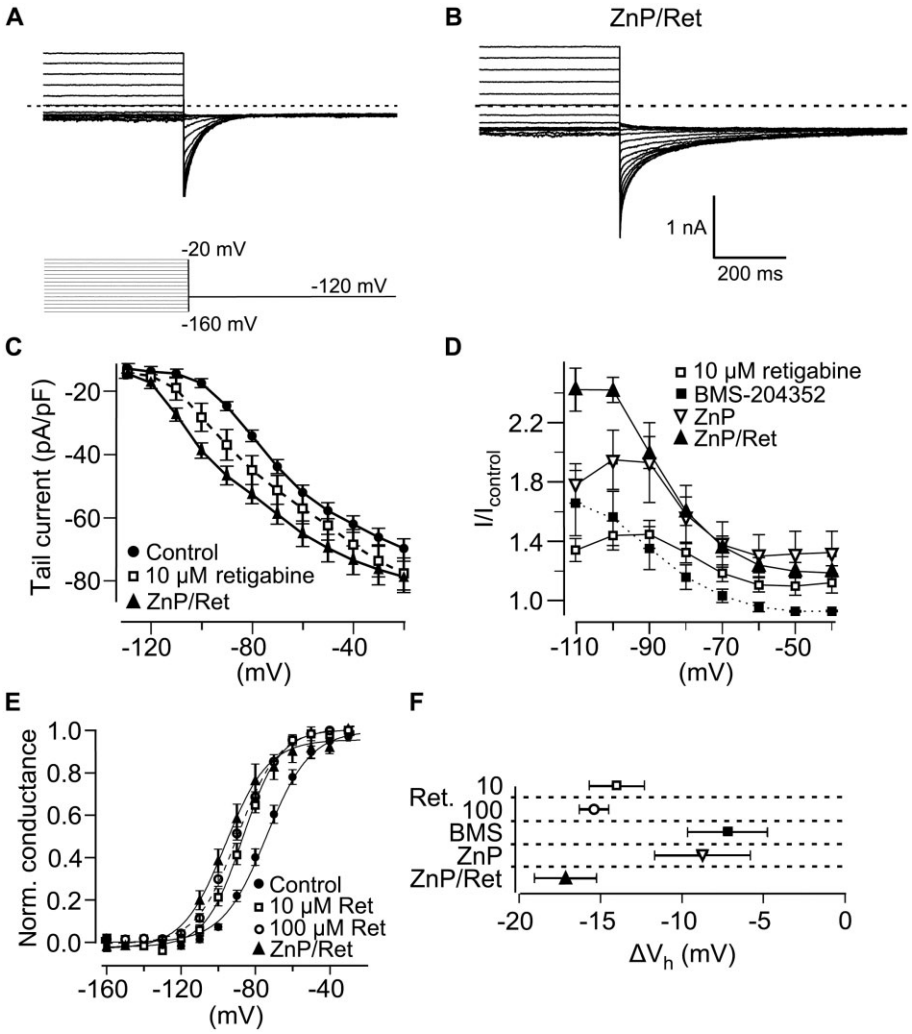

Figure 7

Native $I_{K,n}$ in OHCs is sensitive to chemical KCNQ openers. (A, B) Representative recordings of $I_{K,n}$ currents from an OHC before (A) and during application of ZnP/Ret (10 µM each) (B). Scale bars and voltage protocol apply to (A) and (B). (C) Summary of tail current densities recorded as in (A, B) in presence of retigabine and ZnP/Ret (all 10 µM). Retigabine and ZnP/Ret increased $I_{K,n}$ current amplitudes and shifted voltages of activation to hyperpolarized voltages. (D) Potentiation of $I_{K,n}$ by KCNQ openers (all 10 µM). The relative current potentiation at different holding potentials is presented, calculated from tail currents as in (A–C). (E) Hyperpolarizing shifts in the voltage-dependence of $I_{K,n}$ currents induced by chemical openers. Voltage-dependence was derived from tail currents as in (C), normalized to the maximal current and fitted to a Boltzmann function. Under control conditions V_h was –75.7 ± 1.3 mV (n = 26), and was shifted to –82.0 ± 3.5 mV by BMS-204352 (10 µM; n = 8), to –86.0 ± 3.3 mV by 10 µM retigabine (n = 9), to –92.1 ± 2.0 mV by 100 µM retigabine (n = 8), to –79.7 ± 4.4 mV by ZnP (10 µM; n = 6), and to –92.5 ± 3.29 mV by ZnP/Ret (10 µM; n = 11). (F) Averaged shifts of half-maximal voltages of activation (ΔV_h) upon application of the various KCNQ openers obtained from the data shown in (E).

channel function by chemical channel openers immediately suggests that these compounds may be of clinical use to stabilize the KCNQ conductance and prevent or retard OHC loss. It is worth noting that since DNFA2 is a dominant deafness, most patients present with a heterozygous genotype. Pathology therefore usually results from dominant-negative suppression of channel function by the mutant channel subunits transcribed from the mutated allele (Kubisch *et al.*, 1999; Holt *et al.*, 2007; Smith and Hildebrand, 2008). Here we demonstrated essentially full restoration of currents in this situation, at least in a heterologous expression system. Thus, enhancement of KCNQ4-mediated currents by channel openers might be a therapeutic option for most DFNA2 patients irrespective of the particular mutation in the KCNQ4 gene.

Native $I_{K,n}$ currents were potentiated by chemical openers similar to heterologously expressed KCNQ4, but current gain was substantially smaller. Since the action of the openers includes a substantial shift in the activation voltage range to more hyperpolarized potentials, the degree of current potentiation that can be reached *in vivo* must also depend on the membrane potential (V_M) of OHCs. The V_M of OHCs *in vivo* is not known precisely. While it has been reported as about –80 mV (Mammano and Ashmore, 1996; Marcotti and Kros,

1999), which is close to the half-activating voltage of $I_{K,n}$, V_M might be much more depolarized *in vivo* due to the large depolarizing transducer conductance that is lost with the usual recording conditions *in vitro* (He *et al.*, 2004; Johnson *et al.*, 2011). The lower efficacy of openers on the native channel may impose a limitation to any therapeutic use of channel openers, unless activators with higher potency are found. However, it should be noted that in human DFNA2, hearing loss is slowly progressive (Smith and Hildebrand, 2008) and its progressive nature was recapitulated in a mouse model. Hearing loss in mice heterozygous for the dominant-negative pore mutation G285S progressed significantly slower than after full deletion of KCNQ4 (Kharkovets *et al.*, 2006). Thus, even the minute current levels retained under dominant-negative suppression of KCNQ-mediated currents are obviously sufficient to substantially delay hair cell degeneration. Hence, even a moderate enhancement of native $I_{K,n}$, as observed with the channel openers, may provide significant protection against hair cell loss in DFNA2. We expect that chemical KCNQ openers enhance the current of residual wt channels in the dominant-negative mouse model (Kharkovets *et al.*, 2006) as also seen for native $I_{K,n}$ in OHCs. However, if this current increase is sufficient to delay the onset of hearing loss or even protect OHCs from degeneration, this should be further investigated directly *in vivo*.

Another issue that deserves attention is the finding that channel openers examined here do not only act on KCNQ4 channels within the inner ear but also strongly potentiate M-currents mediated by KCNQ2, –3 and –5 subunits in many central and peripheral neurons (Xiong *et al.*, 2007; Lv *et al.*, 2010). Of note, ZnP alone showed the highest potency for KCNQ4 as compared with other KCNQ isoforms (Xiong *et al.*, 2007). However, the various openers available to date have different potencies towards KCNQ4 and also differ in their potencies towards the different KCNQ isoforms (Schroder *et al.*, 2001; Tatulian *et al.*, 2001; Xiong *et al.*, 2007), suggesting that it may be possible to develop openers with higher specificity for KCNQ4.

Mechanism of current enhancement

It was shown previously that ZnP increases the open probability of KCNQ channels, rather than altering single channel conductance (Xiong *et al.*, 2007). This indicates that ZnP, and most likely other KCNQ openers, are primarily modifiers of channel gating, which is consistent with the shifted voltage-dependence observed here and in previous studies (Schroder *et al.*, 2001; Tatulian *et al.*, 2001; Xiong *et al.*, 2007). DFNA2 mutations may abolish channel function via different mechanisms: most obviously, mutations in the channel pore are expected to impair ion permeation, rendering channels constitutively non-functional independent of channel gating. This is consistent with our observation that KCNQ openers were completely ineffective in uncovering any channel activity in the pore mutants. For the mutation in the proximal C-terminus, G321S, there is no obvious reason to expect an impairment of ion permeation, as this region does not contribute to the pore structure. It seems more likely that this mutation disrupts channel gating, since in KCNQ channels, the C-terminus mediates channel gating by various physiological signals, including phosphatidylinositol(4,5) bisphosphate (Hernandez *et al.*, 2008; 2009), Ca^{2+}-calmodulin (Gamper *et al.*, 2005) and phosphorylation (Li *et al.*, 2004). Consistent with this hypothesis, we found that the gating modifiers, ZnP/Ret, partially restored channel activity in G321S, but not in homomeric or heteromeric pore mutant channels.

Recently, disruption of targeting to the plasma membrane has been proposed as an alternative explanation for the dysfunction of DFNA2 mutants, including the pore mutations and G321S (Mencia *et al.*, 2008; Kim *et al.*, 2011). Such a mechanism would also be consistent with the lack of effect of channel openers on the pore mutants. However, the ZnP/Ret-induced currents through KCNQ4-G321S clearly show trafficking of this mutant channel to the plasma membrane, although we cannot exclude a reduction of surface expression.

Pharmacological properties and the molecular nature of $I_{K,n}$

There is strong evidence indicating that the OHC current $I_{K,n}$ is mediated by KCNQ4 channels (Marcotti and Kros, 1999; Kharkovets *et al.*, 2000; 2006; Holt *et al.*, 2007; Oliver *et al.*, 2003). Nevertheless, the functional properties of $I_{K,n}$ differ from those of heterologously expressed KCNQ4 channels in various aspects. Most strikingly, the activation voltage of $I_{K,n}$ is much more negative and the activation kinetics are faster (Mammano and Ashmore, 1996; Marcotti and Kros, 1999). Here we show that the differences extend to the pharmacological properties. Thus, current potentiation by retigabine, ZnP, and a combination of both openers was much less pronounced with $I_{K,n}$ than with heterologously expressed KCNQ4. Similarly, the drug-induced hyperpolarizing shift in the voltage of activation was smaller for the native channel. The differential sensitivity to channel openers is particularly pronounced for BMS-204352: this opener produced a 10-fold potentiation of recombinant KCNQ4 at saturating voltages, but was essentially ineffective on native $I_{K,n}$.

The molecular basis of these differences has remained elusive so far. An attractive hypothesis, however, is that $I_{K,n}$ characteristics result from the presence of as yet unknown accessory subunits within the native ion channel complex. KCNE β-subunits are obvious candidates for such accessory subunits, as they co-assemble with KCNQ α-subunits and are determinants of biophysical and pharmacological channel properties (McCrossan and Abbott, 2004). KCNE expression has been detected in the cochlea and all KCNE isoforms appear to co-assemble with KCNQ4 upon heterologous expression (Strutz-Seebohm *et al.*, 2006). However, none of the KCNE1-5 isoforms alters the biophysical properties of KCNQ4 currents in a manner consistent with the native current, $I_{K,n}$ (Strutz-Seebohm *et al.*, 2006). Another possibility is that native $I_{K,n}$ channels may be heteromeric KCNQ3/KCNQ4 channels (Kubisch *et al.*, 1999). Among the KCNQ channels, KCNQ3 is least sensitive to channel openers (Xiong *et al.*, 2007; 2008) and might decrease the sensitivity of KCNQ4 in the heteromers. In OHCs, the expression of KCNQ3 cannot be excluded unequivocally (Kubisch *et al.*, 1999; Jentsch, 2000; Kharkovets *et al.*, 2006). Thus, we tested the properties of heteromeric channels in CHO cells co-transfected with KCNQ3 and KCNQ4. Voltage- dependence and sensitivity towards channel openers of KCNQ3/4 heteromers were not different from homomeric KCNQ4

channels, indicating that co-assembly with KCNQ3 in OHCs cannot explain the unusual pharmacological and biophysical properties of $I_{K,n}$ (Supporting Information Figure S5). Thus, the molecular underpinnings of the native current remain unknown.

Mechanistically, one explanation for the relative insensitivity of $I_{K,n}$ might be that endogenous hair cell-specific modifiers of gating increase open probability via the same mechanism that is exploited by the synthetic channel openers, thereby occluding additive effects. In fact, recombinant KCNQ4 in the presence of ZnP/Ret closely resembles the native current in terms of voltage-dependence and kinetics (Supporting Information Figure S2). One prerequisite for the dramatic current enhancement by channel openers is the low open probability of KCNQ channels even at saturating voltages (Li *et al.*, 2005). For recombinant KCNQ4, ZnP was shown to increase the open probability at 0 mV from 0.01 to 0.33 (Xiong *et al.*, 2007). The similarity of $I_{K,n}$ with KCNQ4 currents potentiated by ZnP/Ret may indicate high basal open probabilities of the native channels. For pharmacological restoration of $I_{K,n}$ in DFNA2, a high endogenous open probability may pose a fundamental obstacle, as it limits the enhancement that can be reached. Therefore, it will be interesting to see, if basal open probabilities of native channels mediating $I_{K,n}$ are indeed higher than those of the corresponding channel in an expression system.

In conclusion, we have shown that the dominant-negative suppression of ion channel function underlying hearing loss in DFNA2 can be overcome using synthetic channel openers. Beyond hereditary deafness, KCNQ channel openers may prove useful in forms of deafness that involve hair cell loss, as we have recently shown that ZnP and retigabine can also antagonize the suppression of $I_{K,n}$ by ototoxic aminoglycoside antibiotics (Leitner *et al.*, 2011).

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Conflict of Interest

The authors have no conflict of interest to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Chemical KCNQ openers augment heterologous KCNQ4. (A–D) Dose-response relationships of CHO cells expressing wt KCNQ4 for (A) retigabine (B) BMS-204352 (C) flupirtine and (D) zinc pyrithione. Current amplitudes at 0 mV were normalized to control currents prior the application of the KCNQ agonist, and were fitted to a Hill equation to calculate the concentration at half-maximal effect (EC_{50})

and the Hill coefficient (n). Note different potencies of current augmentation of the substances. (E, F) Voltage-dependence of heterologous KCNQ4 in presence of (E) 10 μM BMS-204352 and (F) 10 μM flupirtine. Conductance-voltage relations were derived from tail current recordings as shown in Supporting Information Figure S2, and currents were normalized to the maximum current and fitted by a two-state Boltzmann function. Continuous lines indicate the Boltzmann fit to the averaged data. Fits to the current-voltage relation of individual cells yielded (E) V_h under control conditions of –19.6 ± 1.9 mV, and in presence of 10 μM BMS-204352 of –31.7 ± 1.2 mV. Note that application of BMS-204352 lowered k from –13.7 ± 3.7 to –6.0 ± 0.5 mV (n = 7). (F) In presence of flupirtine V_h was shifted from –17.2 ± 1.2 mV to –23.2 ± 1.2 mV (n = 11), without affecting the slope. (G) Summarized time course of KCNQ4 current potentiation at 0 mV by ZnP/Ret (10 μM both, n = 8).

Figure S2 ZnP/Ret produces $I_{K,n}$-like KCNQ4 currents in CHO cells. (A–C) Representative recordings of wt KCNQ4 in CHO cells (A), K^+ currents in OHCs (B) and wt KCNQ4 in presence of ZnP/Ret (10 μM both) (C). A and C show recordings from the same cell. Voltage-protocols were as indicated. (D) Current-voltage relationship for native $I_{K,n}$ in OHCs and heterologous KCNQ4 in presence and absence of ZnP/Ret. ZnP/Ret shifted KCNQ4 currents by –40 mV to V_h = –64.0 ± 2.5mV (n = 8), producing an $I_{K,n}$-like voltage range of activation (V_h = –75.7 ± 1.3 mV, n = 24). Note that in presence of ZnP/Ret heterologous KCNQ4 currents showed marked inactivation at voltages more depolarised than +20 mV that was not further investigated in this study. Data are reproduced for comparisons from Figures 1 and 7 in the main text.

Figure S3 KCNQ4-F182L is fully functional and sensitive to ZnP/Ret. (A, B) Representative currents of the same CHO cell expressing KCNQ4-F182L. Voltage command as indicated, scale bar corresponds to A and B. Dashed lines indicate zero current. (C) Current-voltage relationship for F182L under control conditions (*black*) and with ZnP/Ret (*red*) derived from Boltzmann fits to the tail currents as recorded in (A, B). Control F182L currents showed the same voltage-dependence as wt KCNQ4 (F182L: V_h = –18.0 ± 1.8 mV), and ZnP/Ret shifted voltage-dependence as for wt (V_h = –62.9 ± 4.7 mV; n = 8). (D) KCNQ4-F182L current amplitudes at 0 mV in presence and absence of the KCNQ channel opener. For F182L ZnP/Ret-mediated current potentiation and sensitivity to the KCNQ antagonist XE991 were similar as for wt KCNQ4.

Figure S4 KCNQ4 current amplitudes do not depend on the DNA amount used for transfection. (A, B) Co-expression experiments of wt and mutant KCNQ4 (1:1) required the halving of the DNA concentration for each plasmid used for transfection. Using equal DNA amounts in control experiments did not affect homomeric wt KCNQ4 current amplitudes. Thus reduction of current amplitudes by co-expressed mutants was caused by dominant-negative suppression of wt subunits (compare with Figure 5 in the main text). (A) Shows a representative recording of a CHO cell expressing wt KCNQ4 with half[DNA] used for transfection. (B) Current amplitudes using half[DNA] were indistinguishable from control cells using the normal amount of DNA (recordings from the same batch of cells).

Figure S5 KCNQ4 homomeric channels and KCNQ3/KCNQ4 heterotetramers show comparable sensitivity towards chemical openers. (A) Retigabine (*left panel*, 2.5 μM) and ZnP (*right panel*, 2.5 μM) robustly potentiated currents from CHO cells transfected either with KCNQ4 alone or co-transfected with KCNQ4 and KCNQ3. Values shown are current densities at 0 mV. Note that control currents from co-transfected cells are several folds larger than in KCNQ4-tranfected cells, indicating coassembly of KCNQ3/KCNQ4 hetero-tetramers (cg. Kubisch *et al.*, 1999) (recordings from the same batch of cells). The drug concentrations were chosen close to their EC_{50} values (see Supporting Information Figure S1). (B) Current potentiation by retigabine (*left*) and ZnP (*right*) was comparable and statistically indistinguishable for KCNQ4 homomers and KCNQ3/4 heteromers. Values shown indicate relative current increase at 0 mV calculated from the same set of recordings shown in (A). (C) Current-voltage relationship for KCNQ4 homomers and KCNQ3/4 heteromers. KCNQ4 homomeric channels (V_h = –21.7 ± 0.8 mV) show the same voltage-dependence as KCNQ3/4 (V_h = –23.3 ± 1.3 mV; recordings from the same batch of cells).