Mechanism of the defect in gap-junctional communication by expression of a connexin 26 mutant associated with dominant deafness

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ABSTRACT

Gap-junctional channels (connexin oligomers) are large-diameter aqueous pores formed by head-to-head association of two gap-junctional hemichannels, one from each of the adjacent cells. Profound hearing loss of genetic origin is common, and mutations of connexin 26 (Cx26) are the most frequent cause of this disorder. The Cx26 R75W mutant has been associated with disruption of cell-to-cell communication and profound hearing loss, but the mechanism of the gap-junctional defect is unknown. Here, we show that Cx26 R75W forms gap-junctional hemichannels that display altered voltage dependency and reduced permeability, and which cannot form functional gap-junctional channels between neighboring cells. The R75W phenotype is dominant at the gap-junctional channel but not at the hemichannel level. Therefore, the absence of gap-junctional communication caused by R75W expression is due to defective gap-junction formation by functional hemichannels.

Key words: channel • genetic disease
near the tips of the stereocilia, which causes Ca\textsuperscript{2+} and K\textsuperscript{+} influxes and membrane depolarization (14). Targeted ablation of Cx26 in cells of the epithelial gap-junctional network or expression of the Cx26 R75W dominant mutant in transgenic mice causes deafness associated with death of supporting and hair cells (15, 16). Mutations in Cx26 that disrupt cochlear gap-junctional communication may cause an increase in [K\textsuperscript{+}] in the perilymphatic space between the hair and supporting cells, with cell death resulting from chronic hair-cell depolarization or other mechanisms (15, 17–19). Alternatively, Cx26 mutants may still form K\textsuperscript{+}-permeable channels (17), but with other permeability changes such as a decrease in pore size or changes in charge-selective permeability. If this is the case, the mutations would not affect K\textsuperscript{+} recycling in the cochlea but might perturb cell function by altering second messenger transport between cells (1, 20).

The missense mutation that results in a non-conservative amino-acid substitution, R75W, causes autosomal dominant profound hearing loss that has been associated with a mild skin disorder, palmoplantar keratoderma (21). As mentioned above, expression of the R75W mutant in transgenic mice also causes deafness (16). In this work, we show that the bases for the defective gap-junctional communication elicited by expression of the Cx26 R75W mutant is the lack of formation of GJC by functional hemichannels.

**MATERIALS AND METHODS**

**Plasmid engineering**

Human wild-type (WT) Cx26 was amplified from genomic DNA using the following oligonucleotides: forward, 5′-TTAGAATTCCATGGGTCGACGCTGC-3′, and reverse, 5′-TAATCTAGATTATTATCCCACGCAACTGCTTTTTTGAATTTCCAG-3′. The PCR product was digested with EcoRI and XbaI and ligated into the pOcyt-7 plasmid (22) cut with the same enzymes. The WT Cx26 DNA sequence was identical to that deposited in the genebank as NM_004004. The R75W mutant was generated by site-directed mutagenesis using the QuickChange Multisite-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the following mutagenic oligonucleotide: 5′-CCCCATCTCCCACATCTGGCTATGGGCACTGCAGCTGATCTTCG-3′, which also includes a silent mutation to remove an ApaI site for primary screening (base mutations underlined). All sequences were confirmed by DNA sequencing at the Protein Chemistry Core Laboratory of the University of Texas Medical Branch.

**Expression of WT and R75W Cx26 in oocytes**

Isolation of *Xenopus laevis* oocytes, synthesis of cRNA and oocyte injection were performed as described (23). Oocytes were injected with 12.5 ng of antisense Cx38 oligonucleotide to reduce endogenous expression of Cx38 (23–25), alone (control) or with WT Cx26 and/or Cx26 R75W cRNA (generally 6–12 ng for GJC studies and 25 ng for GJH studies). After cRNA injection, the oocytes were maintained in a Barth’s solution (in mM, 88 NaCl, 1.0 KCl, 0.4 CaCl\textsubscript{2}, Ca(NO\textsubscript{3})\textsubscript{2} 0.3, 0.8 MgCl\textsubscript{2}, 10 HEPES/NaOH, pH 7.40), supplemented with 0.1 mg/ml gentamicin, 20 units/ml penicillin and 20 μg/ml streptomycin, and modified by increasing [CaCl\textsubscript{2}] to 5 mM in order to block GJH and prevent cell lysis (23). Most experiments were performed 2 days after injection. To assess the level of expression of WT Cx26 and Cx26 R75W at the plasma membrane, we performed Western blots on biotinylated oocyte plasma membranes (23) by
incubation with a cell-impermeable biotinylation reagent (EZ-Link Sulfo-NHS Biotin, Pierce Biotechnology, Rockford, IL) for 30 min at 4°C. Enrichment of the biotinylated membrane proteins was based on the biotin-streptavidin interaction and was performed as described (23). Blots were probed with a rabbit anti-Cx26 antibody (IC loop epitope, Zymed, South San Francisco, CA), and detection was by chemiluminescence using a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (ECL, Amersham Pharmacia Biotech, Cleveland, OH).

**Measurements of hemichannel and gap-junctional currents**

For GJH current measurements, oocytes in a recording chamber (RC-3Z, Warner Instruments, Hamden, CT) were superfused by gravity at a rate of ~2 ml/min. Voltage clamping and current measurements were performed with a two-microelectrode voltage-clamp amplifier (OC-725C, Warner Instruments), using the bath as reference. The basic bathing solution was ND96 (in mM, 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES/NaOH, pH 7.4), and all experiments were performed at room temperature (22–24°C). Borosilicate glass microelectrodes pulled with a horizontal puller (P-97, Sutter Instruments, Novato, CA) were filled with 3 M KCl, and had tip resistances of 0.5–1.5 MΩ when immersed in ND96 solution. Membrane currents were filtered at 1.0 kHz, digitized, stored, and analyzed with pCLAMP version 8.0 (Axon Instruments, Foster City, CA). Current-voltage (I-V) relationships were obtained by current measurements 100 ms or 5 s after the onset of 5 s square voltage pulses from -80 to +40 mV, in 10 mV steps, with 10 s intervals between pulses.

For the measurements of transjunctional current (Ij) in paired oocytes, the vitelline membrane was manually removed 1 day after cRNA injection (26). The oocytes were then paired with the vegetal poles facing each other, and incubated in Barth’s solution for 2–24 h prior to the electrophysiological measurements using the dual two-microelectrode voltage clamp technique (24, 27). The paired cells were clamped at –60 mV, and a transjunctional potential was generated by stepping the voltage of one cell from –60 mV, while holding constant the voltage of the other cell. The current supplied to the cell clamped at –60 mV is equal in amplitude, but opposite in sign, to Ij. Currents were recorded upon stepping the transjunctional voltage (Vj) from –120 to 120 mV (~80 to 80 mV in some experiments) for 10 s, at 20 mV intervals, and with 15 s intervals between pulses. The voltage of the cell clamped at –60 mV was used as reference.

**Single-channel recordings**

Patch pipettes pulled from borosilicate glass were Sylgard-coated and fire-polished to a resistance of 6–10 MΩ when filled with pipette solution (98 mM NaCl, 2 mM KCl, 5 mM HEPES/NaOH, pH 7.5) and immersed in nominally Ca²⁺/Mg²⁺-free ND96 solution. An Ag/AgCl bath electrode connected to the bath by an agar bridge (2% agar in ND96) was used as reference. Gigaohm seals (150–300 GΩ) were obtained by gentle suction on oocytes with the vitelline membrane removed, and currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Union City, CA). The currents were filtered at 2 kHz with an eight-pole Bessel filter and digitized online at 10 kHz (pClamp 8.2, Axon Instruments).
Statistics

Data are expressed as means ± SEM, and statistical differences were assessed by the Student’s t-test for unpaired data or one-way ANOVA, as appropriate.

RESULTS

Absence of gap-junctional communication mediated by Cx26 R75W

It has been previously shown that the R75W mutant (see Fig. 1A) is expressed at the plasma membrane but does not form gap junctions permeable to small inorganic ions or fluorescent dyes (5, 21, 28–30). The results in Fig. 1B confirm expression of R75W at the plasma membrane. In oocytes injected with equal amounts of WT or R75W Cx26 cRNA, the plasma-membrane expression of R75W Cx26 (estimated from densitometric analysis) was 2.5 ± 0.8-fold (n=4) that of WT Cx26. Figure 2 shows absence of gap-junctional currents in paired oocytes expressing the Cx26 mutant (21).

R75W forms functional GJH with altered voltage dependence

One possibility to explain the absence of R75W gap-junctional currents is that this mutant cannot form GJH or forms GJH that are impermeable. To test this possibility, we decided to study R75W at the hemichannel level. Two observations suggested the presence of functional GJH in oocytes expressing R75W: 1) Expression of WT or R75W Cx26 causes cell lysis that is prevented by increasing extracellular [Ca^{2+}] ([Ca^{2+}]_o). Cell lysis was evident 3 days after injection and probably results from the presence of GJH at the plasma membrane because it was prevented by increasing [Ca^{2+}]_o from 1.8 to 5 mM (data not shown). Others and us have observed oocyte cell lysis in response to heterologous connexin expression (23, 31). 2) Expression of WT or R75W Cx26 caused membrane depolarization in oocytes bathed in ND96 solution containing 1 mM Mg^{2+} and 0.7 mM Ca^{2+} (WT: –14±1 mV, n=17; R75W: –20±3 mV, n=13), compared with the membrane voltage of cells injected with anti-Cx38 antisense oligonucleotide alone (–50 ±3 mV, n=19, P<0.005 vs. either the WT or the R75W values). This is indicative of the presence of poorly selective GJH in the plasma membrane.

To confirm and quantify the presence of functional R75W GJH, we measured hemichannel currents. Figure 3A shows WT Cx26 whole-cell hemichannel currents in response to lowering [Ca^{2+}]_o. The control records shown in Fig. 3A were obtained in oocytes bathed with ND96 solution containing 0.7 mM Ca^{2+} and 1 mM Mg^{2+}. These concentrations were sufficient to block 85 ± 3% (n=7) of WT Cx26 GJH conductance. An additional block was obtained by increasing [Ca^{2+}] to 5 mM (95±2%, n=7). In addition, from a nominally Ca/Mg-free solution, increasing [Mg^{2+}] to 1 or 5 mM reduced the WT Cx26 GJH conductance by 72 ± 6% (n=7) and 94 ± 1% (n=11), respectively. Therefore, the concentration that blocks Cx26 hemichannel currents by 50% is likely in the micromolar range, a value significantly lower than those determined for Cx32 and Cx46, which are in the millimolar range (32, 33).

The WT Cx26 hemichannel currents did not exhibit significant voltage-dependent inactivation in the voltage range studied (–80 to +40 mV, Fig. 2), consistent with the properties of the WT Cx26 GJC (34–36). The WT Cx26 GJH were blocked by 18βG (Fig. 2 and Fig. 3A, B), as expected.
from hemichannel data on other connexins (1, 23, 37). R75W also formed functional GJH blocked by divalent cations (Fig. 3A). However, the voltage dependency of WT and R75W Cx26 GJH currents clearly differed (Fig. 3). WT Cx26 GJH showed a nearly linear instantaneous I-V relationship (Fig. 3A, B). In contrast, the R75W GJH display a rectifying instantaneous I-V relationship, with inactivation at negative voltages and activation at positive voltages (Fig. 3A, C).

**Single R75W GJH have a conductance similar to that of WT Cx26 single GJH**

As shown in Fig. 3, the whole-cell I-V relationships of WT and R75W Cx26 GJH differ significantly. To identify the biophysical bases of these differences, we performed studies on excised, inside-out single GJH. Figure 4A shows typical single-hemichannel records of WT Cx26. The GJH displayed a unitary conductance (γ) of 281 ± 13 pS (Fig. 4C), consistent with ~150 pS single gap-junctional channels (1, 35, 36) formed by two Cx26 GJH in series. The record in Fig. 4B shows open-channel noise similar to that of Cx26 GJC and several subconductive states between the fully open and closed states (35). Smaller subconductive states (near the closed state) were also observed (Fig. 4A). These properties of the WT Cx26 single-GJH are consistent with the available single gap-junctional channel data (36, 37). Single GJH were reversibly blocked by Ca2+ (data not shown).

The whole-cell R75W GJH show lower instantaneous conductance at cell positive voltages (Fig. 3). We performed experiments on single R75W GJH in order to determine whether the whole-cell membrane-conductance rectification and the decrease in conductance and permeability (Fig. 3) were caused by changes in single-hemichannel conductance (γ) and/or open probability (Pₒ). The γ of the fully open R75W single GJH (300 ± 8 pS, see Fig. 4C) was not different from that of the WT Cx26 single GJH (281 ± 13 pS, see above) and did not show significant rectification (Fig. 4C, D). The records in Fig. 4C show that the dwell time of R75W GJH in the fully open state is significantly shorter at +40 mV than at −40 mV. The Pₒ, calculated from >30 s-long records of the experiment shown in Fig. 4A, were 0.59, 0.72, 0.98, and 0.97, for the +40, +20, −20, and −40 mV-records, respectively. The corresponding Pₒ values for the experiment shown in Fig. 4C were 0.02, 0.59, 0.74, and 0.74, for the +40, +20, −20 and −40 mV-records, respectively. Therefore, the single GJH data indicate that the decreased permeability and altered voltage dependence of R75W GJH in the whole-cell studies are caused by changes in the voltage dependence of Pₒ, with no changes in γ.

**Dominant effect of R75W on WT Cx26 at the GJC, but not GJH level**

Figure 5 shows that coexpression of Cx26 R75W inhibits the gap-junctional communication mediated by WT Cx26, WT Cx30, or GJC formed by heteromeric Cx26-Cx30 GJH. These results confirm previous observations (5, 16, 21, 28), extend them to inhibition of GJC formed by heteromeric Cx26-Cx30 GJH and are consistent with the dominant nature of the deafness associated with Cx26 R75W (21). In oocytes injected with equal amounts of cRNA, plasma-membrane expression of R75W Cx26 was higher than that of WT Cx26 (2.5-fold, Fig. 1B). However, the dominant nature of the inhibition of gap-junctional communication by expression of Cx26 R75W was also apparent in oocyte pairs co-injected with WT and R75W Cx26 at a ratio of 12 to 1 (Fig. 5B). Recent studies on another mutant, Cx26 M34T, showed absence of gap-junctional communication between paired oocytes expressing the mutant, but cell-to-cell currents
were detected between an oocyte expressing the mutant and a paired oocyte expressing WT Cx26 (38). This observation suggests that M34T forms GJH that can dock to other GJH forming GJC. Our results with R75W (Fig. 5) differed from those obtained with M34T Cx26 because no functional GJC were formed between an oocyte expressing R75W and another one expressing WT Cx26 (data not shown).

In oocytes injected with equal amounts of WT Cx26 and R75W cRNAs, the hemichannel currents were similar to those of WT Cx26 GJH (Fig. 6). These oocytes, however, cannot form GJC (Fig. 5), and therefore the R75W phenotype is dominant at the GJC but not at the GJH level.

DISCUSSION

These studies confirm that R75W cannot form functional GJC and are consistent with deafness caused by defective cell-to-cell communication (5, 11, 12, 21, 28–30). However, the results presented here indicate that R75W can form functional GJH, although their properties are different from those of WT Cx26 GJH. It has been proposed, based on comparison of detergent-solubilized WT and R75W Cx26, that R75W connexons are not stable (29). Our results clearly show that R75W connexons in membranes are sufficiently stable to form functional GJH and indicate that the absence of gap-junctional communication is not due to lack of GJH formation or to lack of connexon stability.

The macroscopic conductance in oocytes expressing R75W mutant hemichannels (measured between –10 and +10 mV to mimic normal conditions, where the voltage across the GJC is near zero), was ~45% of that measured in WT Cx26-expressing oocytes (144 ± 19 vs. 330 ± 62 μS (n=11, P<0.005). If there were normal docking of R75W GJH, with GJC opening, there should be measurable gap-junctional currents. Therefore, differences in GJH ion permeability between R75W and WT Cx26 GJH cannot explain the absence of gap-junctional communication mediated by R75W. Moreover, coexpression of WT and R75W Cx26 yielded GJH currents indistinguishable from those through WT Cx26 GJH. If there were docking with normal gating, the gap-junctional currents through these WT/R75W connexons would be similar to that through WT Cx26 gap-junctional channels. Therefore, the absence of gap-junctional currents by expression of R75W Cx26 results from defective GJH docking or formation of impermeable gap-junctional channels upon docking (gating defect upon docking). This is consistent with the location of the R75W mutation in the first extracellular loop (EC1), which is critical for GJH docking (1, 39). One possibility is that docking of GJH containing R75W results in formation of impermeable GJC because EC loop mutations and connexin EC loop chimeras affect channel gating (1). Other possibilities are lack of docking or defective docking due to lack of formation of GJC by functional GJH. In the latter, R75W gap-junctional channel behavior will be equivalent to that of a hemichannel, ineffective for cell-to-cell communication. Studies with Cx26 R75W fused to green fluorescent protein showed fluorescent patches consistent with gap junctions (30), whereas electron-microscopy studies suggest reduced gap junction formation (29). Additional studies will be necessary to distinguish between the mechanisms outlined above. In any case, to our knowledge, this is the first report of a disease-associated connexin mutant that can form functional hemichannels but cannot form functional gap-junctional channels.
Defective docking could result in a leak communicating the cytoplasm with the extracellular medium. In the latter scenario, hemichannel-like behavior of the R75W channels could contribute to the death of cochlear cells expressing this mutant. The presence of permeable GJH is associated with cell lysis (see Results), and accumulating evidence indicates a role of Cx43 GJH in the cell damage that occurs in conditions such as hypoxia in a variety of cells, including epithelial, muscle and neural cells (40–42).

In summary, we show that the absence of gap-junctional communication that occurs in the cochlea because of expression of the dominant R75W Cx26 mutant is fundamentally a consequence of the formation of GJH that cannot dock normally, as WT Cx26 GJH, to form functional GJC. The dominant nature of the R75W mutation is evident only by the absence of functional GJC, and not at the level of formation of functional GJH. Because the R75W mutant is well expressed at the plasma membrane and previous observations suggest that R75W can form anatomic plaque-like structures (29, 30), a role of uncoupled R75W GJH in the death of cochlear cells is possible.

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**Figure 1.** Cx26 topological organization and expression. A) Schematic representation of WT Cx26 and the R75W mutant. The transmembrane helices (M1-M4), N-terminal region (NT region), intracellular loop (IC loop), C-terminal region (CT tail), and extracellular loops (EC1 and EC2) are indicated. The filled circle denotes the approximate location of the R75W mutation. B) Plasma membrane expression of Cx26 and R75W. Immunoblots of surface-biotinylated membranes from frog oocytes injected with anti-Cx38 antisense oligonucleotide alone (−) or together with cRNA coding for WT Cx26 (WT) or Cx26 R75W (R75W). The blot was probed with a specific anti-Cx26 antibody and detection was by chemiluminescence. Data representative of three similar blots.
Figure 2. Gap-junctional channel activity. A) Typical gap-junctional currents (Ij) measured in paired oocytes. The records were obtained in oocytes injected with anti-Cx38 antisense oligonucleotide alone (anti-Cx38) or together with WT Cx26 (WT Cx26) or Cx26 R75W (R75W) cRNA. The paired cells were clamped at –60 mV, and a transjunctional potential was generated by stepping the voltage of one cell from –60 mV, while holding constant the voltage of the other cell. Junctional currents (Ij) were recorded upon stepping the transjunctional voltage (Vj) from –80 to 80 mV, at 20 mV intervals. See Materials and Methods for additional details. B) Average gap-junctional current-voltage relationships. Measurements performed 10 s after imposing the transjunctional voltage pulse in anti-Cx38 (■), WT Cx26 (○), and R75W (△) oocyte pairs. Measurements at 0.1 s after the onset of the voltage pulse are also shown for WT Cx26 oocyte pairs (●). Data are means ± SEM of 6–7 experiments.
Figure 3. Whole-cell gap-junctional hemichannel activity. A) Typical hemichannel currents. Records were obtained in single oocytes bathed with ND96 solution containing 0.7 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) (control) and nominally Ca\(^{2+}/\)Mg\(^{2+}\)-free solution in the absence (low Ca) or presence of 20 \(\mu\)M 18\(\beta\)-glycyrrhetinic acid (18\(\beta\)G). The whole-cell currents were recorded upon clamping the voltage to values between –80 and 40 mV, at 10 mV intervals. B) Average WT Cx26 hemichannel current-voltage relationships. Data obtained 0.1 s (○) and 5 s (△) after the pulse in the absence (\(n=9\)) or presence of 18\(\beta\)G (●, \(n=8\)). Data are means ± SEM. C) Average R75W Cx26 hemichannel current-voltage relationships. See (B) for symbols. Data are means ± SEM from \(n = 6\).
Figure 4. Single Cx26 gap-junctional hemichannels. **A)** WT Cx26 GJH current records of an excised inside-out patch at several holding voltages (referred to the pipette) in symmetric nominally Ca\(^{2+}\)/Mg\(^{2+}\)-free solution. The closed and fully open states are labeled c and o, respectively. For display, the records were filtered at 300 Hz. No channels of these characteristics were observed in oocytes injected with anti-Cx38 antisense oligonucleotide alone. **B)** Single-hemichannel record (holding voltage = –40 mV) showing intermediate conductance levels and open-channel noise. Subconductive states are labeled with lines between the fully open (o) and closed (c) states. **C)** R75W Cx26 GJH current records of an excised inside-out patch in symmetric nominally Ca\(^{2+}\)/Mg\(^{2+}\)-free solution. See (**A**) for details. The scale on the right of (**C**) applies to all records. **D)** Current-voltage relationships of the fully open state of single WT Cx26 (●, \(n=7\)) and Cx26 R75W (Δ, \(n=7\)) gap-junctional hemichannels obtained from experiments such as those shown in (**A**) and (**C**). The solid lines represent linear fits to the data with slopes of 276 and 300 pS for WT and R75W Cx26 hemichannels, respectively.
**Figure 5.** Dominant effect of the R75W mutation. A) Inhibitory effect of R75W expression on gap-junctional currents measured in oocyte pairs expressing Cx26 or Cx26 and Cx30 (Cx26-Cx30). The oocytes were injected with equal amounts of Cx26, Cx30, and Cx26 R75W cRNA, alone or in the combinations indicated. The cells were voltage-clamped at –60 mV, and a transjunctional potential was generated by stepping the voltage of one cell to values between –120 and 120 mV, at 20 mV intervals, with the voltage of the cell clamped at –60 mV as reference. B) Effects of R75W on gap-junctional conductance (Gj) calculated between –10 and +10 mV, from the steady-state currents of experiments such as those depicted in (A). The oocytes were injected with equal amounts of Cx26, Cx30, and Cx26 R75W cRNA, alone or in the combinations indicated, except in the case of Cx26-R75W co-injections, where the relative amounts of Cx26 and R75W are shown (1:1, 2:1, and 12:1, with the Cx26 cRNA injection kept constant at 12.5 ng). The data are means ± SEM of 6–12 experiments, except in the case of the oocyte pairs injected with Cx30 cRNA alone (n=4). R75W injection decreased Gj significantly in all cases (P<0.01).
Figure 6. Activity of WT Cx26-R75W hemichannels. A) Gap-junctional hemichannel whole-cell current measured in single oocytes injected with equal amounts of WT Cx26 and Cx26 R75W cRNA. The record was obtained in nominally Ca\(^{2+}\)/Mg\(^{2+}\)-free solution. See legend to Fig. 4A for voltage protocol. B) Average hemichannel current-voltage relationships from experiments such as that shown in (A). Data obtained 5 s after initiation of the voltage pulse are presented as means ± SEM (n=6).