Mutations of connexin 26 at position 75 and dominant deafness: Essential role of arginine for the generation of functional gap-junctional channels

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Abstract

Gap-junctional channels are large intercellular aqueous pores formed by head-to-head association of two gap-junctional hemichannels (connexin hexamers), one from each of the adjacent cells. The mechano-transduction of sound waves into electrical impulses occurs in the cochlea, which houses the organ of Corti. Hereditary deafness is frequent and mutations of connexin 26, the predominant connexin of the cochlea, are its most frequent cause. Mutations of R75 cause deafness and disrupt gap-junctional communication. Here, we determined the effects of substitutions of R75 with different residues (alanine, asparagine, aspartic acid, lysine, phenylalanine, tyrosine or tryptophan) on formation of gap-junctional channels and hemichannels. We show that connexin 26 R75 is essential for the formation of gap-junctional channels. Substitution of R75 with aromatic residues yields functional hemichannels that display altered voltage dependence, whereas substitution with other residues yields non-functional hemichannels. The expression of R75 mutants has a dominant negative effect on gap-junctional communication mediated by wild-type connexin 26, independently of the ability of the mutants to form functional gap-junctional hemichannels. Our results show that the arginine located at position 75 of connexin 26 is essential for function, and cannot be replaced by other residues.

Keywords: Cx26; Hemichannels; Gap junction; Aromatic amino acids; Skin disease; Genetic disease

1. Introduction

Connexins are the proteins that form the gap-junctional channels that mediate cell-to-cell permeation of small inorganic ions and larger hydrophilic molecules, hence underlying electrical and chemical coupling between neighboring cells (Harris, 2001). Gap-junctional channels are formed by end-to-end docking of connexons or gap-junctional hemichannels, one from each of two adjacent cells (Harris, 2001; Sosinsky and Nicholson, 2005). These gap-junctional hemichannels are symmetric connexin hexamers (Unger et al., 1999; Harris, 2001; Sosinsky and Nicholson, 2005).

Mutations of wild-type connexin 26 (WT Cx26) are the most common cause of profound genetic hearing loss (Petit et al., 2001; Harris, 2001; Sosinsky and Nicholson, 2005).

Abbreviations: Cx26, connexin 26; G_{GJH}, Gap-junctional hemichannel conductance; G_{Gj}, Gap-junctional channel conductance; I_{Gj}, Gap-junctional current; WT, Wild-type

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An extensive network of gap junctions that includes all supporting cells communicates several cochlear cell types, but it does not connect with the hair cells (Forge and Wright, 2002; Forge et al., 2003a; Kikuchi et al., 2000). Activation of the hair cells by sound waves opens non-selective cation channels, causing Ca$^{2+}$ and K$^+$ influxes and membrane depolarization (Forge and Wright, 2002). Defective cell-to-cell communication caused by Cx26 mutants can decrease K$^+$ transport via the supporting cells and other cochlear cells to the stria vascularis, decreasing K$^+$ recycling and increasing [K$^+$] in the perilymphatic space between the hair and supporting cells. This could cause death of hair and supporting cells because of chronic depolarization or other mechanisms (Johnstone et al., 1989; Cohen-Salmon et al., 2002; Rizzuto et al., 2003). Alternatively, Cx26 mutants may display selective permeability changes, forming channels permeable to K$^+$, but with limited permeability to larger solutes, affecting cell function by altering, for example, second messenger transport between cells (Beltramello et al., 2005; Zhang et al., 2005).

The missense mutations R75Q (R, arginine; Q, glutamine) and R75W (W, tryptophan) cause autosomal dominant profound hearing loss that has been associated with a mild skin disorder, palmoplantar keratoderma (Richard et al., 1998; Rabionet et al., 2000; Uyguner et al., 2002; Feldmann et al., 2005). Expression of the Cx26 R75W dominant mutant in transgenic mice also causes deafness, which is associated with death of supporting and hair cells (Kudo et al., 2003). These observations, plus the facts that R75 is conserved among connexins and that mutations of R75 are associated with dominant X-linked Charcot-Marie Tooth disease, a hereditary peripheral neuropathy (Yum et al., 2002; Wang et al., 2004), strongly suggest that R75 is important in connexin function. In this work, we studied the functional effects of substitution of R75 of Cx26 with different residues, to elucidate the properties required for the residue at position 75 to yield functional Cx26, and to understand the mechanisms of genetic deafness caused by mutations of R75. We analyzed Cx26 mutants with alanine (A, smaller than arginine, non-polar), asparagine (N, smaller, polar), aspartic acid (D, smaller, anionic), lysine (K, smaller, cationic), phenylalanine (F), tyrosine (Y) or tryptophan (larger, aromatic) at position 75.

2. Materials and methods

2.1. Plasmid engineering

WT Cx26 and the Cx26 mutants were obtained as described (Chen et al., 2005). We generated the other mutants by site-directed mutagenesis (Quick Change Multipsite Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA) using human WT Cx26 (genbank NM_004004 sequence) into the plasmid pOcyt7 (Mo et al., 1999) as template. The mutagenic primers (mutated bases underlined) were: 5’-CCCCATCTCCACACATCGCTATGGGCACTGCAGCTGATCTTCG-3’ (R75A); 5’-CCCCATCTCCACACATCGCTATGGGCACTGCAGCTGATCTTCG-3’ (R75D); 5’-CCCCATCTCCACACATCGCTATGGGCACTGCAGCTGATCTTCG-3’ (R75F); 5’-CCCCATCTCCACACATCGCTATGGGCACTGCAGCTGATCTTCG-3’ (R75K); 5’-CCCCATCTCCACACATCGCTATGGGCACTGCAGCTGATCTTCG-3’ (R75N); 5’-CCCCATCTCCACACATCGCTATGGGCACTGCAGCTGATCTTCG-3’ (R75Q).

Fig. 1. Schematic representation of WT Cx26. The black rectangles represent transmembrane helices, and the arrow shows the approximate location of arginine 75.
2.3. Expression of WT Cx26 and Cx26 R75 mutants

Expression of WT Cx26 and Cx26 mutants at the plasma membrane was assessed from Western blots of biotinylated plasma membranes (Bao et al., 2004a). Biotinylation was performed 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 membrane proteins was based on the biotin–streptavidin interaction, and was performed as described (Bao et al., 2004a). For probing, we employed a rabbit anti-Cx26 antibody (IC loop epitope, Zymed, South San Francisco, CA). Detection was by chemiluminescence using a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (ECL, Amersham Pharmacia Biotech, Cleveland, OH). Analysis of the protein expression was performed by densitometric analysis of Western blots.

2.4. Measurements of hemichannel and gap-junctional currents

Oocytes were injected with 12 ng of antisense Cx38 oligonucleotide to reduce endogenous expression of Cx38 (Ebihara, 1996; Bao et al., 2004a,b), alone (control) or with WT Cx26 and/or Cx26 mutants cRNA (generally 3-6 ng for GJC studies and 12 ng for GJH studies). For GJH current measurements, oocytes were superfused by gravity, at a rate of ~2 ml/min, in a recording chamber (RC-3Z, Warner Instruments, Hamden, CT). Voltage clamping and current measurements were performed with a two-microelectrode voltage clamp amplifier (OC-725C, Warner Instruments), using the bath as reference. The bath solution for the experiments was ND96 (in mM, 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES/NaOH, pH 7.4). The “control” solution was ND96 with 0.7 mM CaCl2, and the “low-Ca” solution was nominally Ca2+- and Mg2+-free ND96. All experiments were performed at room temperature (22–24 °C). Borosilicate microelectrodes were produced with a horizontal puller (P-97, Sutter Instruments, Novato, CA) and had tip resistances of 0.5–1.5 MΩ when immersed in ND96 solution and filled with 3 M KCl. Membrane currents were filtered at 1.0 kHz, digitized, stored and analyzed with pCLAMP version 8.0 (Axon Instruments, Foster City, CA). The whole-cell currents were recorded upon clamping the voltage for 6 s between −80 and 40 mV, in 10 mV steps, with 10-s intervals between pulses. Average gap-junctional hemichannel conductances \( G_{\text{GJH}} \) were calculated from the data obtained 4.5 s after imposing the pulses.

For the measurements of transjunctional current (Ij) in paired oocytes, the vitelline membrane was manually removed 1 day after cRNA injection (Methfessel et al., 1986). The oocytes were then paired (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 (Spray et al., 1981; Bao et al., 2004b, 2005). cRNA injection was less (3–6 ng) than for the GJH measurements because preliminary experiments showed more reproducible Ij at this cRNA injection level. The paired oocytes were clamped at −40 mV, and a transjunctional potential was generated by stepping the voltage of one cell, while holding the voltage of the other cell constant. The current supplied to the cell clamped at −40 mV is equal in amplitude, but opposite in sign, to Ij. Gap-junctional currents were recorded upon stepping the transjunctional voltage to values from −120 to 120 mV, at 20-mV intervals, for 10 s. The voltage of the cell clamped at −40 mV was used as reference. Average gap-junctional conductances \( G_i \) were calculated from the data obtained 10 s after imposing the transjunctional voltage pulse, between transjunctional voltages of ±10 mV (to mimic normal conditions, where the voltage across the gap-junctional channels is near zero). The voltage pulses were longer than those used in the GJH current recordings to show the voltage inactivation at high positive voltages. GJH currents were not measured at these voltages because of the frequent presence of non-hemichannel currents activated at these voltages.

2.5. Data presentation and statistics

Data are expressed as means ± SEM and statistical differences were assessed by the Student t-test for unpaired data or one-way ANOVA, as appropriate.

3. Results

3.1. R75 mutants are targeted to the plasma membrane, but do not form functional gap-junctional hemichannels

All R75 mutants were expressed in the plasma membrane, as shown by Western blots of enriched oocyte plasma membranes purified by biotinylation with an impermeable reagent (Fig. 2a). The relative levels of connexin expression varied among oocyte batches, and the only statistically significant difference, compared to WT Cx26, was a decreased expression of R75N (Fig. 2b). Fig. 3 shows that in oocyte pairs expressing WT Cx26, gap-junctional channel currents are much larger than those in control oocyte pairs. Both WT Cx26 and R75 mutant cRNAs were co-injected with anti-Cx38 antisense oligonucleotide, to reduce endogenous expression of Cx38 (Ebihara, 1996; Bao et al., 2004a,b). Fig. 3a shows representative traces obtained in oocyte pairs injected with R75A, R75K, R75F and R75Y mutant cRNAs. None of these R75 mutants formed functional gap-junctional channels. Similar results were obtained for the mutants R75D, R75N and R75W (not shown). Average gap-junctional channel conductance values are presented in Fig. 3b.
3.2. Substitution of R75 with aromatic residues yields functional gap-junctional hemichannels with altered voltage dependence

We have previously shown that WT Cx26 expressed in frog oocytes forms hemichannels that yield whole-cell and single-hemichannel currents of properties consistent with those of gap-junctional channels (Chen et al., 2005). These hemichannels are blocked by Ca\(^{2+}\), and other known gap-junctional channel and hemichannel blockers (Chen et al., 2005). Relative to the membrane voltage of anti-Cx38 oocytes in control solution, expression of WT Cx26, R75F, R75Y or R75W produced statistically significant depolarizations. Expression of R75N and R75K did not produce depolarization, whereas expression of R75A and R75D slightly depolarized the cell membranes (Fig. 4a). The cell membrane depolarization is consistent with the presence of poorly-selective hemichannels in the plasma membrane of oocytes expressing WT Cx26, and the mutants R75F, R75Y and R75W. However, indirect mechanisms of depolarization cannot be ruled out, and the significance of the small depolarization in oocytes expressing the R75A and R75D mutants is unclear. To directly examine the presence of gap-junctional hemichannels, we measured whole-cell currents sensitive to divalent cations. The records in Fig. 4b show the presence of hemichannel currents activated in low-Ca solution in oocytes expressing WT Cx26 and the mutants R75F, R75Y and R75W. No significant currents were detected in oocytes expressing any of the other R75 mutants or in oocytes injected only with anti-Cx38 antisense oligonucleotides.
Representative current records of oocytes expressing R75A, R75D, and R75N, and control oocytes, are shown in Fig. 4b. These results are summarized in Fig. 4c. WT Cx26 hemichannel currents did not exhibit significant voltage dependent inactivation in the voltage range studied (−80 to +40 mV, Fig. 4b), similar to our previous observations (Ripps et al., 2004; Chen et al., 2005) and consistent with the properties of WT Cx26 gap-junctional channels (Oh et al., 1999; Suchyna et al., 1999; Beltramello et al., 2003). The blocking effect of Ca$^{2+}$ is clearly apparent from the difference between the currents under control and low-Ca conditions. We have previously shown that in oocytes expressing WT Cx26, the hemichannel currents are 85% blocked in the control solution (Chen et al., 2005). R75W
hemichannel currents show instantaneous $I-V$ relationship rectification, with inactivation at negative voltages and slight activation at positive voltages (see Chen et al., 2005 and Fig. 4b). These studies show that R75F and R75Y form gap-junctional hemichannels with similar kinetic properties to those formed by R75W.

3.3. Dominant effect of R75 mutant expression on gap-junctional communication mediated by WT Cx26

The data in Fig. 3 show that none of the R75 mutants studied can form functional gap-junctional channels. Others and us have shown that the R75W mutant has a dominant-negative effect on gap-junctional communication mediated by WT Cx26 (Richard et al., 1998; Forge et al., 2003; Marziano et al., 2003; Chen et al., 2005), consistent with the dominant nature of the deafness caused by the R75W mutation (Richard et al., 1998). The data in Fig. 5 show a marked inhibition of WT Cx26 gap-junctional channel currents in oocytes co-injected with WT Cx26 and two of the R75 mutants. For these experiments, we chose one mutant that does not form functional hemichannels (R75A) and one that does (R75Y). The results show that both mutants display the dominant negative effect, and therefore, this effect is independent on their ability to form functional hemichannels.

We have previously shown that the dominant negative effect of R75W is observed only at the gap-junctional channel level, i.e., WT Cx26/R75W hemichannels behave as WT Cx26 hemichannels (Chen et al., 2005). The effect of R75A is similar to that of R75W regarding a lack of dominant negative effect at the hemichannel level. In oocytes co-injected with equal amounts of WT Cx26 and R75A cRNAs, the hemichannel conductance in low-Ca$^{2+}$ solution was 88 ± 15% of that in oocytes injected with WT Cx26 alone ($n = 6$).

4. Discussion

Our results show that gap-junctional communication mediated by Cx26 is dependent on the presence of arginine at position 75. This is independent of the chemical properties of the side chains because none of the mutants studied could form functional gap-junctional channels between paired oocytes. The lack of gap-junctional channel activity occurred despite the expression of the mutants at the plasma membrane and the ability of R75F, R75Y and R75W to form functional gap-junctional hemichannels. Therefore, arginine at position 75 is essential to generate functional gap-junctional channels formed by Cx26.

The extracellular connexin loops are critical for hemichannel docking to form gap-junctional channels (Foote et al., 1998; Harris, 2001), and the available, albeit indirect, evidence suggests that $\beta$ sheets form a significant part of their structure (Foote et al., 1998; Harris, 2001). However, it seems that the transmembrane helix 1 extend beyond the membrane (Kronengold et al., 2003). It is then possible that R75 is part of an $\alpha$ helix because it is located at or close to the interface between the first extracellular loop and transmembrane helix 2. The region encompassing transmembrane segments 1 and 2 and the first extracellular loop is the most conserved region among connexins (Hua et al., 2003), and the arginine (R75 or equivalent positions) is completely conserved. The importance of R75 is suggested by the dominant deafness associated with the mutations R75W and R75Q (Richard et al., 1998; Rabionet et al., 2000; Uyguner et al., 2002; Feldmann et al., 2005; Maeda et al., 2005; Piazza et al., 2005), and also by the association of the Cx32 mutations R75W, R75Q and R75P with X-linked dominant Charcot-Marie-Tooth disease (Yum et al., 2002; Wang et al., 2004). However, the requirement for the presence of R75 may be unique to Cx26 because functional gap-junctional communication mediated by Cx32 R75Q has been reported (Wang et al., 2004).

Our results showing targeting of all the mutants to the plasma membrane is in agreement with results from others (Forge et al., 2003; Marziano et al., 2003; Thomas et al., 2003; Beltramello et al., 2005; Chen et al., 2005; Piazza et al., 2005), including the observation of gap-junctional plaques formed by the mutants R75A, R75W and R75D (Oshima et al., 2003). It is interesting that the mutants R75F, R75Y and R75W were capable of forming functional hemichannels, and that the dominant negative effect of mutants on WT Cx26-mediated gap-junctional communication is independent on their ability to form functional hemichannels. This was apparent from the observation that R75A, R75Y (in this study) and R75W (Chen et al., 2005) display the dominant negative effect, but R75A cannot form functional hemichannels. The presence of significant currents through R75F, R75Y and R75W hemichannels indicates that the absence of gap-junctional communication mediated by these mutants is not due to lack of hemichannel formation or to lack of connexon stability, as it has been suggested for R75W (Oshima et al., 2003).
Interestingly, the only mutants capable of forming functional GJH were those in which R75 was substituted by the larger aromatic residues phenylalanine, tyrosine or tryptophan. Even the substitution with lysine, which bears a net positive charge at near-neutral pH, like arginine, did not result in functional hemichannels. It is possible that the volume of the side-chain, rather than its charge, is important for the formation of functional hemichannels. However, the amphipatic nature of the arginine side chain (and not that of lysine), may be important. Stacking of the guanidinium arginine group, similar to that found with aromatic residues, is also possible, and may explain the formation of functional hemichannels by WT Cx26, R75F, R75W and R75Y. In this regard, chemical functional rescue of arginine mutations by guanidine/guanidinium has been reported (Rynkiewicz and Seaton, 1996; Boehlein et al., 1997; Williams et al., 2000), but 50 mM guanidine/guanidinium failed to rescue R75A hemichannel activity (not shown). In any case, it is clear that no residue can substitute arginine at position 75 of Cx26 without serious consequences, at a minimum an alteration of the voltage dependence of hemichannels and dominant abolishment of gap-junctional channel function.

The lack of docking without formation of gap-junctions is unlikely because R75W, R75Q, R75A and R75D form gap-junctional plaques (Oshima et al., 2003; Thomas et al., 2003; Piazza et al., 2005). Our results with the R75 mutants are compatible with two possible mechanisms of gap-junctional communication dysfunction: (a) defective docking where gap-junctional channel behavior will be equivalent to that of a hemichannel ("leak") between the cytoplasm and the extracellular medium) that could contribute to the death of cochlear cells expressing the R75W mutant (John et al., 1999; Contreras et al., 2002; Kudo et al., 2003; Vergara et al., 2003), and (b) a gating defect that results in closure of the channels upon docking. Independent of the mechanism of deafness associated with the R75Q and R75W mutants, our results clearly show that R75 is an essential residue for Cx26 function.

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References
