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Conserved glycine at position 45 of major cochlear connexins constitutes a vital component of the Ca^{2+} sensor for gating of gap junction hemichannels

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ABSTRACT

Mutations in gap junction (GJ) family of proteins, especially in the connexin (Cx) 26, are responsible for causing severe congenital hearing loss in a significant portion of patients (30–50% in various ethnic groups). Substitution of glycine at the position 45 of Cx26 to glutamic acid (p.G45E mutation) causes the Keratitis–ichthyosis–deafness (KID) syndrome. Previous studies have suggested that this point mutation caused a gain-of-function defect. However, the molecular mechanism of KID syndrome remains unclear. Since glycine at this position is conserved in many Cxs expressed in the cochlea, we tested the hypothesis that glycine at position 45 is an important component of the sensor regulating the Ca^{2+} gating of GJ hemichannels. Using reconstituted Cx30, 32 and 43 expressed in the HEK 293 cells, we compared the functions of wild type and p.G45E mutant Cxs. We found that G45E in Cx30 resulted in similar deleterious cellular effects as Cx26 did. Cell death occurred within 24 h of transfection, which was rescued by increasing extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Dye loading assay showed that Cx30 G45E, similar to Cx26 G45E, had leaky hemichannels at physiological $[Ca^{2+}]_o$ (1.2 mM). Higher $[Ca^{2+}]_o$ reduced the dye loading in a dose-dependent manner. Whole cell membrane current recordings also indicated that G45E caused increased hemichannel activities. p.G45E mutations of Cx32 and 43 also resulted in leaky hemichannels compared to their respective wild types in lower $[Ca^{2+}]_o$. Our data in this study provided further support for the hypothesis that glycine at position 45 is a conserved Ca^{2+} sensor for the gating of GJ hemichannels among multiple Cx subtypes expressed in the cochlea.

1. Introduction

Connexins (Cxs) are protein subunits that constitute the intercellular gap junction (GJ) channels facilitating direct transfer of small metabolites, cellular messenger molecules and ions between neighboring cells [1]. Six Cx subunits oligomerize to form a connexon in cell membrane (also known as a GJ hemichannel). A fully functional GJ channel is produced when two hemichannels from adjacent cells align to form a complete GJ that allows intercellular communication among neighboring cells. At least twenty different types of human Cx genes have been discovered and they play essential roles in many organs [2,3]. It was once believed that hemichannel must remain in a closed state until they were aligned with another one from an adjacent cell, but recent studies have suggested that hemichannels may play an important role in maintaining homeostasis under different physiological conditions [4,5]. All connexins share a common structural motif in the cell membrane. They all contain four transmembrane (M1–M4), two extracellular (El and E2) with characteristically spaced cysteine residues, three cytoplasmic portions which are the amino-terminal (NT) and carboxy-terminal (CT) domains and the central cytoplasmic loop (CL) (Supplementary Fig. 1B). The variations of the molecular mass among Cxs are accounted for mainly by the different length of the CL and CT sequences [3]. The Cxs usually exhibit complex and overlapping expression patterns [2]. The major Cxs expressed in the cochlea include Cxs26, 30, 32 and 43, and most cochlear GJs are co-assembled from Cxs26 and 30 [6,7].

Mutations in Cx family of genes are involved in a variety of hereditary human diseases (e.g., hearing loss, dermatological disorders and demyelination neuropathy) [8,9]. The most common type of diseases caused by Cx mutations is congenital deafness. Among the different subtypes of Cxs, mutations in Cx26 and Cx30 are the most common cause of autosomal recessive nonsyndromic deafness found in almost all ethnic populations examined so far around the world [10,11]. Among more than one hundred reported disease-causing Cx26 mutations, the consequence of Cx26 G45E mutation is probably the most severe as it has been linked to a fatal form of KID syndrome [12]. This mutation is unique in causing a gain-of-function alternation for Cx26 hemichannels [13].

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Cx26-G45E mice displayed reduced viability, hyperkeratosis, scaling, skin folds, and hair loss [14]. Glycine at position 45 is located in the first extracellular loop next to an aspartic acid, and it is well conserved among all the cochlear Cxs (Supplementary Fig. 1A). The mutation G45E is next to a previously reported Ca^{2+} binding site for the Cx32 [15] and it changes a nonpolar aliphatic side chain to a negatively charged amino acid. Therefore, the location of mutation and the charge alteration make it likely that the G45E mutation could interfere with the Ca^{2+} regulation of the hemichannel gating of cochlear GJs.

It was reported that Cx26 p.G45E mutation causes cell death via leaky hemichannels (a gain-of-function mutation) at physiological extracellular Ca^{2+} concentration [13]. Since G45 locates in the first extracellular loop domain and is conserved in all the Cxs, we hypothesize that glycine at position 45 is a conserved Ca^{2+} sensor for the gating of GJ hemichannels among multiple Cx subtypes expressed in the cochlea. In order to test this hypothesis, we used *in vitro* expression system to investigate the functional effects of p.G45E mutation in Cxs30, 32 and 43.

2. Methods and materials

2.1. Site directed mutagenesis of connexins

Human Cx30, Cx32 and Cx43 cDNA were subcloned in pEGFP – N1 cloning vectors (Clontech Inc, Mountain View, CA) at the *Bgl* II site respectively. Enhanced green fluorescent protein (eGFP) was tagged to the C-terminal of the Cx coding sequence, which allowed direct visualization of Cxs protein in the cells. p.G45E mutations were made from wild-type (WT) Cxs cDNA using a site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene Inc., La Jolla, CA). Following forward and reverse primers containing the Cxs point mutations were synthesized (Sigma Inc., St Louis, MO): Cx30-G45E: (F) 5′-GCCCAGGAAGTGTGGGAAGACGAGCAAGAGGAC-3′, (R) 5′-GTCCTCTTGCTCGTCTTCCCACACTTCCTGGGC-3′. Cx32-G45E: (F) 5′-GGCTGCAGAGAGTGTGTGGGAAGATGAGAAATCTTCCTTC-3′, (R) 5′-GAAGGAAGATTTCTCATCTTCCCACACACTCTCTGCAGCC-3′. Cx43-G45E: (F)5′-GTTGAGTCAGCCTGGGAAGATGAGCAGTCTGCC-3′, (R) 5′-GGCAGACTGCTCATCTTCCCAGGCTGACTCAAC-3′. Thermal cycling protocol for polymerase chain reaction (PCR) was: (1) 94 °C for 30 s, (2) 68–70 °C for 30 s, (3) at 72 °C for 6–10 min extension. Total cycle numbers were 35. The synthesized mutant DNA was transformed into competent *Escherichia coli* (Invitrogen, Carlsbad, CA) and positive colonies were confirmed by sequencing (Anagen Sequencing Inc., Atlanta, GA).

2.2. Transfection into HEK293 cells

The human embryonic kidney (HEK) 293 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). One day before transfection, cells were resuspended by treating them with 0.05% trypsin and 0.02% EDTA and the appropriate seeding density was adjusted to achieve approximately 80% confluence after 24 h in culture. Transfections were done with the Fugene6 Reagent (Roche Diagnostics Co., Indianapolis, IN) according to the manufacturer's instructions. In experiments, we increased $[Ca^{2+}]_o$ to rescue cells transfected with G45E mutant. Extra Ca^{2+} was added up to 10 mM to check its effect on closing the hemichannels at this step.

2.3. Tests of hemichannel and gap junction functions

The fluorescent dye propidium iodide (PI, molecular weight 650, Invitrogen, Grand Island, NY) was used to measure the biochemical permeability of hemichannels with a dye loading assay based on the technique described by Haas [16]. Cells were incubated for 40 min in a modified Hanks balanced salt solution (HBSS, Sigma–Aldrich, St. Louis, MO) containing 0.15 mmol/L PI and various concentrations of extracellular Ca^{2+} as indicated in the text. In Ca^{2+} free HBSS 10 mmol/L EGTA was supplemented. The dye loading in transfected and untransfected cells, as determined by the presence of eGFP, was photographed and the percentage of dye loading for cells bathed in various $[Ca^{2+}]_o$ was calculated.

GJ biochemical coupling was measured with the single cell dye transfer assay. PI (1.5 mmol/L) was injected through a microelectrode by the single-cell electroporation method. Ionic permeability through GJ channels was measured with the intercellular transfer of Ca^{2+} assay according to the methods described by Sun [6].

Apoptotic cells were detected using an in situ cell death detection kit (Roche, Germany). Apoptotic cleavage of genomic DNA in the nuclei was identified by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) following the manufacturer's instructions. Positive controls for apoptosis were obtained by treating cultured cells with DNaseI.

All the tests were repeated four times.

3. Results

3.1. G45E mutation caused cell death, which could be rescued by increasing $[Ca^{2+}]_o$

To identify the functional consequences of G45E mutation, we expressed wild-type and G45E mutant Cxs in HEK 293 cells. Transfected cells were identified by the presence of green fluorescence (eGFP signal) in cells (Figs. 1–3). Cells transfected with G45E mutant Cx30 were swollen and began to detach from the bottom of culture dishes approximately 24 h after plating when bathed in normal HBSS which contained 1.2 mmol/L Ca^{2+}. Nearly all transfected cells died in about 3 days (Fig. 1B). In contrast, untransfected cells in the same culture dish appeared to be morphologically normal after 3 days (Fig. 1B). TUNEL staining of cells one day after transfection with Cx30 G45E showed positive immunoreactivity (Fig. 1C), suggesting ongoing apoptosis induced after expression of G45E mutant. Aberrant hemichannel opening may account for the cell death caused by G45E mutant. Many studies have shown that increasing $[Ca^{2+}]_o$ closes GJ hemichannels [17–19]. Thus, if cells transfected with Cx30 mutant G45E die as result of abnormal hemichannel activity, this outcome may be prevented in the presence of increased extracellular Ca^{2+} concentration. To test this hypothesis, we bathed the cells in 1.2–10 mmol/L $[Ca^{2+}]_o$ to test the differences in viability. As expected, viability of cells transfected with Cx30 G45E mutant was substantially increased (Fig. 1E). Fewer transfected cells (11.6%, $n = 257$) died 3 days after transfection, with 3.2 mmol/L $[Ca^{2+}]_o$ present in the culture medium (Fig. 2C). Survived transfected cells also formed normal GJs between cell pairs one day after transfection (Fig. 1F, indicated by arrow). These results suggested the death of cells transfected with Cx30 G45E was caused by leaky-hemichannel abnormality and can be rescued by extracellular higher $[Ca^{2+}]_o$.

We also found that after 40 min of incubation with a membrane-impermeable dye (propidium iodide [PI]), cells transfected with Cx30 G45E mutant allowed the loading of PI when bathed in normal HBSS which contained 1.2 mmol/L Ca^{2+} (Fig. 2A). In contrast, little dye loading was observed in cells transfected with the WT Cx30. It suggested that the Cx30 G45E may be a gain-of-function mutation similar to that reported for the G45E mutation in the Cx26 [13]. We next bathed the G45E transfected cells in HBSS with different Ca^{2+} concentrations to test the uptake of PI after 1 day of transfection. Results showed that the PI loading was reduced as $[Ca^{2+}]_o$ was increased in a dose dependent manner (Fig. 2). When

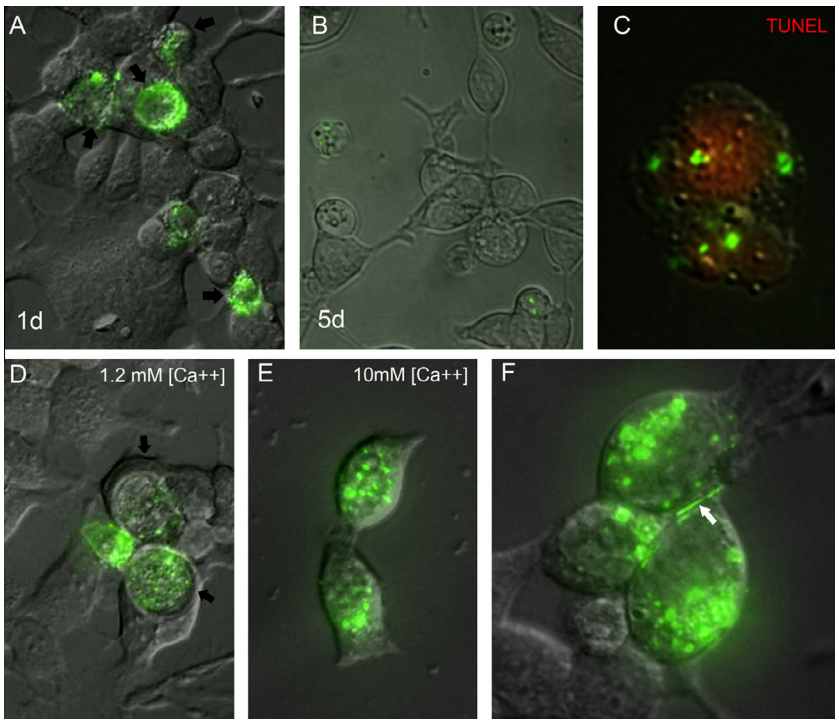

Fig. 1. Cell death caused by Cx30 G45E transfection could be rescued by increasing extracellular $[Ca^{2+}]_o$ (A) HEK293 cells transfected with Cx30 G45E (green eGFP, black arrow) 24 h after transfection, showing the apoptotic cell rounding and shrinkage. (B) All transfected cells were gone 3 days after transfection. Remained were untransfected cells with normal morphology. (C) Transfected cells (green eGFP) were also positive for TUNEL staining (pseudocolored in red in the nuclei of cells) 1 day after transfection, indicating ongoing apoptosis induced by Cx30 G45E transfection. (D) When bathed in physiologic $[Ca^{2+}]_o$ (1.2 mmol/L), HEK 293 cells transfected with Cx30 G45E (green eGFP) showed dysmorphic cellular morphology (black arrow) within 24 h of transfection. (E) Transfected cells survived and show normal morphology when bathed in 10 mmol/L $[Ca^{2+}]_o$. (F) Unlike Cx26 G45E, Cx30 G45E mutant formed very few gap junctions (white arrow) even after rescued by high $[Ca^{2+}]_o$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transfected cells were bathed in regular HBSS (1.2 mM $[Ca^{2+}]_o$), the loading of PI was noticed in 90% transfected cells ($n = 108$) which also displayed abnormal morphology (Fig. 2A) counted from random views of three dishes. In contrast, when the $[Ca^{2+}]_o$ in HBSS was increased to 5 mM, the number of cells loaded with PI decreased to 10% and the morphology of the cells was similar to those transfected with wild type Cx30 (Fig. 2D).

These data suggest that G45E mutation in Cx30 produces similar functional alternation as that found in the Cx26. The glycine at position G45 in Cx30 may have an important role in regulating the binding of extracellular calcium.

3.2. G45E mutant cells displayed increased hemichannel activities

If the increased dye uptake seen in Cx30-G45E mutant cells was mediated by the opening of connexin hemichannels, then one would predict that whole-cell membrane currents reflecting the opening of GJ hemichannels would also be increased. To test this hypothesis, we transfected cells with either wild-type Cx30 or the mutant Cx30-G45E and recorded membrane currents by whole-cell patch clamp electrophysiology. Cells transfected with wild-type Cx30 displayed minimal membrane currents when voltage ramps between −100 and 100 mV were applied (Fig. 2F). However, Cx30-G45E expressing cells exhibited larger whole-cell membrane currents at both hyperpolarizing and depolarizing voltages. The hemichannel opening/closing activities were greatly increased (Fig. 2G).

3.3. G45 is a conserved Ca^{2+} sensor among multiple cochlear Cx subtypes

The above results suggested that the mutation of glycine into glutamic acid at position G45 in Cx30 produced similar functional changes as previously reported in the Cx26. Since G45 is located in the first extracellular loop domain and is conserved in all the connexins, we hypothesized that glycine at position 45 is a conserved Ca^{2+} sensor for the gating of GJ hemichannels among multiple cochlear connexin subtypes. We therefore examined the functional consequences of G45E in Cx32 and Cx43 using the same *in vitro* testing system.

Cells transfected with either Cx32 or Cx43 G45E survived. They exhibited normal morphology, showing no sign of cell death even after 5 days in cultures (Fig. 3A and B). Transfected cells also formed normal gap junctions (Fig. 3A, B, H, and I), which allowed dye diffusion from the PI injected cell to the adjacent coupled cell (Fig. 3H and I). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in the stimulated cell (trace 1 in Fig. 3J and K) were rapidly transferred to the neighboring cell (trace 2 in Fig. 3J and K) through GJs consisting of either Cx32 G45E or Cx43 G45E. The results suggested that G45E mutation in the Cx32 and Cx43 did not affect GJ-mediated biochemical and ionic coupling. It is known that GJ hemichannels are opened by lowering $[Ca^{2+}]_o$ [17]. When cells were bathed in Ca^{2+}-free HBSS, most of the Cx43-G45E mutant cells (81%, $n = 165$) were loaded with PI (Fig. 3D) after 40 min of incubation. In contrast, much fewer WT Cx43 expressing cells (16%, $n = 171$) were loaded with PI (Fig. 3C) at the same time point. The difference of PI loading percentage between Cx43-G45E and WT Cx43 cells gradually increased over time after longer incubation with PI (Fig. 3E). Statistically significant difference was found between the mutation and the wild type by Chi square test ($\chi^2 = 142.27$, $p < 0.01$). The similar but smaller difference also existed between Cx32-G45E and WT Cx32 transfected cells. Percentage of cells loaded with PI was 19% ($n = 179$) and 4% ($n = 183$) for Cx32-G45E and WT Cx32 cells respectively. Chi square test revealed statistically significant difference between Cx32-G45E and WT Cx32 cells

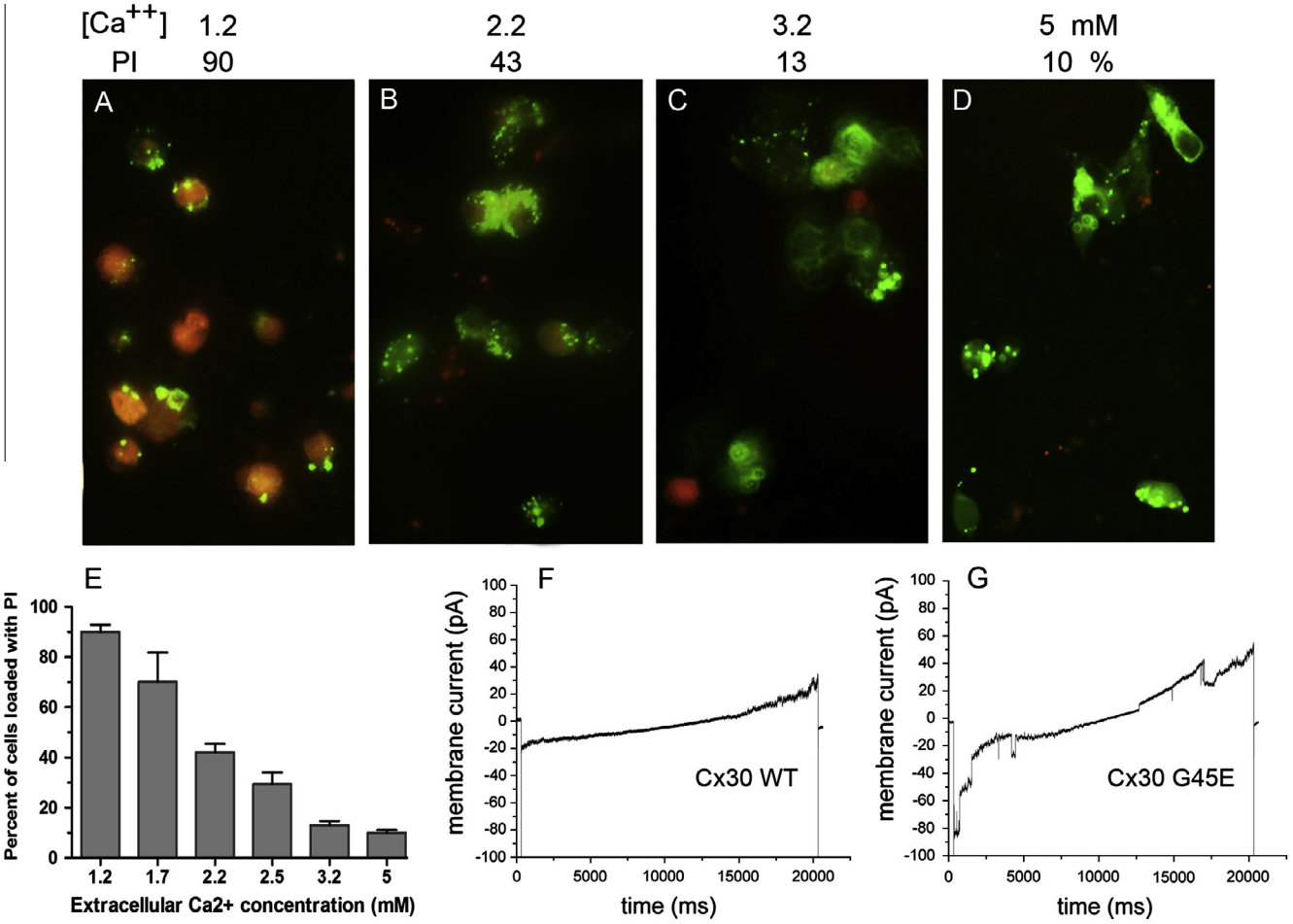

Fig. 2. Dependency of PI loading on $[Ca^{2+}]_o$, and Cx30 G45E mutation induced hemichannel activities. (A–E) Example a membrane impermeable fluorescent dye (propidium iodide [PI]) loading in 1.2 (A), 2.2 (B), 3.2(C), 5(D) mM of $[Ca^{2+}]_o$ are given. (E) The percent of cells trasfected with G45E mutant Cx30 that allowed PI loading is quantified by a bar chart. $N = 4$ independent experiments. (F–G) Hemichannel activities of cells transfected with either Cx30 WT (F) or Cx30 G45E (G) were measured by patch clamp electrophysiology. Increased hemichannel opening/closing activities were observed in cells transfected with Cx30 G45E mutant (G).

($\chi^2 = 20.13$, $p < 0.01$). These results indicated that the mutation of G45E in Cx43 and Cx32 shifted the $[Ca^{2+}]_o$ dependency of hemichannel gating, and induced more leaky hemichannels in lower $[Ca^{2+}]_o$, although it did not cause cell lethality in normal extracellular $[Ca^{2+}]$.

4. Discussion

Gap junctions are composed of oligomeric structures known as connexons or hemichannels, which are in turn formed from a large family of protein subunits called connexins. There is now general, if not universal, agreement that hemichannels can open in response to various physiological and pathological stimuli when they are not apposed to another hemichannels and face the external milieu. Mutations in connexin coding genes are related to many kinds of human diseases including hearing impairment. GJB2 (coding for Cx26) are responsible for more than a large proportion of non-syndromic hearing impairment cases in almost all ethnic populations studied. In addition to Cx26, mutations in Cx30, are known to cause hereditary hearing loss. According to connexin mutation homepage (http://davinci.crg.es/deafness/), there have been more than 100 mutations of connexins related to hearing loss reported. The mutations causing gain-of-function effect are specific, which can produce abnormal hemichannels in resting state resulting in overloading of harmful molecules to threaten the viability of the cells. *In vitro* study revealed that Cx26 G45E belonged to gain-of-function mutation category. It caused the most severe symptoms of KID, which could include the lethal form during the first year of life [20].

The calcium concentration plays an important role in regulate the gating of hemichannels. The divalent cation blockage should be considered as a physiological mechanism to protect the cell from the potentially adverse effects of leaky hemichannels [15]. G45 is located in the first extracelluar loop next to an aspartic acid that is a suspected calcium binding site for Cx32 GJ hemichannels. G45E changes a nonpolar aliphatic side to a negatively charged amino acid and may influence the sensitivity of the hemichannels gating to the calcium. Studies show that this mutation does not affect the formation of Cx26 containing GJs. However, the voltage-sensitive gating was greatly affected to more sensitive side. Mutational G45E hemichannel displayed significantly greater whole cell currents than wild type Cx26, leading to cell lysis and death [13]. This severe phenotype could be rescued in the presence of elevated Ca^{2+} levels extracellularly. This mutation provides a route for excessive entry of Ca^{2+}, which may severely comprise cell integrity and lead to cell death [13]. The leaky hemichannels are also suspected to be the cause of abnormality of epidermal differentiation process and the dysplasia of the cochlear and the saccular neuroepithelium [21].

All connexins share a common structural motif in the cell membrane. Many Cxs expressed in cochlea including Cx26, Cx30, Cx29, Cx31, Cx32, Cx43 and Cx45. The major ones are Cxs26, 30, 32 and

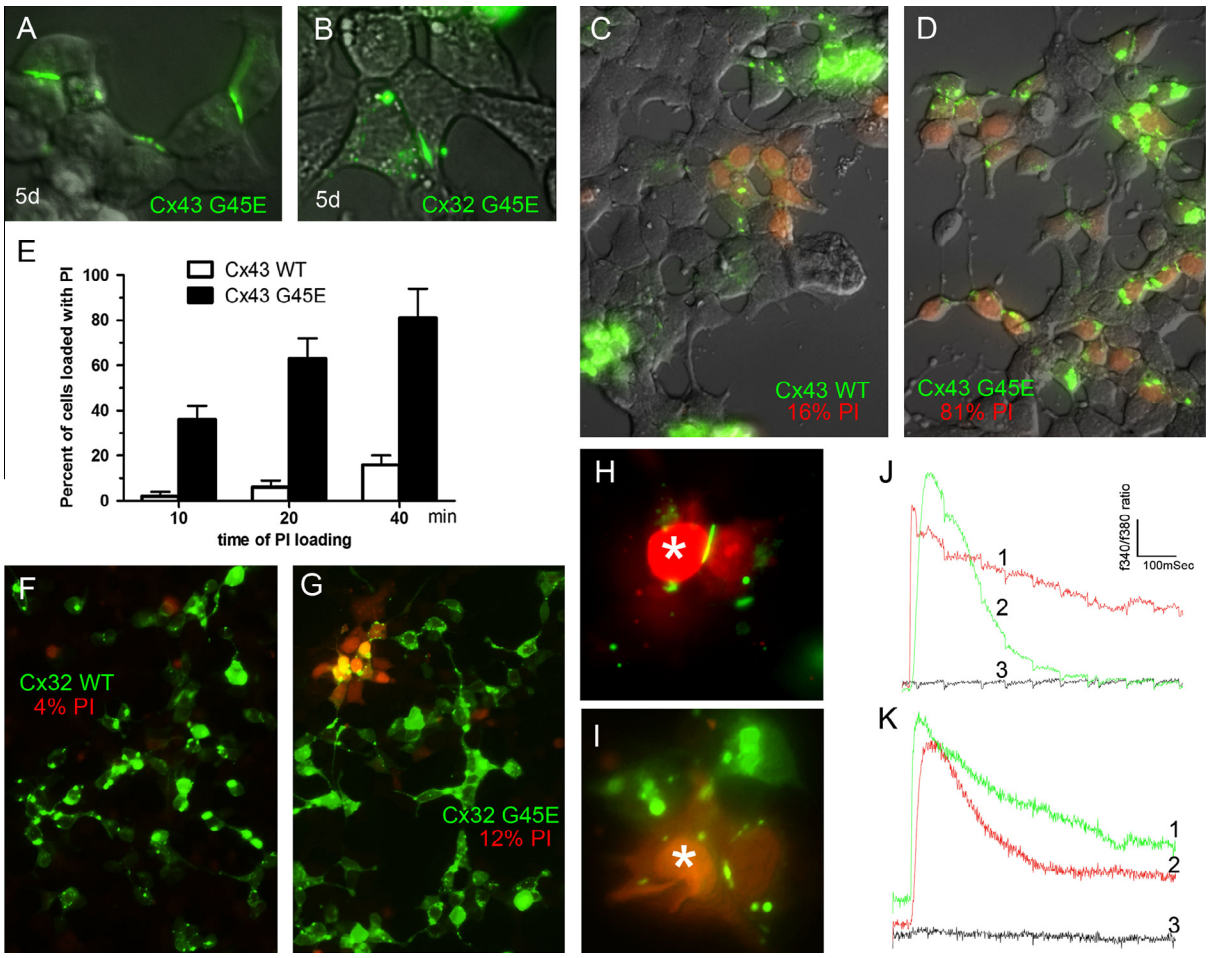

Fig. 3. Characterization of Cx43 G45E, Cx32 G45E mutation effects. HEK293 cells transfected with either Cx43 G45E (A) or Cx32 G45E (B) tagged with eGFP show normal morphology and gap junctions (GJs) 5 days after transfection. Loading of propidium iodide (PI) in Ca^{2+} free HBSS was compared between cells transfected with Cx43 WT (C) and with Cx43 G45E (D), after 40 min of incubation with PI dye. (E) Percentage of loaded cells was quantified at different time point of incubation with PI. $N = 4$ independent experiments. Loading of PI in Ca^{2+} free HBSS was also compared between cells transfected with Cx32 WT (F) and with Cx32 G45E (G). The GJs formed by Cx43 G45E (H) or Cx32 G45E (I) allowed transfer of PI. Injected cell labeled by an asterisk. Ionic coupling of Cx43 G45E (J) and Cx32 G45E (K) GJs measured by intercellular Ca^{2+} transfer assay. Intracellular Ca^{2+} in stimulated cells (labeled 1); GJ-coupled (labled 2) and uncoupled cells (labeled 3) are plotted.

43. The molecular structure of Cx30 resembles that of Cx26. Therefore the function of their protein shares more similarity. In this study we found that Cx30 G45E mutation produced leaky hemichannels to allow the loading of large molecular like PI resulting in cell death in physiologic extracellular $[Ca^{2+}]_o$, in a manner similar to the mutational effect found for Cx26 G45E. Further tests showed that the cell death could be prevented by increasing $[Ca^{2+}]_o$ in a dose dependent manner. The data provided further support for the hypothesis that glycine at position 45 in Cxs is involved in Ca^{2+} dependent dynamic gating of hemichannels formed by Cx26 and Cx30.

Cx32 and Cx43 are also cochlear connexins. All Cxs share a common structure of membrane topology motifs. Their molecular mass is differed mainly by the different length of the CL and CT sequences. Glycine at position 45 is located in the first extracellular loop and it is well conserved among all the cochlear Cxs. Cx32 is the closest relation with Cx26 and Cx30 in the phylogenetic tree of chordate connexin genes [3] and can functionally replace Cx26 in the mouse cochlea resulting in almost normal hearing [22]. In Cx32 a ring of 12 Asp residues within the external vestibule of the pore is responsible for the binding of Ca^{2+} that accounts for both pore occlusion and blockage of gating [15]. G45 is near this area which is implied that mutation at this position may interfere with the binding of calcium. Gap junction protein connexin43 (Cx43), is the abundant connexin in many tissues. It was reported that Cx43 may have a very important function in facilitating the recycling of potassium ions from hair cells in the cochlea back into the cochlear endolymph during auditory transduction processes [23]. Recently Cx43 immunofluorescence was found evident within type III fibrocytes' intercellular plaques in guinea pig spiral ligament, and the cells were coupled via dye-permeable gap junctions [24]. Cx43 belongs to α group in the phylogenetic tree of chordate connexin genes next to β one which includes Cx26, Cx30 and Cx32 [3]. Therefore the replacement of Glycine in positon 45 with Glutamic acid in Cx32 and Cx43 may produce similar effect *in vitro* as Cx26 and Cx30. But our findings indicated that G45E in Cx43 and Cx32 did not cause immediate cell lethality when cultured cells were bathed in solutions containing normal amount of extracellular $[Ca^{2+}]_o$. However, the dependency on $[Ca^{2+}]_o$ for the hemichannel gating was shifted to the more sensitive side. It is demonstrated that the Ca^{2+}-induced closure of gap junction hemichannels was the result of a change in conformation in the pore opening at the extracellular surface. The fexibility of the C-terminal end has been postulated to relate to the function of the cytoplasmic domains being involved in the potential dependent gating of gap junctions [19]. It is suggested that the G45E mutation changed the conformation of the connexin, which resulted in sensitivity changes to calcium. Different C-terminal and cytoplasmic domain

among Cx26, Cx30, Cx32 and Cx43 may play an important role in the little different results produced by G45E mutaions.

In conclusion, the Cx30-G45E mutation resulted leaky hemichannel and the death of cells as Cx26-G45E did. The mutation of G45E in Cx43 and Cx32 shifted the $[Ca^{2+}]_o$ dependency of hemichannel gating, and induced more leaky hemichannels in lower $[Ca^{2+}]_o$. Overall, the data in this study provided further support for the hypothesis that glycine at position 45 is a conserved Ca^{2+} sensor for the gating of GJ hemichannels among multiple Cx subtypes expressed in the cochlea.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.118.

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