Aberrant hemichannel properties of Cx26 mutations causing skin disease and deafness

Dwan A. Gerido,1* Adam M. DeRosa,2* Gabriele Richard,3 and Thomas W. White1,2

1Department of Physiology and Biophysics and 2Graduate Program in Genetics, State University of New York, Stony Brook, New York; and 3GeneDx Incorporated, Gaithersburg, Maryland

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Gerido DA, DeRosa AM, Richard G, White TW. Aberrant hemichannel properties of Cx26 mutations causing skin disease and deafness. Am J Physiol Cell Physiol 293: C337–C345, 2007. First published April 11, 2006; doi:10.1152/ajpcell.00626.2006.—Mutations in the human GJB2 gene, which encodes connexin26 (Cx26), underlie various forms of hereditary deafness and skin disease. While it has proven difficult to discern the exact pathological mechanisms that cause these disorders, studies have shown that the loss or abnormal function of Cx26 protein has a profound effect on tissue homeostasis. Here, we used the Xenopus oocyte expression system to examine the functional characteristics of a Cx26 mutation (G45E) that results in keratitis-ichthyosis-deafness syndrome (KIDS) with a fatal outcome. Our data showed that oocytes were able to express both wild-type Cx26 and its G45E variant, each of which formed hemichannels and gap junction channels. However, Cx26-G45E hemichannels displayed significantly greater whole cell currents than wild-type Cx26, leading to cell lysis and death. This severe phenotype could be rescued in the presence of elevated Ca2+ levels in the extracellular milieu. Cx26-G45E could also form intercellular channels with a similar efficiency as wild-type Cx26, however, with increased voltage sensitive gating. We also compared Cx26-G45E with a previously described Cx26 mutant, A40V, which has an overlapping human phenotype. We found that both dominant Cx26 mutants elicited similar functional consequences and that cells coexpressing mutant and wild-type connexins predominantly displayed mutant-like behavior. These data suggest that mutant hemichannels may act on cellular homeostasis in a manner that can be detrimental to the tissues in which they are expressed.

Connexins have been implicated in a variety of hereditary human diseases including deafness and skin disease (1, 42, 44). The most common connexin-related disease is genetic deafness [sensorineural hearing loss (SNHL)], which can be nonsyndromic or syndromic when associated with skin disorders and other ectodermal abnormalities, such as palmoplantar keratoderma, Vohwinkel syndrome, Bart-Pumphrey syndrome, or keratitis-ichthyosis-deafness syndrome (KIDS) (13, 30). In most cases, SNHL is due to a mutation in the GJB2 gene that encodes connexin26 (Cx26), although mutations in other connexins also have been described (27). Functional analyses of specific Cx26 mutations associated with syndromic and nonsyndromic SNHL have been conducted to decipher the molecular mechanisms underlying these different disorders. Autosomal recessive hearing loss without skin involvement (nonsyndromic) is often due to a simple loss of channel function altering cochlear intercellular communication (5, 43, 46). The lack of skin involvement in these cases suggests that loss of Cx26 function alone is not detrimental for the development and function of the epidermis. Thus, it has been hypothesized that mutant connexins resulting in syndromic SNHL associated with skin disorders must acquire novel activities compared with wild-type Cx26 channels to alter the epidermal differentiation.

Connexins are the basic subunits of a gap junction channels and consists of four membrane-spanning domains with two extracellular loops and cytoplasmic NH2 and COOH termini. Six connexins oligomerize within the cell membrane to form a hemichannel, or one-half of an intercellular channel. A complete gap junction channel arises from the alignment of two hemichannels from adjacent cells in the extracellular space, creating a direct communication pathway between cytoplasm of neighboring cells (18). It was once believed that hemichannels must remained in a closed state until they were aligned with another hemichannel from an adjacent cell, but recent studies (16, 35) have suggested that hemichannels may play important roles in maintaining homeostasis under different physiological conditions.

If hemichannels play a role in normal physiology, it is possible that some connexin mutations may result in abnormal hemichannel activity that could contribute to disease pathology. This idea has been supported by studies of connexin mutations causing syndromic deafness associated with skin disease. One recently described example is the A40V mutation of Cx26, which is linked to a severe form of KIDS (25). A40V is located within the first extracellular domain of Cx26, a region in which multiple Cx26 mutations linked to syndromic deafness have been found (29). Injection of A40V cRNA into Xenopus oocytes resulted in a disorganization of cell pigmentation followed by cell death. Moreover, the induction of large membrane currents not seen in wild-type Cx26-expressing oocytes suggested the presence of aberrant A40V hemichannel activity (25), leading the authors to conclude that constitutively active hemichannels could contribute to the pathophysiology of this mutation.

Functional evaluation of additional KIDS mutations in Cx26 would help determine if aberrant hemichannel activity is a general pathological mechanism for this syndromic disorder. Here, we report the functional characteristics of a Cx26 mutation (G45E) that was observed in two previously described infants with a rare fatal form of KIDS. The children harboring the G45E mutation in GJB2 had congenital deafness, hyper-
keratosis of the skin, and recurrent severe skin infections, which eventually lead to septicemia and death within the first year of life (14, 17, 21). Using an in vitro expression assay, we show that Cx26-G45E hemichannels display a significant increase in membrane current flow that results in cell death. Elevated Cx26-G45E hemichannel currents can be attenuated by increased extracellular Ca\(^{2+}\) levels that prevent cell death. Moreover, when oocytes are cultured in high Ca\(^{2+}\), we demonstrate that Cx26-G45E can assemble into functional intercellular channels with coupling levels identical to those observed for Cx26. While the intercellular conductance was nearly identical, the voltage gating properties of Cx26-G45E and wild-type Cx26 gap junction channels displayed significant differences, further indicating a fundamental change in channel gating due to this mutation.

MATERIALS AND METHODS

**Molecular cloning.** Human wild-type Cx26 was cloned into the BamHI restriction site of the pcS2+ expression vector for functional experiments in Xenopus laevis oocytes. Mutant Cx26-G45E was prepared by site-directed mutagenesis. Wild-type Cx26 was amplified by PCR using primers 5’-TGT TGT GGA TCC ATG GAT TGG GGC ACG CTCAGACG-3’ and 3’-CTG CTC ATC TTC CTT CCA CAC CTC TTGTGC-5’ as well as 5’-AAG CAG GAG TGT TGG GAA GAT GAG CAG GGC GAC-3’ and 3’-TGT CTT GGA TCC TTA AAC TGG CTT TTT TGA TCT CCC AGA-5’ and gene splicing by the overlap extension method (20) to create the glycine to glutamic acid substitution. Following amplification, G45E was initially cloned into pBlueScript and sequenced on both strands prior to being subcloned into the pCS2+ expression vector for Xenopus laevis expression experiments. Mutant Cx26-A40V was directly amplified from patient genomic DNA as previously described (25).
In vitro transcription, oocyte microinjection, and pairing. Human Cx26, A40V, and G45E were linearized using the Not I restriction site of pCS2+ and transcribed using the SP6 mMessage mMachine RNA protocol (Ambion, Austin, TX). Adult Xenopus females were anesthetized with ethyl-3-aminobenzoate methanesulfonate, and the ovarian lobes were surgically removed and digested for 1.5 h in a solution containing 50 mg/ml collagenase B and 50 mg/ml hyaluronidase in modified Barth’s (MB) medium without Ca2++. Stage V–VI oocytes were collected and injected first with 10 ng of antisense Xenopus Cx38 oligonucleotide to eliminate endogenous connexins (2, 4). The aforementioned antisense oligonucleotide-treated oocytes were then injected with wild-type Cx26, A40V, or G45E cRNA transcripts alone or a combination of wild-type and mutant Cx26 at a 1:1 ratio (total concentration of 5 ng/cell in all cases) or H2O as a negative control. cRNA-injected oocytes were then cultured in Ca2+-free MB medium or MB medium with elevated Ca2+ (2 mM CaCl2). For measurements of gap junctional conductance, the vitelline envelopes were removed in a hypertonic solution (200 mM aspartic acid, 10 mM HEPES, 1 mM MgCl2, 10 mM EGTA, and 20 mM KCl; pH 7.4), and oocytes were manually paired with the vegetal poles apposed in MB medium with elevated Ca2+.

Preparation of oocyte samples for Western blot analysis and quantification. Oocytes were collected in 1 ml of buffer containing 5 mM Tris (pH 8.0), 5 mM EDTA, and protease inhibitors and lysed using a series of mechanical passages through needles of diminishing caliber. Extracts were centrifuged at 1,000 g at 4°C for 5 min. The supernatant was then centrifuged at 100,000 g at 4°C for 30 min. Membrane pellets were resuspended in SDS sample buffer (2 μl/oocyte), and samples were separated on 15% SDS gels and transferred to nitrocellulose membranes. Blots were blocked with 5% BSA in 1× PBS with 0.02% NaN3 for 1 h and probed with a polyclonal Cx26 antibody at a 1:500 dilution (Zymed Laboratories, San Francisco, CA) followed by an incubation with alkaline phosphatase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Band intensities were quantified using Kodak 1D Image Analysis software (Eastman Kodak, Rochester, NY). Values from three independent experiments were normalized to the mean value of band intensity of the wild-type Cx26 sample.

Electrophysiological recording of hemichannel currents. Macroscopic recordings of hemichannel currents were recorded from single Xenopus oocytes using a GeneClamp 500 amplifier controlled by a personal computer (PC)-compatible computer through a Digidata 1320 interface (Axon Instruments, Foster City, CA). pCLAMP 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms. To obtain hemichannel current-voltage curves, cells were initially clamped at −40 mV and subjected to 5-s depolarizing voltage steps ranging from −30 to +60 mV in 10-mV increments. To test the effect of added Ca2+ on hemichannel currents, cells were transferred between 35-mm dishes containing MB media without Ca2+ or MB media supplemented with either 2 or 4 mM CaCl2. Hemichannel currents were recorded within 1–2 min of oocyte transfer.

Dual whole cell voltage clamp. Gap junctional coupling between oocyte pairs was measured using the dual whole cell voltage-clamp technique (36). Current and voltage electrodes (1.2 mm diameter, omega dot, Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with solution containing 3 M KCl, 10 mM EGTA, and 10 mM HEPES (pH 7.4). Voltage-clamp experiments were performed with an Axopatch 200B amplifier (Axon Instruments), through a Digidata 1320A interface (Axon Instruments, Foster City, CA), using pCLAMP 8.0 software (Axon Instruments). A large holding potential of −80 mV was used to inactivate the voltage-dependent K+ channels, and oocytes were mechanically paired using a glass micropipette containing 3 M KCl, 10 mM EGTA, and 10 mM HEPES (pH 7.4). Steady-state currents were measured by a voltage step of −40 mV for 5 s.

Fig. 4. Increased extracellular Ca2+ inhibits Cx26-G45E and CX26-A40V hemichannel currents. A: the large whole cell currents observed in G45E oocytes were suppressed by the addition of 2 and 4 mM Ca2+ to the medium. B: mean i ms observed in G45E cells in the absence of Ca2+ were reduced in the presence of 2 and 4 mM Ca2+ (n = 3–6). C: similar to G45E-injected cells, A40V hemichannels displayed large i ms that were suppressed by the addition of 2 and 4 mM Ca2+ (n = 3–4). Data are means ± SE.
performed using two GeneClamp 500 amplifiers controlled by a PC-compatible computer through a Digidata 1320A interface (Axon Instruments).

For measurements of junctional conductance ($G_j$), both cells in a pair were initially clamped at −40 mV to eliminate any transjunctional potential ($V_j$). One cell was then subjected to alternating pulses of ±20 mV while the current produced by the change in voltage was recorded in the second cell. The current delivered to the second cell was equal in magnitude to the junctional current ($I_j$), and $G_j$ was calculated by dividing the measured current by the voltage difference as follows: $G_j = I_j/(V_1 - V_2)$, where $V_1$ and $V_2$ are the voltages in the first and second cells, respectively.

To determine voltage gating properties, $V_j$s of opposite polarity were generated by hyperpolarizing or depolarizing one cell in 20 mV steps (range = ±120 mV) while clamping the second cell at −40 mV. Currents were measured at the end of the voltage pulse, at which time they approached steady state ($I_{ms}$). Macroscopic conductance ($G_{ms}$) was calculated by dividing $I_{ms}$ by $V_j$ (normalized to the values determined at ±20 mV) and plotted against $V_j$. Data describing the relationship of $G_{ms}$ as a function of $V_j$ were analyzed using Origin 6.1 software (Microcal Software, Northampton, MA) and fit to a Boltzmann relation of the following form: 

$$G_{ms} = \frac{(G_{max} - G_{min})}{1 + \exp[(V_j - V_0)/k]} + G_{min},$$

where $G_{max}$ (normalized to unity) is maximum conductance, $G_{min}$ is the residual conductance at large values of $V_j$, and $V_0$ is the $V_j$ at which $G_{ms} = (G_{max} - G_{min})/2$. The constant $A = nq/kT$ and represents the voltage sensitivity in terms of gating charge as the equivalent number ($n$) of electron charges ($q$) moving through the membrane, $k$ is the Boltzmann constant, and $T$ is the absolute temperature. To analyze channel closure kinetics, the initial 1,000 ms of current decay were plotted against time and fit to a monoexponential function to determine the time constant ($\tau$) using Origin 6.1.

RESULTS

G45E induces gap junction hemichannel currents in single Xenopus oocytes. The G45E mutation in the GJB2 gene results in a single amino acid substitution in the first extracellular domain of Cx26 and causes KIDS, with a reported lethal outcome in two unrelated infants. To identify the functional consequences of mutation G45E, wild-type Cx26 and Cx26-G45E were expressed in Xenopus oocytes. Initially, single cells were subjected to depolarizing voltage pulses and membrane currents were recorded (Fig. 1). Control oocytes (H2O injected) displayed negligible current flow over a range of voltages from −30 to +60 mV. Cx26-injected cells exhibited larger outward currents than control cells, confirming previous reports (15, 34) of low levels of hemichannel activity. In contrast, oocytes expressing the G45E mutation displayed currents of much greater magnitude in response to depolarization than either control or Cx26-injected cells. Thus, expression of G45E correlated with a markedly decreased plasma membrane electrical resistance, suggesting that G45E formed hemichannels that were functionally distinct from wild-type Cx26.

To quantitatively compare hemichannel currents produced by G45E and Cx26, mean $I_{ms}$ values were plotted as a function of membrane potential (Fig. 2). Control oocytes displayed small currents at all voltages tested. Cx26-injected cells were initially indistinguishable from controls but showed greater current magnitudes at higher voltages with a maximum current approximately fourfold higher than control at the largest voltage tested (+60 mV, $P < 0.05$ by Student’s t-test). G45E-expressing oocytes had much greater membrane currents than either H2O- or Cx26-injected cells at all tested voltages. At +60 mV, the G45E membrane current was 30-fold higher than control cells and more than 7-fold greater than Cx26-injected cells ($P < 0.05$). This statistically significant increase in membrane current may result from G45E forming hemichannels that are either fixed in the open configuration or gate open more easily than wild-type Cx26, leading to much greater ionic flux.

G45E induces cell death that can be rescued by elevated extracellular Ca2+ levels. We (25) have previously shown that another dominant Cx26 mutation associated with KIDS and the follicular occlusion triad (A40V) produced abnormal hemichannel currents when expressed in Xenopus oocytes and eventually led to lysis and death of the injected cells. Numerous studies (9, 28) have shown that increased extracellular Ca2+ suppresses gap junction hemichannel activity. Hence, if Xenopus oocytes expressing Cx26 mutants A40V and G45E die as result of abnormal hemichannel activity, this outcome may be prevented in the presence of increased Ca2+ levels. To test if Cx26-G45E indeed can affect cell viability in a Ca2+-dependent manner, oocytes were cultured in medium in the presence or absence of Ca2+ (Fig. 3). Control and Cx26-expressing oocytes remained healthy as indicated by visual inspection when incubated in MB medium without Ca2+ (Fig. 3, A and B). In contrast, cells injected with G45E displayed pigment disorganization and blebbing of the ooplasm into the culture medium within 8 h (Fig. 3C), which preceded lysis and cell death within 12–24 h. Incubation of G45E-injected cells in MB media supplemented with ±2 mM extracellular Ca2+ rescued this phenotype and preserved cell viability (Fig. 3D). These data suggested that the increased Ca2+ level suppressed G45E hemichannels and maintained cell viability.

If the addition of Ca2+ preserved cell viability by suppressing hemichannel activity, then whole cell membrane currents should be greatly reduced in elevated Ca2+. To test this idea, we measured hemichannel currents from G45E- and A40V-expressing oocytes. The results are presented in Fig. 5. Wild-type and mutant connexins are equally expressed in Xenopus oocytes. 

**Fig. 5.** Wild-type and mutant connexins are equally expressed in Xenopus oocytes. A: equal amounts of membrane extracts were probed with antibodies for Cx26. H2O-injected controls did not express Cx26, as expected. Cx26, G45E, and A40V were readily detected in lanes corresponding to each injection condition. B: quantitation of Cx26 expression by densitometry revealed that Cx26, G45E, and A40V were expressed in equal amounts ($n = 3$). Data are means ± SD.
expressing cells in media containing 0, 2, or 4 mM Ca²⁺ (Fig. 4). Oocytes expressing G45E in the absence of extracellular Ca²⁺ produced large outward currents that were reduced by 2 mM Ca²⁺ and further reduced by 4 mM Ca²⁺ (Fig. 4A). To quantitate these findings, Iₘₛ values of G45E oocytes in the presence and absence of Ca²⁺ were plotted as a function of voltage. The addition of 2 and 4 mM Ca²⁺ produced a significant decrease in current flow at all tested voltages, with the maximum voltage displaying a greater than twofold decrease at 2 mM Ca²⁺ and a sixfold decrease in hemichannel current in 4 mM Ca²⁺. In addition, the threshold of voltage activation was shifted to more positive potentials with increased Ca²⁺ concentration (Fig. 4B).

Like G45E, the A40V Cx26 mutation has been previously shown to induce hemichannel-mediated cell lysis and death (25); however, the ability of elevated Ca²⁺ to rescue A40V had not been tested. To determine whether elevated Ca²⁺ could also suppress A40V activity, we measured membrane currents from A40V hemichannels in the presence and absence of elevated extracellular Ca²⁺. In the absence of Ca²⁺, A40V-injected oocytes produced large currents similar to those observed in G45E cells (Fig. 4C). The addition of Ca²⁺ to the media produced a dramatic reduction in hemichannel current flow consistent with the results observed in G45E cells. Again, quantitation of Iₘₛ values revealed a significant decrease in hemichannel activity in the presence of Ca²⁺ and a positive shift in the threshold of voltage activation (Fig. 4D). This decrease in current flow was detected at all voltages tested, with the maximum decline being twofold in 2 mM Ca²⁺ and fourfold in 4 mM Ca²⁺ at +60 mV. Taken together, these data indicate a significant reduction in the number of active hemichannels with the elevation of extracellular Ca²⁺ for both G45E and A40V and that these two mutations share similar functional properties.

G45E and A40V may contribute to cellular pathology through abnormal hemichannel activity; however, wild-type Cx26 also induced low levels of hemichannel current, and the differences in current magnitude between the wild type and mutants could have resulted from different levels of protein expression in each condition. To examine the levels of connexin protein expressed in oocytes, we performed Western blot analysis with cell extracts from control (H₂O injected) and Cx26-, G45E-, and A40V-injected cells (Fig. 5). Antibodies for Cx26 failed to detect protein expression in control cells but readily detected expression of Cx26, G45E, and A40V following injection of their respective cRNAs (Fig. 5A). To quantify expression levels, we measured the band intensities of G45E and A40V and compared them to the value obtained for Cx26 (Fig. 5B). Cx26, G45E, and A40V were all expressed in equal amounts, and thus the differences in hemichannel current magnitude were not due to the relative overexpression of mutant protein.

Previous reports have described the G45E mutation as dominant in the Caucasian population; however, this mutation has
also been linked to recessive deafness in some Asian populations (26). Given the conflicting nature of these data, we performed experiments to examine the impact of the mutant allele on wild-type Cx26 function. To test for dominant activity, we injected oocytes with both wild-type Cx26 and G45E in a 1:1 ratio and measured hemichannel currents in the absence and presence of elevated extracellular Ca$^{2+}$. Oocytes coexpressing G45E and wild-type Cx26 displayed large membrane currents similar to those observed with expression of G45E alone (Fig. 6A). The large membrane currents seen in coinjected oocytes were reduced with the addition of 2 mM Ca$^{2+}$ in a manner also consistent with the results from cells only expressing the G45E variant of Cx26. Quantitation of $I_{\text{mss}}$ values revealed a significant decrease in membrane current with the addition of Ca$^{2+}$, displaying a threefold decrease at the maximum voltage tested (Fig. 6B).

Given that oocytes injected with A40V exhibited properties similar to G45E, we also examined the dominance of A40V by coinjecting cells with A40V and wild-type Cx26. Oocytes expressing A40V in conjunction with wild-type Cx26 again displayed large membrane currents that were reduced by the addition of 2 mM Ca$^{2+}$ (Fig. 6C). Again, quantitation revealed a significant decrease with the addition of calcium with a threefold difference at a membrane potential of +60 mV (Fig. 6D). Together, these data show that both G45E and A40V exhibited dominant, Ca$^{2+}$-sensitive hemichannel activity when coexpressed with wild-type Cx26.

G45E also forms gap junction channels in paired Xenopus oocytes. We and others (5–7, 24, 38, 41, 45) have previously shown that not all disease-causing mutations in Cx26 result in the complete loss of gap junction channel activity. The ability to rescue viability in cells expressing G45E with elevated extracellular Ca$^{2+}$ allowed us to examine its ability to form intercellular channels using the dual cell voltage-clamp technique in paired oocytes (Fig. 7). After G45E or Cx26 cRNA injection, oocytes were paired and incubated in medium with 2 mM Ca$^{2+}$. H$_2$O-injected cells were used as negative controls. Wild-type Cx26 formed functional channels with $G_j$ that was 20-fold higher than background levels recorded in control pairs. Oocyte pairs injected with G45E also had a mean conductance 20-fold higher than the background level and...
indistinguishable from wild-type Cx26 \((P > 0.05)\). Thus, the G45E mutation retained the ability to form functional gap junction channels when hemichannel activity was suppressed by elevated extracellular Ca\(^{2+}\).

**G45E intercellular channels have altered voltage gating properties.** The ability of G45E to form functional gap junction channels in elevated extracellular Ca\(^{2+}\) allowed its voltage gating properties to be compared with wild-type Cx26 channels. Although the macroscopic conductance measurements were indistinguishable, we found that the voltage gating properties of Cx26 and G45E channels were markedly different. Figure 8 shows the gap junctional currents \((I_j)\) for wild-type Cx26 and G45E recorded for a series of different \(V_j\) s. Cx26 currents were consistent with previous data showing an asymmetric decay at higher voltages with greater closure for positive \(V_j\) s (Fig. 8A) \((2, 24)\). In contrast, G45E gap junctions exhibited a more symmetric current decline in response to \(V_j\) s of either polarity (Fig. 8B). In addition to the increased symmetry, G45E currents decayed more rapidly than wild-type Cx26. For example, at \(V_j = -120 \text{ mV}\), G45E currents decayed with a mean \(\tau\) value of 0.19 ± 0.05 \(s\) \((n = 6)\), whereas Cx26 currents decayed six times slower with a mean \(\tau\) value of 1.15 ± 0.38 \(s\) \((P < 0.05, n = 5)\).

To analyze the equilibrium voltage gating properties, \(G_j\) vs values were normalized, plotted against \(V_j\), and fitted to a Boltzmann equation (Fig. 8C). This analysis revealed that for Cx26, \(V_0\) (which corresponds to the midway point between \(G_{\text{max}}\) and \(G_{\text{min}}\)) was asymmetric with values of 99 and −117 mV for positive and negative \(V_j\) s, respectively (Table 1). Conversely, \(V_0\) values for G45E channels were more symmetrical and much lower, with positive and negative \(V_0\) values of +56 and −61 mV. These data demonstrate that, in addition to increased hemichannel function, the magnitude and kinetics of the voltage gating properties of gap junction channels made from G45E differed quantitatively from those of wild-type Cx26.

We also tested for a dominant impact of G45E on wild-type Cx26 in gap junction channels between paired oocytes (Fig. 9). Cell pairs co-injected with equal amounts of wild-type Cx26 and G45E cRNA were able to form functional gap junctions that exhibited significantly increased conductance compared with \(H_2O\)-injected negative controls (Fig. 9A) but were not statistically different from the coupling levels in oocyte pairs injected with Cx26 or G45E alone (see Fig. 7). An examination of the voltage-induced current decay revealed that co-injected pairs displayed voltage gating properties more like those of gap junctions composed of G45E alone rather than Cx26 alone. Specifically, the voltage sensitivity of the currents was more symmetrical than Cx26 with faster gating kinetics (Fig. 9B). Analysis of the equilibrium voltage gating properties con-

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**Table 1. Boltzmann parameters for wild-type Cx26 and G45E mutant channels**

<table>
<thead>
<tr>
<th>Cell Injection</th>
<th>(V_1)</th>
<th>(V_0)</th>
<th>(G_{\text{min}})</th>
<th>(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26</td>
<td>+</td>
<td>99</td>
<td>0.35</td>
<td>0.10</td>
</tr>
<tr>
<td>Cx26</td>
<td>−</td>
<td>−117</td>
<td>0.44</td>
<td>0.03</td>
</tr>
<tr>
<td>G45E</td>
<td>+</td>
<td>56</td>
<td>0.23</td>
<td>0.14</td>
</tr>
<tr>
<td>G45E</td>
<td>−</td>
<td>−61</td>
<td>0.21</td>
<td>0.09</td>
</tr>
<tr>
<td>Cx26 + G45E</td>
<td>+</td>
<td>61</td>
<td>0.24</td>
<td>0.10</td>
</tr>
<tr>
<td>Cx26 + G45E</td>
<td>−</td>
<td>−63</td>
<td>0.21</td>
<td>0.07</td>
</tr>
</tbody>
</table>

\(V_1\) is the transjunctional membrane potential, with + and − indicating polarity. \(V_0\) indicates the voltage measured midway through the conductance decline, \(G_{\text{min}}\) represents the minimum conductance value, and \(A\) denotes the cooperativity constant, which reflects the number of charges moving through the transjunctional field. Cx26, connexin26.
firmed that coinjected cell pairs were dominated by G45E-like behavior (Fig. 3C). The equilibrium properties of cell pairs coexpressing G45E and Cx26 were deduced from a Boltzmann fit and found to be nearly identical to those of pairs injected with G45E alone (Table 1). These data showed that the voltage gating properties of gap junction channels made from an equal mixture of Cx26 and G45E did not differ markedly from those composed of G45E alone and suggested that G45E exerted a dominant gating phenotype on wild-type Cx26.

**DISCUSSION**

Numerous studies have linked mutations in the GJB2 gene to syndromic deafness associated with skin disease. In the case of KIDS, all pathogenic mutations cluster in regions coding for the first extracellular domain and the NH2-terminal of Cx26, implying common functional defects. In the present study, we showed that the G45E and A40V mutant forms of Cx26 produced greatly increased hemichannel activity that ultimately led to cell death. This severe phenotype could be rescued by elevated extracellular Ca2+, which allowed Cx26-G45E intercellular channels to be evaluated. G45E mutant gap junction channels also displayed significantly altered voltage-sensitive gating. These findings are consistent with the hypothesis that aberrant hemichannel activity might be a common feature of some KIDS mutations and may contribute to severe epidermal pathology in these cases.

While the rare fatal form of KIDS has been associated with Cx26-G45E in two unrelated Caucasian patients (14, 17, 21), it has also been linked to recessive nonsyndromic deafness in some Asian populations. In a large study of Japanese patients with nonsyndromic SNHL, G45E was detected in 16% of the GJB2 disease alleles (26). Among Japanese patients, G45E was classified as a recessive allele as it was always present homozygously or as compound heterozygotes with other GJB2 mutations in deaf individuals. In addition, it was never detected in control individuals or classified as causing syndromic deafness associated with skin disease (12, 26).

Our data are consistent with a dominant gain of function for G45E, which rapidly killed Xenopus oocytes through aberrant hemichannel activity. Further support for the cellular lethality of Cx26-G45E comes from a report of hemichannel activity in transfected HEK-293 cells that appeared while this article was being written (37). Stong et al. (37) reported that Cx26-G45E expression facilitated dye uptake, resulting in apoptosis within 24 h of cell transfection, and cell death could be rescued by increasing the extracellular Ca2+ concentration. Thus, in two different functional expression systems, Cx26-G45E led to aberrant hemichannel formation and cell lysis, functional properties that we show were shared by a second severe KIDS mutation in GJB2, Cx26-A40V. To date, the A40V mutation has only been associated with KIDS and the follicular occlusion triad (25).

Nine connexin genes, including Cx26, are expressed during epidermal morphogenesis, and gap junctional communication plays an important role in keratinocyte growth and differentiation (8, 22). Mutations in Cx26 are the leading cause of hereditary deafness, which can be associated with a variety of skin diseases (23, 31–33, 40). The presence of Cx26 mutations in syndromic and nonsyndromic deafness suggests that different functional consequences of distinct mutations may correspond to unique pathological states. For example, many nonsyndromic mutations in the GJB2 gene have resulted in altered protein trafficking, loss of channel function, or alteration of channel permeability, without causing cell death (43, 46). In contrast, we and others (25, 37) have shown that two separate KIDS mutations exhibited lethal hemichannel activity that may have contributed to both hearing loss and the epidermal pathology. Further support for this aberrant hemichannel hypothesis comes from studies of mutations in the GJB6 gene (which encodes Cx30) causing hidrotic ectodermal dysplasia (HED). Two HED-associated Cx30 mutants, G11R and A88V, induced cell death in Xenopus oocytes, which could have resulted from the presence of functional hemichannels, an idea supported by the detection of large voltage-activated currents in single oocytes expressing mutant proteins that were not seen in cells injected with wild-type Cx30 (10). Furthermore, transfected cells expressing the mutant channels had a two- to threefold higher ATP leakage than control cells, suggesting that ATP release through unregulated hemichannels may play a role in the HED phenotype (10). In addition to causing cell depolarization and death, hemichannels could induce the release of metabolites into the extracellular space in the epidermis and influence the regulation of keratinocyte growth and differentiation.

Extracellular Ca2+ plays an important role in normal epidermal differentiation, regulating cell proliferation, terminal differentiation, and cell-to-cell adhesion. In addition, altered Ca2+ regulation has been implicated in the pathogenesis of some epidermal diseases (3, 11, 19, 39). An increase in the extracellular Ca2+ concentration is thought to drive the developmental switch from keratinocyte proliferation to terminal differentiation by providing a reservoir of Ca2+ that influences intracellular Ca2+-dependent signaling processes. However, it is not known if this developmentally regulated rise in extracellular Ca2+ achieves concentrations sufficiently high to inhibit G45E or A40V mutant hemichannels. While we have not tried other hemichannel blockers, future studies may identify novel treatment strategies seeking to modulate epidermal Ca2+ concentrations pharmacologically or seeking novel blocking agents that specifically act on Cx26 hemichannels.

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**REFERENCES**