Gap junction-mediated intercellular biochemical coupling in cochlear supporting cells is required for normal cochlear functions

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Edited by Michael V. L. Bennett, Albert Einstein College of Medicine, Bronx, NY, and approved August 23, 2005 (received for review March 8, 2005)

Dysfunction of gap junctions (GJs) caused by mutations in connexin26 (Cx26) and Cx30 accounts for nearly half of all cases of hereditary nonsyndromic deafness cases. Although it is widely held that GJs connecting supporting cells in the organ of Corti mainly provide ionic pathways for rapid removal of K⁺ around the base of hair cells, the function of GJs in the cochlea remains unknown. Here we show that GJs were not assembled in the supporting cells of the organ of Corti until 3 days after birth in mice and then gradually matured to connect supporting cells before the onset of hearing. In organotypic cochlear cultures that were confirmed to express GJs, GJs mediated the propagation of intracellular Ca²⁺ concentration waves in supporting cells by allowing intercellular diffusion of inositol 1,4,5-trisphosphate. We found that a subset of structurally mild Cx26 mutations located at the second transmembrane region (V84L, V95M, and A88S) and a Cx30 mutation located at the first cytoplasmic segment (T5M) specifically affect the intercellular exchange of larger molecules but leave the ionic permeability intact. Our results indicated that Cx26 and Cx30 mutations that are linked to sensorineural deafness retained ionic coupling but were deficient in biochemical permeability. Therefore, GJ-mediated intercellular exchange of biochemically important molecules is required for normal cochlear functions.

Connexins (Cx) are a family of membrane proteins that form intercellular channels known as gap junctions (GJs). Cx26 and Cx30 are the two major types of Cxs found in the cochlear supporting cells and fibrocytes (1–4). Genetic linkage studies demonstrate that Cx mutations are found in approximately half of deaf patients with nonsyndromic sensorineural hearing loss (5, 6). So far, >100 Cx26 mutations and ≈5 Cx30 mutations are linked with human hearing impairments (6). One leading hypothesis proposes that GJs in the supporting cells provide pathways to allow rapid removal of K⁺ away from the base of hair cells, and the K⁺ ions are recycled back to the endolymph (7). This implies that Cx mutations cause deafness by disrupting intercellular ion movement among supporting cells. Consistent with the K⁺ recycling theory, some Cx26 mutations are found to nullify the GJ conductance. However, some deafness-linked Cx mutants (T8M, V84L, V95M, and N206S) do not significantly affect GJ-mediated ionic coupling (8–10). A recent report further shows that the V84L mutant impairs GJ permeability to inositol 1,4,5-trisphosphate (IP₃) (11). These emerging data suggest that cochlear GJs mediate diverse forms of intercellular signaling other than simply allowing K⁺ ions to diffuse intercellularly.

In addition to electrically coupling cells, such as in the propagation of action potentials through the myocardiun and at electrical synapses, GJs also mediate biochemical coupling that contributes to slower homeostatic cellular processes. Biochemical coupling by GJs, involving the intercellular transfer of signaling molecules and metabolites, is particularly vital for avascular embryonic as well as adult tissues (e.g., lens in the eye; ref. 12). Specific alteration of biochemical coupling results in cataracts (13). Whether changes in GJ-mediated biochemical coupling in the cochlea are sufficient to cause hearing impairment is unclear. We have used two-electrode patch–clamp and imaging techniques to systemically compare the effect of deafness-linked Cx mutations on GJ-mediated ionic and biochemical coupling. We found that several deafness-linked Cx26 (A84L, V95M, and A88S) and Cx30 (T5M) mutants specifically altered the permeation of GJs for molecules larger than simple ions [IP₃, propidium iodide (PI)]. By using long-term organotypic cochlear cultures in which supporting cells were confirmed to be coupled by GJs, we demonstrated that intercellular biochemical coupling in the cochlea-mediated IP₃ diffusion initiated Ca²⁺ waves, which is one type of long-range intercellular signaling known to contribute to many important cellular homeostatic processes (14). Together, our results indicate that intercellular biochemical coupling, mediated by GJs between supporting cells in the organ of Corti, is required for normal hearing.

Materials and Methods

Immunolabeling of Cxs in Cochlear Cryosections and Organotypic Cultures. CD-1 and Math1-eGFP mice were used in this study. Segments of the cochlea were dissected from 1-day-old (postnatal day 1 (P1)) mouse pups and cultured on glass coverslips coated with poly(l-lysine). The culture medium was 90% DMEM/10% FBS supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml). The medium was changed the day after plating and every other day thereafter. Cochlear cryosections and organotypic cultures were processed for fluorescent immunolabeling according to our published protocol (15). Samples were mounted with a SlowFade anti-fading mounting kit (Electron Microscopy Sciences, Hatfield, PA). The immunolabeling procedures were repeated at least four times at each developmental stage. The exposure time for digital photographing was adjusted such that the brightest labeling area in the field of view had a registered pixel intensity of 4,096 (we used a 12-bit camera). This prevented overexposure of samples and enabled us to compare the staining intensity among difference cell groups in the same cochlear section.

Creation of Point Mutations in the Cx Coding Sequence and Reconstitution of GJs in Human Embryonic Kidney (HEK)293 Cells. The Cx coding sequences were subcloned into the pEGFP vector (BD Biosciences, Palo Alto, CA) to create Cx-eGFP fusion proteins (Cx26-eGFP and Cx30-eGFP). Cx point mutations were created with the QuikChange II site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. GJ-deficient HEK293 cells (American Type Culture Collection, Manassas, VA) grown to ≈80% confluence were transfected with plasmids expressing eGFP-fused mutants. Six hours after transfection, cells were fixed and immunolabelled with anti-eGFP (Molecular Probes) and anti-Cx26 or anti-Cx30 antibodies. Immunolabeling was performed according to our published protocol (15).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GJ, gap junction; Cx, connexin; [Ca²⁺], intracellular Ca²⁺ concentration; IP₃, inositol 1,4,5-trisphosphate; PI, propidium iodide; Pn, postnatal day n.

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confluence were transfected with plasmid DNA (purity > 1.8) by using the FuGene6 transfection kit (Roche Diagnostics, Indianapolis) according to the protocol provided by the manufacturer.

Measurement of GJ-Mediated Intercellular Ionic Coupling by Two-Electrode Patch-Clamp and Ratio Imaging Methods. For in vitro characterizations of reconstituted GJs, we used cell pairs visually identified to be connected by GJs by the presence of EGFP at the apposing cell membrane. Functional conductance (Gf) was recorded by the two-electrode patch-clamp method. While holding the membrane potential steady at −20 mV for one cell (nonstimulated cell), the junctional voltage (ΔV) was changed from −110 to +110 mV by increasing the membrane potential of the other cell (the stimulated cell) in 10-mV steps. The current needed to hold the membrane potential stable in the nonstimulated cell (I0) reflected the junctional current. Gf was calculated by the equation: Gf = I0/ΔV.

Cells cultured on coverslips were loaded with the cell-permeant form of fura-2 [fura-2 acetoxymethyl ester (fura-2 AM)] (5 μM for 30 min) or Na+-binding benzofuran isophthalate-AM (10 μM for 120 min) to measure the intracellular concentrations of Ca2+ and Na+, respectively. Both indicators were purchased from Molecular Probes. Coverslips were placed in a perfusion chamber mounted on a Zeiss upright fixed-stage microscope (AxioSkop II, Zeiss). High concentrations of Ca2+ in an EGTA-free internal solution (120 mM KCl/10 mM Hepes/10 mM d-glucose, pH 7.2) or a high concentration of Na+ (137 mM, Hanks’ balanced salt solution was used) were introduced into the cells by forming the whole-cell recording configuration. During optical recordings, Ca2+ or Na+ concentration-related fluorescent signals (at 510 nm) were recorded by exciting the cells with a fast wavelength switcher (Lambda DG-4, Sutter Instruments, Novato, CA) alternatively at 340 and 380 nm. Images were collected by a ×40 objective (Zeiss, water achroplan, numerical aperture 0.8) and digitized with an intensified charged-coupled device camera (PTI-IC200, Photon Technology International, Lawrenceville, NJ). Experimental protocols were programmed and executed with the AXON IMAGE WORKBENCH software (Ver. 2) (Axon Instruments, Union City, CA). The standard ratio imaging technique (16) was used to record intracellular ion concentration changes. Details were given in our previous publications (15). The speed of Ca2+ wave propagation was calculated as the time difference in the onset of the Ca2+ waves divided by the distance that the wave traveled.

Assessment of GJ-Mediated Intercellular Biochemical Coupling with Dye Transfer Assay and IP3 Initiated Intercellular Ca2+ Signaling. For characterizing dye diffusion pattern and IP3 injection-induced Ca2+ waves in organotypic cochlear cultures, we confirmed the presence of GJs after experiments by performing immunolabeling procedures as outlined above. The cell-impermeable fluorescent dye (PI) was injected into cells by the single-cell electroporation method based on the technique described by Haas et al. (17). D-myo-inositol 1,4,5-trisphosphate hexapotassium salt (IP3, 250 μM) was dissolved in normal pipette internal solution (130 mM CsCl/10 mM EGTA/0.5 mM CaCl2/3 mM MgATP/2 mM Na2ATP/10 mM Hepes, pH 7.2). IP3 was introduced intracellularly by forming the whole-cell recording configuration. Cells were normally bathed in Hanks’ balanced salt solution supplemented with 20 μM ATP and 200 μM 8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid (suramin) to eliminate Ca2+ waves mediated by purinergic receptors (15).

Results

Developmental Expressions of Cochlear GJs Paralleled the Time Course of Postnatal Functional Development of the Organ of Corti. Supporting cells in the mature organ of Corti are well coupled by GJs consisting of Cx26 and Cx30 (3, 4, 18, 19). Because mice are born with an immature hearing organ, and their cochleae continue to develop postnatailly (20), we first examined the temporal and spatial expressions of GJs in the postnatal organ of Corti (Figs. 1 and 2). At birth, cells at the tip of the spiral limbus and bordering the basal cells in the stria vascularis were strongly labeled by antibodies recognizing Cx26 and Cx30 (Fig. 1). In contrast, Cx26 or Cx30 were not detected in the supporting cells in the organ of Corti before P3 (Fig. 2A–C and A′–C′). Cx26 and Cx30 immunoreactivity first appeared in the Deiter cells (small arrows in Fig. 2C) and some Claudius cells near the spiral ligament (arrowheads in Fig. 2C) at P3. At P5, immunoreactivity corresponding to Cx26 and Cx30 was found in larger number of Deiter cells than that at P3 (Fig. 2D and D′). Cx expression, however, was still absent in many supporting cells, including most Hensen’s cells, at P5. Not until P8 was Cx immunoreactivity detected in almost all supporting cells in the organ of Corti (Fig. 2E). In the adult cochlea (Fig. 2F), Cx26 were strongly expressed in the cell membrane of all supporting cells but not in the hair cells (labeled green in Fig. 2F) by an antibody against myosin7a.

Similar to results observed from cochlear sections (Fig. 2), Cx expression in the supporting cells showed a similar developmental profile in cochlear organotypic cultures (Fig. 3). Cx immunoreactivity in the spiral limbus region near the inner hair cells was intense from the beginning of the culture (P1 cochleae were used). Examples are indicated by small arrows in Fig. 3A and B. In contrast, very little Cx expression was detected in the supporting cell region (illustrated by a diagram in Fig. 1B) in cultures maintained up for 5 days (Fig. 3A, A′, B, and B′). Sparse Cx immunoreactivity started to appear in the supporting cell region after 5 days in culture (Fig. 3C and C′). The immunolabeling was more visible in the Deiter cell area under the outer hair cells (arrows in Fig. 3C′). After 8 days in culture (Fig. 3D), outgrown supporting cells were extensively coupled by GJs, as shown by the strong immunoreactivity at the cell membrane (Fig. 3D′) and diffusion of a cell-impermeable fluorescent dye (PI) intercellularly (data not shown). These results show that supporting cells in cultured organ of Corti developed intercellular coupling in vitro with a temporal sequence similar to that found in vivo (Fig. 2).

GJs in Supporting Cells Mediated the Propagation of Ca2+ Waves by Allowing Intercellular Diffusion of IP3. Based on our observations that cochlear supporting cells were not coupled by GJs until P8, we used P1 cochleae cultured for 8 days for functional studies. Fig. 4A shows the extensive expression of Cxs at the cell membrane of supporting cells in such cultures. The cell marked by an asterisk was injected with IP3 (250 μM), and the first-to-sixth-tier neighboring cells are indicated by numbers (Fig. 4A). Intracellular Ca2+ concentration ([Ca2+]i) increased quickly in injected cells, and the increases were transmitted to neighboring cells successively in the form of Ca2+ waves (n = 18, Fig. 4B). The Ca2+ waves moved at an average speed of 28 ± 5.2 μm/sec and over a mean distance of 245 ± 24 μm.

We next investigated the mechanism underlying the propagation of Ca2+ waves between supporting cells. The propagation of Ca2+ waves could have been mediated by cellular release of ATP and subsequent purinergic receptor activation (15). However, blockade of this pathway by combined application of suramin and ATP did not affect the properties of Ca2+ waves initiated by IP3 injections (Fig. 4C, average speed = 24 ± 6.2 μm/sec, average distance traveled = 231 ± 44 μm, n = 12). In addition, we found that a GJ channel blocker (flufenamic acid, 100 μM) (21) greatly reduced the range of Ca2+ wave propagation to 45 ± 7 μm (n = 8, Fig. 4D and F). The Ca2+ waves were not a consequence of direct mechanical stimulation during patch-clamping, because changes in [Ca2+]i were observed only after forming the whole-cell recording configuration and when IP3 was included in the pipette internal solution. The direct introduction
of Ca\textsuperscript{2+} into cells by adding up to 20 mM CaCl\textsubscript{2} in the pipette internal solution failed to initiate the propagation of Ca\textsuperscript{2+} waves, which showed that IP\textsubscript{3}-initiated Ca\textsuperscript{2+} waves were not due to intercellular Ca\textsuperscript{2+} diffusion. To further determine the involvement of GJs in Ca\textsuperscript{2+} wave propagation, we tested whether IP\textsubscript{3} injection induced Ca\textsuperscript{2+} waves in supporting cells of P1 cochlea. Consistent with the absence of Cx immunoreactivity in supporting cells at this time point (Fig. 3), intercellular propagation of Ca\textsuperscript{2+} waves after IP\textsubscript{3} injections was not observed at this developmental stage (n = 5, Fig. 4E). Moreover, intercellular Ca\textsuperscript{2+} waves were not detected upon IP\textsubscript{3} injection in HEK293 cells, which are not coupled by GJs (data not shown). Taken together, our findings support a mechanism where IP\textsubscript{3} diffusion through GJs underlies intercellular signal transmission for IP\textsubscript{3}-initiated Ca\textsuperscript{2+} waves in supporting cells.

Differential Effects of Cx Mutations Affecting Ionic and Biochemical Permeabilities. The effect of Cx mutations on the properties of GJs was analyzed by two-electrode patch–clamp recordings. HEK293 cells deficient in GJs were used to prevent contamination by endogenous GJs. These results, summarized in Table 1, which is published as supporting information on the PNAS web site, showed that most Cx26 mutations we studied completely disrupted intercellular electrical coupling. These included (i) mutations located in the extracellular loops: delE42, W44C, R75W, D179N, and R184Q (22–25); (ii) mutations located in the first transmembrane region: A40G, V37I, M34T, and I20T (26, 27); and (iii) one Cx26 mutation located in the second transmembrane domain (L90V) (28). In contrast, structurally mild Cx26 mutations located in the second transmembrane domain [V84L (29), V95M (29), and A88S (30)] specifically affected biochemical coupling. These mutants displayed little change in the intercellular conductance (Table 1) and voltage-dependent gating properties (Figs. 5 Left and 6 and Table 1). One Cx30 point mutation (T5M) (31) also did not significantly affect intercellular electrical coupling (Figs. 5 and 6 and Table 1) (see Fig. 7, which is published as supporting information on the PNAS web site).

Additional ratio imaging experiments were carried out to
characterize the Cx mutants that apparently showed normal intercellular conductance. Injecting high concentrations of \( \text{Ca}^{2+} \) (20 mM) or \( \text{Na}^{+} \) (137 mM) triggered sustained increases in \( [\text{Ca}^{2+}]_i \) and intracellular \( \text{Na}^{+} \) concentration \( ([\text{Na}^+]_i) \) in the injected cells (dark traces in Fig. 5 Center and Right). Consistent with data obtained with two-electrode patch-clamp recordings showing that wild-type GJs and mutant GJs (V84L, V95M, A88S, and Cx30T5M) showed similar ionic permeability (Fig. 5 Left, data traces), \( [\text{Ca}^{2+}]_i \), and \( [\text{Na}^+]_i \), in follower cells coupled to the source cell by these mutant GJs equilibrated with the source cells (Fig. 5 Center and Right, gray data traces). We used R75W GJ as a negative control based on two-electrode patch-clamp recordings data indicating a total loss of ionic coupling (Fig. 5). In contrast, IP3 injections elicited only transient \( [\text{Ca}^{2+}]_i \) elevations in the injected cells (cell 1 in Fig. 6, \( n = 10 \) for each mutant) if the cell pairs were coupled by mutant GJs containing V84L, V95M, or T5M (Fig. 6 B, C, and E). Interestingly, follower cells (cell 2 in Fig. 6) coupled to the source cell by A88S mutant GJs still showed transient rises in \( [\text{Ca}^{2+}]_i \). Apparently the A88S mutant GJs still allow IP3 to pass. However, the \( [\text{Ca}^{2+}]_i \) in follower cells started to increase only after the \( [\text{Ca}^{2+}]_i \) in the source cells returned to the baseline. Differences in the onset of the two responses changed from 3.6 ± 0.6 sec in wild-type GJs \( (n = 6) \) to 12.8 ± 3.1 sec in A88S mutant GJs \( (n = 6) \). These results were consistent with our previous findings (15) and suggest that altering the kinetics of GJ-mediated intercellular signaling may profoundly disrupt normal cochlear functions.

### Discussion

Congenital hearing loss is one of the most prevalent inherited human birth defects, affecting \( \approx 1 \) in 1,000 births. A strikingly high proportion \( (\approx 50\%) \) of nonsyndromic childhood deafness cases have been linked to mutations in the GJB2 coding for the Cx26, which included > 100 mutations (6). Cx mutations could disrupt normal GJ functions by affecting the stability, transport,
and membrane targeting of Cxs, as well as the assembly and permeability of GJs. Mutations that result in no formation of GJs in the cell membrane include R143W, V153I and L214P, W77R (10, 32). Many more Cx26 point mutants, e.g., V37I, L90P, S113R, delE120, M163V, R184P, 235delC, W44S, G59A, D66H, R75W, and R127H (8, 9, 33), are incorporated into GJs in the membrane. They interfere with either alignment of connexons or GJ permeability to abolish normal intercellular communications. Our previous work demonstrated that Cx26 and Cx30 are coassembled in the cochlea to form heteromeric GJs (15), although it is not clear exactly what proportion of cochlear GJs exist in homomeric and heteromeric forms. For mutant Cx26 or Cx30 coassembled in the cochlea to form heteromeric GJs (15), although it is not clear exactly what proportion of cochlear GJs exist in homomeric and heteromeric forms. For mutant Cx26 or Cx30 assembled into heteromeric GJs, mutant Cx may interact with the other partner Cx resulting in a transdominant mutational effect, as demonstrated by Forge et al. (34). In addition to the details underlying the molecular assembly of GJs in the cochlea, a central issue for understanding the function of GJs in the cochlea is how exchanges of molecules important for cellular signaling and metabolic activities are affected by Cx mutations. To address this question, we studied whether structurally mild Cx missense point mutations showed differential effects in disrupting ionic and biochemical permeabilities of GJs.

We used supporting cells in organotypic cultures as a model system to investigate intercellular coupling in the organ of Corti. Based on data observed from cochlear sections and cultures, we concluded that supporting cells were not coupled by GJs in the first 3 postnatal days. Extensive intercellular coupling did not appear until a few days before mice started to hear. These results were consistent with earlier findings obtained from Cx26 and Cx30 knockout mice showing that the absence of either Cx26 or Cx30 does not affect normal development of the organ of Corti (18, 19). In contrast to negative results obtained from 1-day-old cultures, IP3 injection elicited reliable Ca2+ concentration changes in the source cell (cell 1, black curves) and recipient cell (cell 2, gray curves) are shown in Right. Arrows under data traces mark the time points when whole-cell recording configuration was established with micropipettes containing 250 μM IP3.

Fig. 5. Effects of Cx mutations on ionic permeability of GJs reconstituted in HEK293 cells. Ionic permeability was measured by (i) double-electrode patch-clamp recordings (data traces, Left); (ii) intercellular transfer of Ca2+ ions measured by fura-2 ratio imaging (Center); and (iii) intercellular transfer of Na+ ions measured by SBFI ratio imaging (Right). Ratio signal representing ionic concentration changes in the cell injected (source cells, dark curves) and the neighboring cell coupled with GJs (follower cells, gray curves) are given.

Fig. 6. Effects of Cx mutations on the permeability of GJs to fluorescent dye (PI) and IP3. Data obtained from wild-type GJs (A) are shown as controls. Pictures in Left show dye diffusion patterns in the injected (cell 1) and recipient (cell 2) cells. IP3 injection elicited intercellular Ca2+ concentration changes in the source cell (cell 1, black curves) and recipient cell (cell 2, gray curves) are shown in Right. Arrows under data traces mark the time points when whole-cell recording configuration was established with micropipettes containing 250 μM IP3.
Biochemical coupling was not restricted to Cx26 mutations in that a Cx30 mutant (T5M) also demonstrated similar effect, and that the A88S mutant had even more subtle effects on biochemical coupling of GJs by slowing the kinetics of IP3 diffusion. Although we did not analyze the biophysical mechanism underlying these different results, these Cx mutations could alter GJ functions by causing channels to dwell longer in subconductance than in closed states, which block the passage of larger molecules (e.g., fluorescent tracer molecules and cAMP) while permitting a reduced permeation of inorganic ions [to <20% of the main open state (39)]. Our characterization of the developmental expression of GJs in the cochlea also strengthened our conclusion that biochemical coupling is important for propagating Ca2+ waves in supporting cells near the onset of hearing. Given that Cx mutations that specifically affect biochemical coupling cause human hereditary diseases (40, 41) and considering the results presented here and the recently available data (8–11), we conclude that GJ-mediated permeability to cytoplasmic signaling molecules and metabolites is required for normal cochlear function.

We thank Dr. Amy Lee for critically reading and Mrs. Kristen Radde-Kallwitz for proofreading the manuscript. This study was supported by grants from the Woodruff Foundation and the National Institute on Deafness and Other Communication Disorders (RO1-DC04709). Calcium ratio imaging equipment was supported partially by funds provided by the American Tinnitus Association.