Functional Coassembly of KCNQ4 with KCNE- β -Subunits in *Xenopus* Oocytes

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Key Words

Gating • Kv7 • Kinase • Channel kinetics • K⁺ channel • MinK • Mirp

Abstract

The KCNQ gene family comprises voltage-gated potassium channels expressed in epithelial tissues (KCNQ1, KCNQ5), inner ear structures (KCNQ1, KCNQ4) and the brain (KCNQ2-5). KCNQ4 is expressed in inner and outer hair cells of the inner ear where it influences electrical excitability and cell survival. Accordingly, loss of function mutations of the KCNQ4 gene cause hearing loss in humans and functional k.o.-mice show progressive degeneration of outer hair cells (OHCs). However, characteristic electrophysiological features of the native KCNQ4-carried current $I_{K,n}$ in OHCs are not recapitulated by expression of KCNQ4 channels in heterologous expression systems. This might suggest modulation of KCNQ4 by interacting KCNE β -subunits, which are known to modify the properties of the closely related KCNQ1. The present study explored whether transcripts of the KCNE isoforms could be identified in OHC mRNA and whether the subunits modulate KCNQ4 function. RT-PCR indeed yielded transcripts

of all five KCNEs in OHCs. Coexpression of the KCNE- β -subunits with human KCNQ4 in the *Xenopus laevis* oocyte expression system revealed that all KCNEs modulate KCNQ4 voltage dependence, protein stability and ion selectivity of hKCNQ4 in *Xenopus* oocytes. The deafness-associated Jervell and Lange-Nielsen syndrome (JLNS) mutation KCNE1(D76N) impairs KCNQ4-function whereas the Romano-Ward syndrome (RWS) mutant KCNE1(S74L), which shows normal hearing in patients, does not impair KCNQ4 channel function. In conclusion, KCNEs are presumably coexpressed with KCNQ4 in hair cells from the organ of Corti and might regulate KCNQ4 functional properties, effects that could be important under physiological and pathophysiological conditions.

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Introduction

Voltage-gated K⁺ channels are composed of homo- or heterotetrameric assembly of pore-forming α -subunits and additional regulatory β -subunits (e.g. KCNE, KChIP, Kv β). KCNQ channels (KQT, Kv7) comprise a distinct family of five voltage-dependent α -subunits. Mutations

in specific KCNQ genes can cause epilepsy cardiac arrhythmia, and or deafness [1-6]. KCNQ4 was first identified by a homology cloning approach to KCNQ1-3 channels [4]. The gene maps to chromosome 1p34, the DFNA2 (deafness, autosomal dominant nonsyndromic sensorineural 2) locus for a form of nonsyndromic dominant deafness, and KCNQ4 mutations were reported to be associated with this disease [4, 7, 8].

KCNQ-like currents have been identified in both inner (IHC) and outer (OHC) hair cells [4, 9, 10], where they seem to serve different functions. In OHCs, the large KCNQ-like currents are the dominating K^+ currents both at rest and at depolarized voltages and thus form the major exit route for K^+ ions entering the cells through the transduction channels. In IHCs, the relatively small KCNQ currents set the resting membrane potential to about -70 mV and determine the membrane time constant at rest [10]. These currents maybe carried by KCNQ4 channels, whose RNA and protein were detected and whose lack of functional KCNQ4 channels in DFNB 2 most likely leads to OHC degeneration and deafness [4, 11, 12]. The deafness may result from use-dependent depolarization causing Ca^{2+} influx through voltage-gated Ca^{2+} channels [13] and ultimately excitotoxicity of IHCs, OHCs and afferent fibres [4]. This scenario is supported by the phenotype of KCNQ4 k.o. mice and dominant-negative-KCNQ4-G286S mutant mice. These mice lack the KCNQ4-carried I_{K_n} current of outer hair cells which leads to progressive OHC-degeneration in high frequency generating cochlear turns [12]. On the other hand, no morphological changes were found in IHCs or vestibular hair cells of those mice.

The native I_{K_n} current of hair cells is distinguished by an activation curve shifted to exceptionally negative potentials. Heterologous expression of KCNQ4 channels in *Xenopus* oocytes or mammalian cell lines induces a slowly activating, non-inactivating potassium selective current with half maximal activation at about -10 mV to -25mV [12, 14, 15]. Thus, the basic biophysical properties of heterologously expressed KCNQ4 channels are distinct from the I_{K_n} known from outer hair cells [8-10, 12]. The reasons for this discrepancy remained elusive but may include unknown interacting proteins like prestin or other scaffolding proteins, KCNE β -subunits, or regulation by ions, kinases, PIP_2 and/or further signaling mechanisms. Indeed, regulation of KCNQ4 by prestin has recently been suggested [14]. Heterologously expressed KCNQ4 channels are down-regulated by activation of M1-receptors and upregulated by activation of dopamine D2 receptors [16, 17]. Furthermore, KCNQ4

channel function has been reported to be regulated by cell volume, protein kinases (SGK1, PKA and PKC), G proteins and intracellular Ca^{2+} [13, 18].

The present study has been performed to explore the influence of KCNE- β -subunits on current properties of human KCNQ4. We show that coexpression of KCNEs in *Xenopus* oocytes modulates KCNQ4 kinetics, protein stability and ion selectivity. Further, we show that the deafness-associated Jervell and Lange-Nielsen syndrome (JLNS) mutation KCNE1(D76N) impairs KCNQ4-function whereas the Romano-Ward syndrome mutant KCNE1(S74L), which shows normal hearing in patients, does not impair KCNQ4 channel function.

Materials and Methods

Molecular Biology

The molecular biological procedures were the same as described before [18]. The KCNQ4, KCNE4 and KCNE5 cDNAs were expressed in *pXOOM* expression vector. The restriction enzyme used for linearization was XbaI [19]. KCNE1, KCNE2, KCNE3 were expressed in pSGEM and DNA was linearized with NheI. *In vitro* cRNA synthesis was done with mMessage mMachine T7-polymerase kit (Ambion, Cambridgeshire, UK).

RT-PCR analysis

mRNA from heart (1 ventricle) was prepared from NMRI mice at P18. Heart tissue was homogenized with a sonicator and mRNA was isolated using Dynabeads® (DynaL Biotech) following the protocols of the manufacturer. For cDNA synthesis, 10 μ l of RT-mix were added to each 2 μ l of mRNA [20]. Each microcentrifuge tube was incubated at 42 °C for 60 min followed by 10 min at 70 °C, and the cDNA was stored at -20 °C.

For hair-cell specific RT-PCR, an apical turn of an organ of Corti (P17) was fixed on a coverslip with Cell-Tak (Becton Dickinson, Bedford, MA, USA) and placed under a ZEISS Axioskop upright microscope with a 40x water immersion objective. After Hensen and Claudius' cells had been removed with cleaning pipettes of 10 - 15 μ m tip diameter, about 80 OHCs were harvested with pipettes of 10 μ m tip opening and frozen in liquid N_2 . Prior to cDNA first strand synthesis, samples (10 μ l) were treated with 1 μ l RNase-free DNase I (Ambion Bioscience) for 10 min at 37 °C. Subsequently, RNA samples were mixed with 1 μ l dNTP (10 mM, Roche) and 1 μ l oligo dT₂₀ Primer (50 μ M, Invitrogen) and incubated for 5 min at 65 °C. An RT mix consisting of 4 μ l reverse transcription buffer (5 x, Invitrogen), 2 μ l DTT (0,1 M, Invitrogen), 1 μ l RNase OUT (40 U/ μ l, Invitrogen) and 1 μ l reverse transcriptase superscript III (Invitrogen) was added and the reaction mixture was incubated for 45 min at 50 °C. The reaction was stopped by heating the samples for 15 min at 70 °C.

The cDNA was stored at -80°C until PCR analysis. PCR analysis was then performed with 1 μ l of the reverse transcription product in a total volume of 25 μ l of a PCR mix containing

22 µl sterile H₂O, 1 µl of sense primer (100 pmol/µl) and antisense primer (100 pmol/µl), and 1 puReTaq Ready-To-Go PCR bead (Amersham Biosciences) through 40 cycles (30 s at 94°C; 30 s at 60°C; 45 s at 72°C). Primers used for amplification were as follows: KCNQ4 sense *gcagaaagctggagcttca*, antisense *gtgtctgtctgaataactgtct*; KCNQ1 sense *tgtaccacttcaccgtcttc*, antisense *acgcagaggacaaccataga*; KCNE1 sense *atgagcctgcccattccacga*, antisense *ttctctgcccagcactctga*; KCNE2 sense *aggaacacgacagccgagga*, antisense *atgttctcatggatgggtggc*; KCNE3 sense *actgagacctggtacatgag*, antisense *cggttcttgatgtacacatg*; KCNE4 sense *ctgatcggaatcatgctggg*, antisense *aggtgcacatcaggagagga*; KCNE5 sense *tgctgctggagctgcacat*, antisense *ttctgtctcagcaaccagag*. PCR products were analysed by agarose gel electrophoresis.

Oocyte expression

Xenopus laevis frogs were anaesthetised with tricaine, ovarian lobes were harvested, digested with collagenase (collagenase Type II, Worthington, CellSystems GmbH, D-53562 St. Katharinen, Germany) and healthy looking stage V-VI oocytes were collected. Frogs were humanely killed after the final collection in accordance with the German law. The oocytes were injected with 40 nl or 60 nl of cRNA-solution with a nanoliter injector 2000 (World Precision Instruments, Berlin, Germany) and incubated at 17-18°C for 3 to 4 days as previously described [18]. Individual oocytes were injected with 4 ng KCNQ4 cRNA or with 4 ng KCNQ4 RNA plus 4 ng KCNE cRNA.

Electrophysiology

Standard two-electrode voltage/current-clamp techniques [21] were used to record whole cell currents in *Xenopus* oocytes at room temperature (22-24°C) using a Turbo TEC-10CX amplifier (NPI, Tamm, Germany) combined with AD/DA-board Digidata 1322A (Axon Instruments Inc. /Molecular Devices, California, USA). The recording pipettes were filled with 3M KCL and had a tip resistance of 0.4-1 MΩ. Currents were recorded using the amplifiers integrator and controlling the integrity of the recording by the PI-controller. Data were acquired with the Clampex program of pCLAMP 8.0 software (Axon Instruments Inc. /Molecular Devices, California, USA) and analyzed with Clampfit 8.0 and Origin 6.0 (Microcal, Friedrichsdorf/Ts, Germany). For voltage clamp experiments, oocytes were bathed in ND96 solution containing (in mM): 96 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES; pH 7.6.

Labeling of cell surface proteins using biotinylated ConA

To identify the fraction of receptor protein inserted in the plasma membrane, surface proteins were tagged with Sulfo-NHS-LC-Biotin (PIERCE, IL, USA), and then isolated by streptavidin-sepharose-mediated NeutrAvidin Biotin-Binding Protein (PIERCE, IL, USA) precipitation of the biotinyl-ConA/protein complex as described elsewhere [22]. Briefly, intact oocytes were incubated in 10 µM Sulfo-NHS-LC-Biotin for 30 min at room temperature. After five 10 min washes in ND-96 buffer, intact oocytes were homogenized with a Teflon pestle in H-buffer (20 µl/oocyte; 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 % Triton X-100, plus a mixture of proteinase inhibitors

(Complete; Boehringer Mannheim, Mannheim, Germany) and were kept at 4 °C for 1 hr on a rotating rod. A 20 µl aliquot was kept as a Total protein sample (T). After centrifugation of the remaining homogenate for 1 min at 16,000g, the supernatants were supplemented with 20 µl of washed Neutr Avidin beads and incubated at 4 °C for 3 hrs on a rotating rod. The beads were then pelleted by a 2 min spin at 16,000 g and washed three times in H-buffer. 20 µl of the supernatant was kept as supernatant protein sample (SN). The final pellets (P) were boiled in 20 µl of SDS-Page loading buffer (0.8 M β-mecaptoethanol, 6 % SDS, 20 % glycerol, 25 mM Tris-HCl, pH 6.8, and 0.1% bromphenol blue).

Gel electrophoresis and Western blotting

Proteins from homogenized oocytes were separated by SDS electrophoresis and transferred to nitrocellulose filters. Blots were blocked in 1x PBS containing 5% milk powder for at least 1 hour at room temperature. For the detection of KCNQ4, primary antibody (kindly provided by Marlies Knipper) and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:1000 dilution, Amersham Bioscience, Freiburg, Germany) were used. For verification of protein levels, Ponceau Red staining was performed.

Data analysis

The activation curves obtained by plotting normalized tail current amplitudes (I_{tail}) versus test potential were fitted to standard Boltzmann equation of the form:

$$I_{tail} = 1/(1 + \exp[(V_{1/2} - V_t)/k]) \text{ (Equ. 1)}$$

where $V_{1/2}$ is the voltage of half maximal activation and k is the slope factor. The time independent component was offline subtracted.

Statistical analysis

Students t- test with independent populations was used to test for statistical significance which was assumed at $p < 0.05$.

Results

In a first series of experiments, we searched for KCNE-RNAs in outer hair cells (OHCs) of mice, the cells in which KCNQ4 is predominantly expressed, to check if KCNEs are possible candidates for regulation of KCNQ4 currents *in vivo*. We harvested around 45 - 80 OHCs and used RT-PCR to show the presence of KCNE-specific mRNA in OHCs. We could amplify KCNE- specific transcripts from OHC cDNA. We also detected KCNQ4 but not KCNQ1 mRNA in OHCs and applied RT-PCR to samples from murine heart where all KCNEs, KCNQ1 and KCNQ4 are expressed (Figure 1) as control.

To determine possible functional effects of the

Fig. 1. RT-PCR demonstrating KCNQ4 and KCNE1-5 mRNA in hair cells and in addition KCNQ1 mRNA in heart cells. About 80 outer hair cells (OHC) and heart (ventricle) tissue were harvested. Cell numbers used in each OHC PCR were estimated at about two OHCs. Primers were designed to amplify ~270 bp fragments corresponding to the respective clones.

different KCNEs on KCNQ4 we expressed KCNQ4 alone or coexpressed KCNQ4 with the individual KCNE- β -subunit in *Xenopus* oocytes. Functional testing was performed by standard two-electrode voltage-clamp. Expression of KCNQ4 channels in oocytes produced the characteristic KCNQ4-currents (Figure 2). Coexpression of KCNQ4 with KCNEs resulted in differences in current amplitudes. Whereas KCNE1, KCNE2 and especially KCNE4 increased currents, KCNE3 strongly decreased KCNQ4-currents. Effects of KCNE5 on KCNQ4 current amplitudes were relatively mild (Figure 2). Thus, KCNEs can modulate current amplitudes.

KCNQ4 channels activated with a half point of voltage-dependent activation of $V_{1/2} = 10.9 \pm 0.8$ mV, $k = 20.8 \pm 0.7$ (n = 14). The voltage dependence was shifted by the respective KCNEs, yielding for KCNQ4-KCNE1: $V_{1/2} = 1.9 \pm 0.6$ mV, $k = 18.2 \pm 0.5$ (n = 16); KCNQ4-KCNE2: $V_{1/2} = -6.9 \pm 1.2$ mV, $k = 14.0 \pm 1.0$ (n = 16), and KCNQ4-KCNE5: $V_{1/2} = 3.3 \pm 0.9$ mV, $k = 18.0 \pm 0.8$ (n = 12) (Figure 2). The strongest effects on the voltage dependence of KCNQ4 were found in coexpressions with KCNE3 and with KCNE4 (very flat activation curves). Data for KCNQ4-KCNE3 and KCNQ4-KCNE4 could

not properly be fitted by equation Eq. 1 because the fits were very variable and the curves were far away from being saturating (slope factor k was > 50). Additionally, KCNE4 clearly blunted the voltage-dependence of KCNQ4 (Figure 2A,B). KCNQ4 was reported to inactivate [15] and the channels seemed to inactivate to different degrees depending on KCNE coexpression. However, this effect was largely dependent on the batch of oocytes and was not analyzed in detail.

KCNQ4 and KCNQ4-KCNEs reversed close to -70 to -80 mV, which is around the potassium reversal potential, except for KCNQ4-KCNE4 channels whose currents reversed at about -30 mV. The relatively positive reversal potential of KCNQ4-KCNE4 could have been caused by changes in the selectivity. To test this hypothesis we expressed KCNQ4 alone or in combination with the respective KCNE, and perfused the oocytes with solutions containing 100 mM of either monovalent ions Li^+ , Na^+ , K^+ , Rb^+ or Cs^+ . Using the shift of reversal potentials relative to K^+ we calculated relative K^+ -conductances for the respective ion species (Figure 3). Whereas KCNE5 seemed to decrease slightly the relative Rb^+ conductance, the other KCNEs did not modify the

Fig. 2. Effect of coexpression of KCNQ4 with KCNE isoforms on KCNQ4 channel current amplitude, voltage dependence of activation and activation kinetics. A. Human KCNQ4 plus H₂O or KCNQ4 plus KCNEs were expressed in *Xenopus* oocytes and currents measured in extracellular solution ND96. Currents were elicited from a holding potential of -80 mV by pulses applied in 20 mV increments to potentials ranging from -140 mV to +80 mV (pulse protocol indicated above current traces). These depolarizing test pulses were followed by a pulse to -120 mV to elicit tail currents. B. The current amplitudes at the end of the test pulses were measured, normalized to the mean values of KCNQ4 at +80 mV and plotted against the respective voltage. C. The initial tail current amplitude at -120 mV was analyzed, normalized to the minimal current at +60 mV and plotted vs. the voltage of the preceding test pulse. The data were fitted to the Boltzmann equation (Eq. 1); values are given in the text. Error bars represent $\pm$ SEM. Symbols used as indicated where Q4 = KCNQ4 and Ex = KCNEx.

Fig. 3. KCNQ4 relative selectivity towards monovalent ions is modulated by KCNE4 and KCNE5. Oocytes were coinjected with KCNQ4 plus H₂O or KCNEs. The cells were depolarized to activate currents and then repolarized to close channels. The reversal potential in different solutions containing 100 mM Li⁺, Na⁺, K⁺, Rb⁺ or Cs⁺ were determined from the initial phase of tail currents. The shift of reversal potentials relative to the reversal potential determined in 100 mM K⁺ was used to determine the relative conductance for the respective ion species. Data are shown with the corresponding $\pm$ SEM and n = 3-6.

relative conductances. The conductance series was K⁺ = Rb⁺ > Cs⁺ > Na⁺ = Li⁺. However, in the case of KCNQ4-KCNE4 coexpression, the relative selectivities towards Cs⁺ > Na⁺ = Li⁺ were reduced. These data show

that KCNEs do not only modulate current amplitudes and kinetics but also the channel's selectivity.

The KCNE1 mutations KCNE1(S74L) and KCNE1(D76N) were reported to decrease KCNQ1

Fig. 4. Disease-associated mutations in KCNE1 differently modulate KCNQ4 current amplitude. A. Oocytes were coinjected with KCNQ4 plus H₂O or KCNQ4 plus KCNE1 or KCNE1 mutants. Representative current traces are shown. B. Wild type KCNE1 and the Romano-Ward syndrome associated mutant KCNE1(S74L) activate KCNQ4 by about 75 % and 100 %, whereas the Jervell and Lange-Nielsen syndrome associated mutant KCNE1(D76N) suppresses channel function by about 40 %. Error bars represent $\pm$ SEM, $n = 43 - 56$. C. Initial tail currents were determined and normalized to the maximal current at 80mV and plotted versus the potential. D. Oocytes were coinjected with KCNQ4 plus H₂O or KCNEs. The channels were activated and the reversal potentials were determined in different solutions (containing 100 mM Li⁺, Na⁺, K⁺, Rb⁺ or Cs⁺). The shift of reversal potentials relative to the reversal potential determined in K⁺ was used to determine the relative conductance for the respective ion species. Data are shown with the corresponding $\pm$ SEM and $n = 3-6$.

channel currents which results in LQT syndrome. However, only the Jervell and Lange-Nielsen Syndrome (JLNS) associated KCNE1(D76N) but not Romano-Ward Syndrome (RWS) associated KCNE1(S74L) causes hearing impairment. Hearing impairment solely by reduction of KCNQ1/KCNE1 channel current in the stria vascularis of the inner ear by the mutations seems not to explain the differences. As KCNE1 mildly increases KCNQ4 channel currents (Figure 2) the differences could be a result of differences in KCNQ4-modulation by KCNE1 or KCNE1(S74L) and KCNE1(D76N). To test this hypothesis we coexpressed KCNQ4 with KCNE1,

KCNE1(S74L) or KCNE1(D76N). KCNQ4 coexpressed with KCNE1 or KCNE1(S74L) increased channel currents, whereas coexpression with KCNE1(D76N) suppressed KCNQ4 channel function (Figure 4). KCNE1(S74L) and KCNE1(D76N) did not change ion selectivity and kinetics seemed not to be markedly changed (Figure 4). KCNQ4 channels activated with a half point of voltage-dependent activation of $V_{1/2} = 10.9 \pm 0.8$ mV, $k = 20.8 \pm 0.7$ ($n = 14$). The voltage dependence was not markedly affected by the respective KCNE1. The precise values are KCNQ4-KCNE1: $V_{1/2} = 1.9 \pm 0.6$ mV, $k = 18.2 \pm 0.5$ ($n = 16$), KCNQ4-KCNE1(S74L): $V_{1/2} = 10.4$

Fig. 5. Protein expression of KCNQ4 in *Xenopus* oocytes. A. Oocytes were coinjected with KCNQ4 plus H₂O or KCNQ4 plus KCNEs or KCNE1(D76N) mutant. Protein expressed in the plasma membrane (P) and in total (T = plasma membrane plus intracellular protein) was detected from three batches of oocytes. One representative blot is shown. KCNQ4 protein has an apparent molecular weight of ~77 kDa. B. The bar graph shows relative abundance of KCNQ4 plasma membrane protein. The band intensity of plasma membrane expressed protein (P) was quantified by arithmetic analysis using the software Scion image. The values were normalized to the band intensity of KCNQ4 alone.

± 1.4 mV, $k = 19.9 \pm 1.2$ (n = 15) and KCNQ4-KCNE1(D76N): $V_{1/2} = 1.2 \pm 0.7$ mV, $k = 17.6 \pm 0.7$ (n = 17) (Figure 4).

KCNEs including KCNE1(D76N) modulate KCNQ4 current amplitudes (Figure 2, 4), which could be the result of differences in surface membrane expression of the channels. To explore this possibility we expressed KCNQ4 alone or together with the individual KCNE- β -subunits in *Xenopus* oocytes. We performed Western blotting to differentiate between plasma membrane expressed KCNQ4 and total KCNQ4 protein. KCNE1-3 and KCNE1(D76N) did not markedly change the amount of KCNQ4 expressed in plasma membranes or in total cell lysates. However, KCNE4 and KCNE5 decreased KCNQ4 in the plasma membrane and in total cell lysates, an effect possibly due to decreased KCNQ4 protein stability.

Discussion

KCNQ4 channels underly the potassium current $I_{K,n}$ in OHCs. DFNA2-mutations in KCNQ4 lead to loss of function and degeneration of OHCs. Expression of KCNQ4 in oocytes generates a channel with right-shifted activation curves compared to the native KCNQ4-carried $I_{K,n}$. Here, we tested the possibility that KCNEs may modulate KCNQ4 channels. We show expression of KCNE1-5 RNA by RT-PCR. Even though the presence of RNA does not conclusively prove the existence of KCNE-protein it is suggestive for the expression of KCNEs in KCNQ4-expressing OHCs.

If KCNEs are present in OHCs, they may interact with KCNQ4. It has even been suggested that all voltage-gated channels form complexes with KCNE β -subunits [23]. KCNEs modulate the biophysical properties of

several voltage-gated cation channels in such a way that the channel characteristics represent the features of native currents in various tissues. Among the KCNE-modulated channels are KCNQ1 [24, 25], ERG [26], HCN4 [27], Kv1.1, Kv1.3 [28], Kv2.1, Kv3.1 [29], and Kv4.3 [30]. We confirm the previous observation that KCNQ4 currents are suppressed by KCNE3 in *Xenopus* oocytes [31]. The present observations reveal that further members of the KCNE β -subunit family modulate KCNQ4. KCNEs modulate several properties of KCNQ4 channels including voltage-dependence of activation, current amplitude, ion selectivity and protein abundance in *Xenopus* oocytes. In part, these alterations are dramatic. For example, KCNE4 reduces selectivity and shifts the voltage-dependence significantly to the left. Whereas KCNE4 is inhibitory for Kv1.1, Kv1.3 and KCNQ1, it has an activating effect on KCNQ4 in oocytes [28, 32]. Nevertheless, KCNE4 could be responsible for the marked rightward shift of the activation curve of $I_{K,n}$. The other KCNEs do not affect KCNQ4 selectivity. Combinations of KCNEs might partially mimic features of native $I_{K,n}$ and contribute to the characteristics of $I_{K,n}$ in OHCs.

Although the coexpression of KCNE4 markedly increases the current amplitude of KCNQ4 channels, it did not lead to enhanced plasma membrane expression of KCNQ4. Possibly, KCNE4 changes single channel current amplitude or open probability of KCNQ4 similar to what was shown for KCNE1 on KCNQ1 [33, 34]. Coexpression of KCNE5 with KCNQ4 similarly yielded large current amplitudes despite relatively low protein abundance in the cell membrane. Again, single channel current amplitude or open probability of KCNQ4 could have been changed by KCNE5.

Among the most dramatic effects of KCNEs on a voltage-gated channel is the modulation of KCNQ1 channels by KCNE1. KCNQ1 (KvLQT1, Kv7.1) channels activate and deactivate relatively fast whereas coassembly of KCNQ1 with KCNE1 (MinK, IsK) results in slowly activating, non-inactivating, K^+ -selective channels that activate at more positive potentials with increased single channel conductance [24, 25, 35, 36]. The resulting current represents the cardiac potassium current I_{Ks} which contributes to terminal repolarization of the cardiac action potential. Further, KCNQ1/KCNE1 channels are important for K^+ -secretion by marginal cells into the scala media of the stria vascularis to generate the high endolymph K^+ concentration and endocochlear potential. Mutations in *KCNE1* and/or *KCNQ1* reduce I_{Ks} and cause long QT syndrome (LQTS) predisposing

affected individuals to arrhythmia and sudden cardiac death. The mutations KCNE1(S74L) and KCNE1(D76N) reduce I_{Ks} whereas KCNE1(D76N) causes an additional leftward shift of the activation curve by several mV. However, not all *KCNE1* mutations causing LQTS lead to hearing impairment. The reason for these phenotypical differences is not well understood. Possibly the different KCNE1 mutants modulate not only KCNQ1 but also other channels vital for hearing like KCNQ4 in a mutation- and phenotype-characteristic way.

Kubisch et al. [4] showed no significant effects of KCNE1wt on KCNQ4 channels. They concluded from their electrophysiological measurements in *Xenopus* oocytes that KCNE1wt is probably not coexpressed with KCNQ4. Further, they showed that KCNQ4 is expressed in outer hair cells, whereas KCNE1 could not be detected by immunohistochemistry in those cells by Sakagami et al. [37]. We provide evidence for mRNA of KCNE1 in outer hair cells and verify the existence of KCNQ4 RNA in outer hair cells by RT-PCR.

The effect of the disease-linked mutations KCNE1(D76N) and KCNE1(S74L) on KCNQ4 current had not been determined in the study of Sakagami et al. [37]. We found loss of KCNQ4 function when coexpressed with KCNE1(D76N) but not with KCNE1wt or KCNE1(S74L). No pronounced leftward shift of the activation curve was found in KCNQ4-KCNE1(D76N) currents compared to KCNQ4 expressed alone (Figure 4). The effect of KCNE1(D76N) is a functional effect on the channel itself and not caused by differences in channel expression or ion selectivity (Figure 4, 5). Outer hair cells are key regulators for dampening under very loud conditions and they amplify signals at sound pressure levels below 60 dB SPL. Impairment of KCNQ4 function leads to degeneration of OHCs and hearing impairment with positive recruitment [12, 38]. The reduction of KCNQ4-currents by KCNE1(D76N) may have implications for JLNS patients carrying KCNE1(D76N). These patients suffer from hearing impairment in addition to LQT syndrome. Patients harboring the RWS associated KCNE1(S74L) do not show the hearing phenotype but only the LQT syndrome.

Endogenous KCNQ1 α -subunits form functional channels with the exogenous KCNE1 when KCNE1-RNA is injected into oocytes. The affinity of this α -subunit- β -subunit interactions is believed to be relatively high and specific. Interestingly, coexpression of KCNE1 with KCNQ4 does not induce any of these slowly activating channels suggesting that the affinity of KCNQ4 for KCNE1 is even higher than for KCNQ1 (Figure 4).

We also coexpressed KCNQ1 plus KCNE1 together with or without KCNQ4. Without KCNQ4 KCNQ1/KCNE1 channels produced large, very slowly activating K⁺ currents. When KCNQ4 was coexpressed with KCNQ1/KCNE1 characteristic slowly activating currents were completely absent and currents appeared, which activated like KCNQ4 alone with current amplitudes about as large as the sum of KCNQ4 expressed alone plus KCNQ1 expressed alone (data not shown). These observations again suggest high affinity of KCNQ4 for KCNE1. Such a high affinity would explain the adverse effects by the mutant KCNE1(D76N).

Disease-associated mutations in KCNQ1 or KCNE1 might either directly or by an allosteric mechanism prevent interactions of KCNE1 with the interaction site of KCNQ1 [18]. However, direct interaction of the transmembrane domains of KCNQ1 with KCNE1 was suggested but the precise molecular basis of interactions and even the gross location remained controversial [39-44]. Nothing is known about the molecular interactions leading to functional alterations of the other channels. Taking into account the high homology of KCNQ1 and KCNQ4, KCNEs may interact within the transmembrane region with each other and this interaction could be impaired by the mutation KCNE1(D76N). Of course, it

is possible, that also other KCNQ proteins modulate KCNQ4 channel properties in outer hair cells, as for example KCNQ3, which has been shown to form heteromeric channels with KCNQ4 in oocytes [4]. We do not rule out possible effects of KCNEs on heteromeric KCNQ channels in outer hair cells.

In conclusion, KCNE transcripts could be identified in OHCs and KCNEs are regulators of KCNQ4 kinetics, ion selectivity, protein abundance and current amplitude in *Xenopus* oocytes. This regulation may be of physiological and pathophysiological relevance.

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