Insights on the mechanisms of Ca²⁺ regulation of connexin26 hemichannels revealed by human pathogenic mutations (D50N/Y)

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Because of the large size and modest selectivity of the connexin hemichannel aqueous pore, hemichannel opening must be highly regulated to maintain cell viability. At normal resting potentials, this regulation is achieved predominantly by the physiological extracellular Ca²⁺ concentration, which drastically reduces hemichannel activity. Here, we characterize the Ca²⁺ regulation of channels formed by wild-type human connexin26 (hCx26) and its human mutations, D50N/Y, that cause aberrant hemichannel opening and result in deafness and skin disorders. We found that in hCx26 wild-type channels, deactivation kinetics are accelerated as a function of Ca²⁺ concentration, indicating that Ca²⁺ facilitates transition to, and stabilizes, the closed state of the hemichannels. The D50N/Y mutant hemichannels show lower apparent affinities for Ca²⁺-induced closing than wild-type channels and have more rapid deactivation kinetics, which are Ca²⁺ insensitive. These results suggest that D50 plays a role in (a) stabilizing the open state in the absence of Ca²⁺, and (b) facilitating closing and stabilization of the closed state in the presence of Ca^{2+} . To explore the role of a negatively charged residue at position 50 in regulation by Ca^{2+} , this position was substituted with a cysteine residue, which was then modified with a negatively charged methanethiosulfonate reagent, sodium (2-sulfanoethyl) methanethiosulfonate (MTSES). D50C mutant hemichannels display properties similar to those of D50N/Y mutants. Recovery of the negative charge with chemical modification by MTSES⁻ restores the wild-type Ca²⁺ regulation of the channels. These results confirm the essential role of a negative charge at position 50 for Ca²⁺ regulation. Additionally, charge-swapping mutagenesis studies suggest involvement of a salt bridge interaction between D50 and K61 in the adjacent connexin subunit in stabilizing the open state in low extracellular Ca²⁺. Mutant cycle analysis supports a Ca²⁺-sensitive interaction between these two residues in the open state of the channel. We propose that disruption of this interaction by extracellular Ca²⁺ destabilizes the open state and facilitates hemichannel closing. Our data provide a mechanistic understanding of how mutations at position 50 that cause human diseases are linked to dysfunction of hemichannel gating by external Ca²⁺.

INTRODUCTION

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Connexins constitute a family of transmembrane proteins, with at least 21 members in humans, and are found in almost all cell types (Willecke et al., 2002). They form gap junction channels (GJCs) that mediate intercellular communication crucial in diverse processes including development and physiology, as well as response to trauma, inflammation, and disease (Sáez et al., 2003; Contreras et al., 2004). Newly synthesized connexins are assembled in the endoplasmic reticulum and/or Golgi apparatus to form hexamers known as hemichannels. The hemichannels are transported to the plasma membrane where they reside until they dock with hemichannels in apposed cells to form GJCs. Hemichannels and GJCs are typically permeable to ions and molecules up to \sim 12 Å in diameter (Harris, 2001). Release of small metabolites, such as ATP and glutamate, by unapposed hemichannels at the plasma

membrane seems to play an important role in autocrine/paracrine signaling in many cell types and tissues (Bennett et al., 2003; Ebihara, 2003; Sáez et al., 2010; Wang et al., 2013). Exacerbated opening of hemichannels, however, leads to loss of electrochemical gradients and cytoplasmic metabolites, causing cell death. There is compelling evidence that metabolic inhibition, hypoxia, inflammation, and oxidative stress increase the opening of plasma membrane hemichannels, thereby aggravating cell damage and accelerating cell death (Contreras et al., 2002, 2004; Decrock et al., 2009; Orellana et al., 2011a,b). In addition, a large number of human connexin mutations produce exacerbated opening of unapposed hemichannels, causing the cellular dysfunction and death that are pivotal in the development of connexin-associated pathologies (Abrams et al., 2002; Liang et al., 2005; Stong et al., 2006; Dobrowolski et al., 2007; Gerido et al., 2007; Matos et al., 2008; Lee

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Abbreviations used in this paper: GJC, gap junction channel; hCx26, human connexin26; MTSES, sodium (2-sulfanoethyl) MTS; MTSET, [2-(trimethylammonium) ethyl] MTS bromide.

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et al., 2009; Minogue et al., 2009; Sánchez et al., 2010; Tong et al., 2011).

Proper control of the opening and closing of unapposed hemichannels is essential and is achieved by physiological levels of extracellular Ca²⁺ (~1.8 mM), which drastically reduce hemichannel activity. Opening of unapposed hemichannels was first observed in *Xenopus* laevis oocytes expressing lens connexins; osmotic swelling rapidly killed the oocytes unless Ca2+ was raised to keep the hemichannels closed (Paul et al., 1991). Reduction of extracellular Ca²⁺ below physiological levels increases the opening of most, if not all, connexin hemichannels (e.g., Cx26, Cx30, Cx32, Cx43, Cx46, and Cx50) (Paul et al., 1991; Zampighi et al., 1999; Valiunas and Weingart, 2000; Beahm and Hall, 2002; Contreras et al., 2003; Ebihara et al., 2003; Gómez-Hernández et al., 2003; Ripps et al., 2004). Despite the important role of external Ca²⁺ in regulating hemichannel opening, little is known about the molecular mechanisms underlying this process. Recently, a high resolution structure of the human connexin26 (hCx26) GJC was solved (Maeda et al., 2009), providing crucial information to guide functional studies to elucidate the molecular gating mechanisms of connexin channels. Here, we investigate the mechanism by which extracellular Ca²⁺ regulates the opening and closing of unpaired hCx26 hemichannels in the plasma membrane. We found that aspartate to asparagine or tyrosine substitutions at position 50 (D50N/Y)-human mutations that cause syndromic deafness- severely compromise the ability of hCx26 hemichannels to be regulated by extracellular Ca²⁺. Analysis of the kinetic and steady-state data strongly suggests that D50 stabilizes the open state of Cx26 hemichannels by interacting with a positively charged residue (K61) in the adjacent connexin subunit. We propose that disruption of this interaction by extracellular Ca2+ facilitates destabilization of the open state and promotes hemichannel closing.

MATERIALS AND METHODS

Channel expression and molecular biology

cDNA for hCx26 was purchased from OriGene. Wild-type Cx26 was subcloned in the pGEM-HA vector (Promega) for expression in *Xenopus* oocytes. Mutations of hCx26 were produced with Quik-Change II Site-Directed Mutagenesis kits (Agilent Technologies). DNA sequencing performed at the New Jersey Medical School Molecular Resource Facility confirmed the amino acid substitutions. Nhe1-linearized hCx26 wild-type and mutant DNAs were transcribed in vitro to cRNAs using the T7 Message Machine kit (Ambion).

Electrophysiology

Electrophysiological data were collected using the two-electrode voltage-clamp technique. All recordings were made at room temperature (20–22°C). The recording solutions contained (mM) 118 NaCl, 2 KCl, and 5 HEPES, pH 7.4, with a range of Ca²⁺ concentrations from 0.01 to 20. Currents from oocytes were

recorded 1–3 d after cRNA injection using an oocyte clamp (OC-725C; Warner Instruments). Currents were sampled at 2 kHz and low-pass filtered at 0.2 kHz. Microelectrode resistances were between 0.1 and 1.2 M Ω when filled with 3 M KCl. All recordings were performed using agar bridges connecting bath and ground chambers.

Measurement of Ca²⁺ dose-response curves

In *Xenopus* oocytes, lowering Ca^{2+} below ~ 0.15 mM activates endogenously expressed Cx38 hemichannels, which can interfere with analysis of heterologously expressed hCx26 currents. To efficiently reduce endogenous Cx38 expression, antisense oligonucleotide against Cx38 (1 mg/ml; using the sequence from Ebihara, 1996) was injected 4 h after harvesting the oocytes. After 1 d, the same oocytes were coinjected with 18-50 nl cRNA (0.5-1 mg/ml) hCx26 or mutants plus the Cx38 (1 mg/ml) antisense. Before performing any Ca²⁺ dose-response curves in oocytes expressing hCx26 or mutant hemichannels, we tested for the levels of Cx38 endogenous current in oocytes injected only with Cx38 antisense; only batches that showed no or low endogenous Cx38 currents at the lowest extracellular Ca²⁺ concentration (0.01 mM) were used to perform the Ca²⁺ dose–response curves. Because of the slow kinetics of hCx26 activation and deactivation, Ca²⁺ dose-response measurements were obtained by assessing the tail current peaks after reaching current saturation during a depolarizing pulse from -80 to 0 mV. Tail current measurements include the steady-state "holding" currents caused by the opening of hemichannels at -80 mV induced by reduction of extracellular Ca²⁺ concentrations. The peak tail current thus measured reflects the number of channels that are open at 0 mV, not only those that opened during the pulse to 0 mV. To minimize the compromise of cell viability produced by exacerbated opening of hemichannels under these experimental conditions, no more than three to four different Ca2+ concentrations, including 0.01 mM Ca²⁺, were assessed per oocyte. This significantly improved oocyte-to-oocyte reproducibility of the currents in response to Ca²⁺ changes. Deactivation time constants from tail currents were determined by fitting tail current, up to 10 s after reaching steady state, to exponential functions using Clampfit 11 software (Molecular Devices).

Mutant cycle analysis

Mutant cycle analysis was performed on the apparent affinities of Ca^{2+} from steady-state currents and on binding rates obtained from deactivation time constants. Macroscopic parameters from apparent affinities have been used previously to support side chain interactions and establish coupling coefficients in ligand-gated channels (Kash et al., 2003; Price et al., 2007; Gleitsman et al., 2008). Coupling energy ($\Delta\Delta G$) was calculated as:

$$\Delta\Delta G = RT \ln \left(k_{\text{[wildtype]}} \times k_{\text{[double mutant]}} / k_{\text{[mutant1]}} \times k_{\text{[mutant2]}} \right), (1)$$

where R is the ideal gas constant, and T is the absolute temperature. If two mutations are functionally independent with respect to Ca^{2+} sensitivity, the coupling energy will be close to $0 \operatorname{kcal/mol}$. Significant coupling is indicated by any value that deviates from zero, but the accepted cutoff for nonadditivity is $0.5 \operatorname{kcal/mol}$ (Laha and Wagner, 2011).

Online supplemental material

The supplemental text and Fig. S1 describe and validate the use of tail current measurements as an indication of hCx26 hemichannel activation in *Xenopus* oocytes. Fig. S2 demonstrates isolation of hCx26 hemichannel currents from endogenous Ca²⁺-activated chloride currents. Fig. S3 shows the Ca²⁺ regulation of D50C mutant hemichannels. Fig. S4 shows the interplay between

Ca²⁺ and voltage regulation of wild-type and D50N/Y mutant hemichannels. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201210893/DC1.

RESULTS

Ca²⁺ regulation of hCx26 hemichannels

The Ca²⁺-regulated gating properties of hCx26 hemichannels expressed in Xenopus oocytes were explored using the two-electrode voltage-clamp technique. We observed that the tail currents that followed depolarizing pulses were more accurate and useful measures of hCx26 hemichannel activation and kinetics than were the outward currents that developed during the pulses (see supplemental text and Figs. S1 and S2 for a complete description). We found that with depolarizing pulses to 0 mV, the peak tail currents increased as a function of pulse duration, reaching a maximum with pulses of 40 s (Fig. S1). Therefore, our standard protocol for assessment of hCx26 hemichannel activation and deactivation was to examine the peak tail currents and their relaxation kinetics after 40-s pulses from -80 to 0 mV. Even though depolarization pulses above 0 mV would activate more hemichannels, limiting depolarizations to 0 mV allowed us to minimize the deleterious effects of massive hemichannel opening during the long pulses required to reach steady-state activation, as well as eliminate contributions from endogenous currents. The tail currents thus measured reflect the steady-state channel activation at 0 mV at any given Ca²⁺ concentration.

Using this method, the hCx26 hemichannel tail currents in response to changes in external Ca²⁺ concentration were examined. Fig. 1 A shows current traces obtained at 10, 1.8, 0.5, and 0.1 mM of extracellular Ca²⁺ from the same oocyte expressing a moderate level

of hCx26 currents. The peak tail currents increase with reduction of external Ca²⁺, showing that external Ca²⁺ inhibits activation of hCx26 hemichannels. The holding current before depolarization is significantly increased at 0.1 mM Ca²⁺, indicating that low Ca²⁺ causes an increase in open hemichannels, even at -80 mV. The activation of the hemichannels as a function of Ca²⁺ concentration, normalized to the maximal activation of the tail current at the lowest Ca²⁺ concentration, is shown in Fig. 1 B. The data are fit to a Hill equation of the form:

$$I/I_{\text{max}} = 1/1 + ([Ca^{2+}]/K_D)^n,$$
 (2)

where the fractional current is I/I_{max} , I is the tail current at a particular Ca^{2+} concentration, I_{max} is the maximal tail current activation at 0.01 mM Ca^{2+} , K_D is the apparent affinity, $[Ca^{2+}]$ is the concentration of Ca^{2+} applied to the bath, and n is the Hill coefficient. The best-fit parameter values for K_D and n are 0.33 mM and 1.38, respectively. Fig. 1 C displays the deactivation time constants of tail currents at different Ca^{2+} concentrations. The deactivation kinetics are accelerated as a function of Ca^{2+} concentration.

Consistent with previous reports based on currents obtained during depolarizing pulses, the tail current data indicate that external Ca²⁺ causes a relative stabilization of closed hemichannels. The kinetic data indicate that external Ca²⁺ also accelerates transitions to the closed state.

Aberrant gating by extracellular Ca²⁺ in D50N/Y mutant hemichannels

Several hCx26 mutations produce exacerbated opening of hemichannels, the closure of which requires high extracellular Ca²⁺ (5–10 mM), unlike wild-type channels,

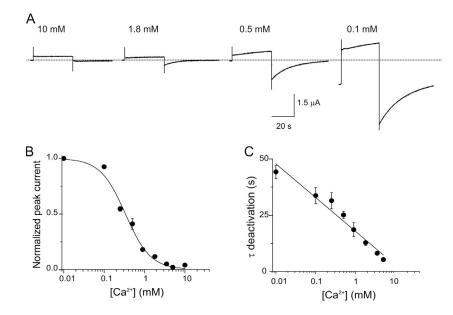


Figure 1. Ca²⁺ modulates gating in hCx26 hemichannels. (A) Current traces elicited by a voltage pulse from -80 to 0 mV from oocytes expressing hCx26 hemichannels in the presence of different Ca²⁺ concentrations. (B) [Ca²⁺] dose-response relation determined from the peak tail current after a voltage pulse from -80 to 0 mV. The solid line represents the best fits of the data to a Hill equation (Eq. 2). (C) Deactivation time constants as a function of Ca²⁺ concentration. The solid line corresponds to a linear fit to the data. The data points represent mean ± SEM of at least three independent measurements.

for which 1.8 mM of extracellular Ca²⁺ is sufficient. Using tail current analysis, the effects of extracellular Ca²⁺ on hCx26 hemichannels containing a single human mutation replacing the aspartate at position 50 with an asparagine (D50N) or a tyrosine (D50Y) were examined. These substitutions remove the negative charge at this position. Fig. 2 A shows current traces in response to depolarizing pulses from -80 to 0 mV in the presence 0.25, 1.8, and 10 mM Ca²⁺ for oocytes expressing D50N or D50Y mutant hemichannels. For both mutations, the holding currents and peak tail currents significantly increase as extracellular Ca²⁺ is reduced, and at high Ca²⁺ concentrations, the tail currents are greater than for wild-type channels. For example, at 10 mM Ca²⁺, tail currents are completely absent in wildtype channels (Fig. 1 A), but in both D50N and D50Y mutants, they are substantial, suggesting that these mutations decrease the ability of Ca²⁺ to stabilize the closed state.

Given the enhanced hemichannel opening of the mutants at high extracellular Ca²⁺, we were concerned that Ca²⁺ influx through the increased number of open hemichannels could activate endogenous Ca²⁺-activated chloride currents, contaminating the measured macroscopic currents. Ca²⁺-activated chloride currents can be eliminated by preinjection of oocytes with ~120 μM BAPTA (see Fig. S2). We obtained [Ca²⁺] dose–response curves in oocytes expressing the D50N/Y mutants preinjected with BAPTA. No significant differences were observed in the currents in the absence or presence of intracellular BAPTA for oocytes expressing moderate levels of D50N/Y mutants (not depicted), indicating that even with the increased hemichannel activity at

high extracellular Ca²⁺, there was no significant contamination by Ca²⁺-activated chloride currents.

Fig. 2 B shows the [Ca²⁺] dose–response relations for D50N and D50Y. The calculated values for K_D are 1.5 and 1.3 mM for D50N and D50Y mutants, respectively. There is a significant rightward shift in the K_D of D50N/Y mutants with respect to that observed in wildtype hemichannels. At physiological Ca²⁺ concentration (1.8 mM), although D50N/Y mutant hemichannels reach ≥40% of the maximal response, the wild-type channels reach only $\sim 15\%$. As suggested by the traces in Fig. 2 A, these results confirm that the charge-removing substitutions of the aspartate at position 50 with asparagine or tyrosine interfere with the ability of extracellular Ca²⁺ to favor the closed state of hCx26 hemichannels. There is a decrease in the slope of the relation as well, which could suggest fewer sites of Ca²⁺ action, reduced cooperativity, or less effective transduction of Ca²⁺ binding to effect gating changes.

In contrast to wild-type channels, Fig. 2 C shows that these mutations render the deactivation time constants rapid and nearly completely unresponsive to changes in extracellular Ca^{2+} concentration. Interestingly, even though the mutations favor the open state at high Ca^{2+} concentrations relative to wild type, the deactivation (i.e., closing) rate of the channels is increased, even at low Ca^{2+} , to that of wild-type channels. Whereas D50N mutant hemichannels show a single-exponential decay component of $\sim 11 \pm 0.4$ s, D50Y mutants display two components, a fast component of 2.6 ± 0.5 s and a slow component of 10 ± 0.4 s. Intracellular injection with $120 \,\mu\text{M}$ BAPTA did not affect the exponential components for D50Y mutant hemichannels, suggesting that

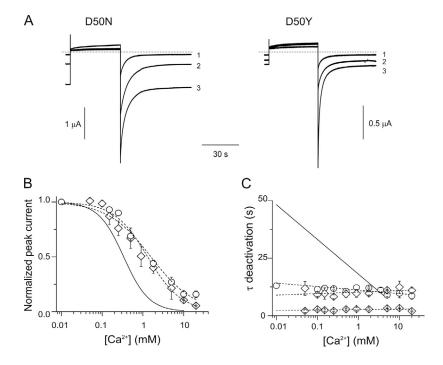


Figure 2. Gating by Ca²⁺ is altered by D50N/Y mutations in hCx26 hemichannels. (A) Representative current traces elicited by a pulse to 0 mV from a holding potential of -80 mV for an oocyte expressing D50N or D50Y mutant hemichannels. Numbers 1, 2, and 3 correspond to current traces obtained in the presence of 10, 1.8, or 0.25 mM Ca²⁺, respectively. (B) Ca²⁺ doseresponse curve for oocytes expressing D50N (open circles) or D50Y mutant (open diamonds) hemichannels. The solid and dotted lines represent the best fits to a Hill equation (Eq. 2) for wild-type (from Fig. 1 B) and D50N/Y mutant hemichannels, respectively. (C) Deactivation time constants of the corresponding tail currents at different Ca2+ concentrations for D50N (open circles) or D50Y mutants (fast and slow time constants are shown as two sets of diamonds). Dotted lines are the best linear fit to the D50N/Y mutant hemichannel data. The solid line corresponds to the linear fit of the average data for wild-type hemichannels (from Fig. 1 C). The data represent mean \pm SEM of at least three independent measurements.

the additional component is not caused by contaminating chloride currents activated by Ca²⁺ influx, and that it is a component of Cx26 channel closure specifically induced by the D50Y mutation.

These mutations at D50 can be seen to have two effects: (1) from the steady-state measurements, they significantly reduce (but do not entirely eliminate) the ability of Ca²⁺ to stabilize the closed state relative to the

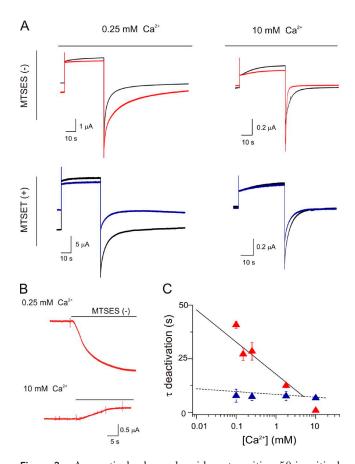


Figure 3. A negatively charged residue at position 50 is critical for regulation by Ca²⁺. (A) Hemichannel currents from oocytes expressing D50C mutant hemichannels in low and high extracellular Ca²⁺ to assess the effects of chemical modification with MTS reagents. Currents were elicited by a pulse to 0 mV from a holding potential of -80 mV before (black trace) or in the presence of MTSES⁻ (red traces) or MTSET⁺ (blue traces). (B) Holding currents obtained at -80 mV for oocytes expressing D50C mutant hemichannels incubated in low (0.25 mM) or high (10 mM) external Ca²⁺. The addition of MTSES⁻ increased the holding current in low extracellular Ca2+ (top trace) and decreased the holding current in the presence of high Ca²⁺ (bottom trace). (C) Deactivation time constants for D50C mutant hemichannels at different Ca²⁺ concentrations before (dotted line; from Fig. S3) and after chemical modification with MTSES- (red triangles) or MTSET+ (blue triangles). Time constants obtained after MTSES⁻ modification coincide with the solid line (from Fig. 1 C) that corresponds with the linear fit for wild-type hemichannels. Conversely, the time constant obtained after MTSET+ overlaps with the linear fit for D50C mutants with no modification. The data represent mean \pm SEM of at least three independent measurements.

open state (rightward shift in Fig. 2 B); and (2) from the tail current kinetics, they eliminate the Ca²⁺ effect on the deactivation time constant and increase the deactivation time constant to that seen at high Ca²⁺ in wild-type channels (at 1.8 mM Ca²⁺; Fig. 2 C). The more rapid deactivation kinetics suggest that elimination of the negative charge at position 50 decreases the dominant energy barrier for channel closing, just as high Ca²⁺ does in wild-type channels. The fact that mutations that remove the negative charge at position 50 reduce Ca²⁺ sensitivity of the currents, accelerate deactivation kinetics, and render them effectively insensitive to Ca²⁺ lead to the idea that in wild-type channels, Ca²⁺ may disrupt electrostatic interactions that involve D50.

A negatively charged residue at position 50 is critical to enhance opening and closing of the channel at low and high Ca²⁺, respectively

To further explore the role of a negatively charged residue at position 50 in Ca²⁺ regulation, D50 was substituted with a cysteine residue (D50C), which was then chemically modified with negatively or positively charged MTS reagents, sodium (2-sulfanoethyl) MTS (MTSES)⁻ and [2-(trimethylammonium) ethyl] MTS bromide (MTSET)⁺. As expected, substitution of the Cys residue at this position yielded hemichannels with gating properties similar to those described above for D50N/Y mutants. Fig. S3 shows the [Ca²⁺] dose–response relation and deactivation time constants for D50C hemichannels. The best-fit parameter values for K_D and *n* were 4 mM and 1.38, respectively. As for D50N/Y, deactivation time constants were fast and essentially unresponsive to changes in extracellular Ca²⁺ concentration.

Fig. 3 (A and B) shows representative hemichannel current traces from D50C mutant hemichannels obtained at 0.25 and 10 mM of extracellular Ca2+ in the absence (black traces) or the presence (colored traces) of 100 µM MTS reagents. At low extracellular Ca²⁺, modification of D50C channels with negatively charged MTSES⁻ (Fig. 3 A, red trace) increased the holding and tail currents and slowed the deactivation time constant (Fig. 3, A, top left panel, and B, top trace). Conversely, at high extracellular Ca²⁺, modification with MTSES⁻ reduced the holding currents and accelerated the deactivation time constant (Fig. 3, A, top right panel, and B, bottom trace). Fig. 3 C shows that modification of D50C with MTSES-, which reinstates the wild-type negative charge at this position, reproduces wild-type Ca²⁺-sensitive deactivation kinetics (Fig. 3 C, red symbols).

These results point to the importance of a negative charge at position D50 to both stabilize the open state relative to the closed state at low Ca²⁺ and to favor and facilitate transitions to the closed state at high Ca²⁺. We note that at high Ca²⁺, the tail currents are larger after modification with MTSES⁻, rather than smaller as

would be expected if full wild-type behavior was recovered. These increased tail currents are not affected by intracellular injection with 120 mM BAPTA, eliminating the potential contribution of endogenous chloride currents (not depicted). Given the clear recapitulation of Ca²⁺ effects on the deactivation kinetics (Fig. 3 C), and the decrease in holding current at high Ca²⁺ (Fig. 3 B, bottom trace), we infer that the increase in tail currents at high Ca2+ with MTSES modification arises from an effect on a different process. A likely effect is on activation of the channels by voltage, perhaps caused by the negative charge of the MTSES⁻ moiety having a different relation to the transmembrane voltage field than that of the native aspartate. This region is known to be involved in voltage-induced rearrangements at the extracellular loop (Tang et al., 2009; Verselis et al., 2009).

D50C mutants modified with the positively charged MTSET⁺ show a decrease in the holding currents and tail currents at low Ca²⁺ (opposite of the effect of MTSES⁻) but no change in the deactivation time constants (Fig. 3 A, bottom panels, and C, blue symbols).

These data show that chemical modification with a negatively charged reagent largely reinstates wild-type behavior in D50C hemichannels, indicating that a negative charge at this position is critical to the effects of Ca²⁺ on relative stability of the open and closed states, and the transitions between them. When the charge at this position is removed or made positive, the deactivation is independent of the extracellular Ca²⁺ concentration. We do not know whether all six of the D50C residues in a hemichannel need to be modified to have these effects, or if only a subset is modified by the MTS reagents.

Molecular interactions of D50 that stabilize the open state in low external Ca^{2+}

To better understand the molecular mechanism by which D50 stabilizes the open-channel conformation at low extracellular Ca²⁺ concentrations, we investigated

possible electrostatic interactions between D50 and neighboring residues, using the recent hCx26 crystal structure (Maeda et al., 2009) as a guide. This structure was obtained in the absence of Ca²⁺ and seems to represent the channel with open gates. D50 is in the outer part of the ion permeation pathway. The only positively charged residue near the carboxyl group of D50 is lysine 61 (K61) in the adjacent connexin subunit, at ~4.4 Å (Fig. 4). Although this distance is just beyond that of a typical salt bridge interaction (<4 Å), the moderate 3.5-Å resolution of the structure and the proximity of these oppositely charged residues led us to speculate that these residues interact electrostatically in the open conformation to provide the enthalpy that helps to stabilize the open conformation.

As a first approach to test this idea, we reasoned that if a salt bridge interaction between D50 and K61 favors the open state of the channels and is involved in regulation by Ca²⁺, then swapping the positively and negatively charged residues should retain a possible electrostatic interaction between these residues and produce channels sensitive to Ca²⁺, whereas the individual charge reversals should not (of course, there could be other effects of both the individual and combined mutations). A similar approach was applied successfully to CNG channels to establish the role of salt bridges in gating (Craven and Zagotta, 2004).

The single charge reversal mutations, D50K and K61D, do not yield functional hemichannel currents (Fig. 5 A, top two traces). However, as predicted, hemichannel currents and sensitivity to Ca²⁺ were rescued in the double mutant D50K/K61D (Fig. 5, A, bottom trace, and C), which swaps the charges at the two positions. The Ca²⁺ dependence of the deactivation kinetics was only modestly recovered, yet the kinetics were slower at all Ca²⁺ concentrations than in the D50N/Y mutants, particularly at low Ca²⁺ (Fig. 5 B, symbols). This suggests that a negatively charged residue is specifically required at position 50 for the full Ca²⁺ effect on deactivation, and that a negative charge at position 61

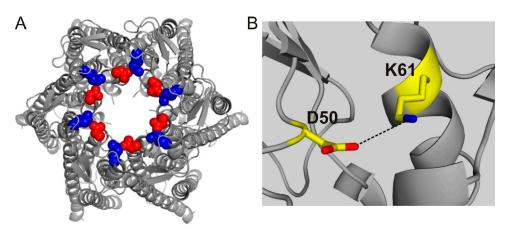


Figure 4. Possible intersubunit salt bridge interaction between positions D50 and K61 in the open conformation. (A) Top (extracellular) view of the hCx26 hemichannel from the crystal structure (Protein Data Bank accession no. 2ZW3; Maeda et al., 2009). Positions D50 and K61 are highlighted in red and blue (in all subunits), respectively. (B) Enlargement showing the proximity between position D50 in one subunit and position K61 in the adjacent subunit. The average distance for the six pairs of D50–K61 residues is \sim 4.4 Å.

cannot serve this function. For the $[Ca^{2+}]$ dose–response relations of D50K/K61D double mutants, the best-fit parameter values for K_D and n were 0.5 mM and 0.6, respectively (Fig. 5 C). There is a decrease in the slope of the relation with respect to wild-type hemichannels, suggesting fewer sites of Ca^{2+} action, less cooperativity, and/or less efficient transduction of Ca^{2+} binding to effect gating changes. Overall, reversing the polarity of charged residues at positions 50 and 61 produced functional channels and partially rescued the wild-type hCx26 sensitivity to Ca^{2+} , suggesting that having opposite charges at these two positions is important both to stabilize open channels and to respond to Ca^{2+} .

To obtain further validation of an interaction between D50 and K61 and its relation to regulation of Ca²⁺, we performed thermodynamic analysis based on mutagenesis, a method known as double mutant cycle analysis, which quantifies the coupling energy between two mutated residues and thus indicates the likelihood that the two residues interact (Faiman and Horovitz, 1996).

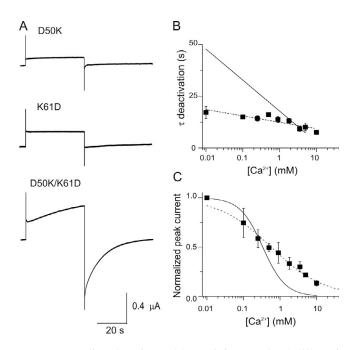


Figure 5. Exchanging the positions of the negative (D50) and positive (K61) residues partially rescues the wild-type hCx26 regulation by Ca²⁺. (A) Current traces elicited by a pulse to 0 mV from a holding potential of -80 mV are shown for oocytes expressing D50K, K61D, or D50K/K61D mutant hemichannels in 1.8 mM Ca²⁺. (B) Deactivation time constants for oocytes expressing D50K/K61D mutant hemichannels (closed squares). The solid line corresponds to best fits to the average data for wild-type hemichannels (from Fig. 1 C). The dotted line is the best linear fit to the data from double mutant D50K/K61D hemichannels. (C) [Ca²⁺] dose-response relations for oocytes expressing D50K/K61D mutant hemichannels (closed squares). The solid and dotted lines correspond to the best fits to the data of a Hill equation (Eq. 1) for wild-type and double mutant D50K/K61D hemichannels. The data represent mean ± SEM of at least three independent measurements.

Ideally, each mutation should eliminate the interaction being studied. For an electrostatic interaction, the full coupling energy can be derived from substitutions that completely eliminate the interaction; however, a partial coupling energy can be derived from mutants that alter but do not eliminate the interaction. For position D50, we used the D50N mutation because it eliminates the electrostatic interaction, yet the channels show robust hemichannel currents and simple kinetics (Fig. 2). However, at position K61, substitution of neutral amino acids (alanine, cysteine, and serine) or histidine did not lead to observable hemichannel currents. However, it has been shown previously that mutant cycle analysis can be performed using a substitution with the same charge that also alters the channel property being studied (e.g., NaChBac channels; Paldi and Gurevitz, 2010). We found that replacing K61 with an arginine (K61R), which retains the charge at this position, results in functional hemichannels. As expected, this channel has kinetic properties similar to those of wild-type channels (Fig. 6), but it has somewhat different parameters of Ca²⁺ sensitivity, enabling it to be used in the cycle analysis. [Ca²⁺] dose-response curves in oocytes expressing the K61R mutant channels show values for K_D and n of 0.4 mM and 0.85, respectively. Interestingly, the double mutant D50N/K61R shows steady-state currents similar to those of the K61R mutant but with two deactivation time constants nearly insensitive to extracellular Ca²⁺, similar to the D50Y mutants. The best-fit parameter values for K_D and n from the $[Ca^{2+}]$ doseresponse curves for double mutant D50N/K61R were 0.51 mM and 0.7, respectively (Fig. 6 B).

The apparent affinities for Ca^{2+} from wild-type, D50N, and K61R single mutants, and D50N/K61R double mutant channels (Fig. 6 B), were used to perform mutant cycle analysis. The pairwise interaction energy between positions was estimated using Eq. 1. This analysis yielded a coupling energy ($\Delta\Delta G$) of -0.75 kcal/mol (cutoff for noninteraction is below ± 0.5 kcal/mol). Because this pair of substitutions, in which the charge at one position was preserved, can yield only a portion of the coupling energy of a salt bridge still yields a significant $\Delta\Delta G$, this result suggests that side chains of residues D50 and K61 interact in a Ca^{2+} -sensitive manner.

Even though the above result indicates a significant Ca^{2+} -sensitive interaction between D50 and K61, an apparent K_D can depend on multiple microscopic reactions that involve both ligand affinity and channel gating (Colquhoun, 1998; Gleitsman et al., 2008). These factors may affect the values calculated for the coupling energies that exist between two states (Laha and Wagner, 2011). The fact that charge-eliminating substitutions at position D50 render the deactivation kinetics nearly completely insensitive to Ca^{2+} suggests that essentially all of the Ca^{2+} sensitivity of the deactivation time constant is caused by Ca^{2+} -mediated disruption of the D50–K61

interaction that exists in the open state. This idea—the effect of Ca²⁺ on deactivation kinetics is exclusively on open channels with a D50–K61 interaction—can be assessed by how well the Ca²⁺ dependence of the deactivation kinetics can be modeled as if they arise from simple open-channel "block" by Ca²⁺ using an equation of the form:

$$\tau = 1/k_{\text{off}} + k_{\text{on}} \left\lceil Ca^{2+} \right\rceil, \tag{3}$$

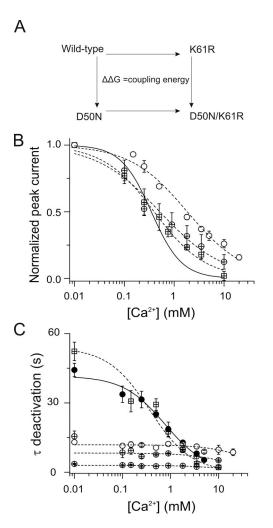


Figure 6. Mutant cycle analysis of K_D indicates that D50 and K61 residues are coupled in a Ca²⁺-sensitive manner. (A) Scheme for mutant cycle analysis of wild-type and mutant hemichannels. (B) Graph shows the [Ca²⁺] dose–response relations for oocytes expressing D50N (open circles) and K61R (crossed squares) mutants, and D50N/K61R (crossed circles) double mutant hemichannels. The solid and dotted lines represent the best fits to the Hill equation for wild-type (from Fig. 1 B) and mutant channels, respectively. (C) Deactivation time constants for oocytes expressing wild-type (closed circles), D50N (open circles), and K61R (crossed square) mutants, and D50N/K61R (fast and slow time constants are shown as two sets of crossed circles) double mutant hemichannels. The solid line corresponds to the best fits to the average data for wild-type hemichannels using Eq. 3. The dotted line is the best fits to the data to Eq. 3 for single and double mutant hemichannels.

where k_{on} is a Ca^{2+} -dependent "closing rate," and k_{off} is a Ca^{2+} -independent "opening rate." If the system can be accurately described in this way, this analysis can reveal how the apparent Ca^{2+} affinity of open channels changes with the mutations that preserve and do not preserve the D50–K61 interaction.

In Fig. 6 C, the Ca^{2^+} dependence of deactivation time constants for wild-type, D50N, and K61R single mutants, and the D50N/K61R double mutant, were fit using Eq. 3. The data from the channels that preserve the opposing charges at positions 50 and 61 (wild-type and K61R mutant) were well fit by Eq. 3, strongly suggesting that the Ca^{2^+} effect on deactivation kinetics of channels with this charge pair is on open channels. The derived values for the apparent Ca^{2^+} on-rates (k_{on}) and off-rates (k_{off}) are summarized in Table 1. An apparent affinity (K_D) of Ca^{2^+} of open channels can then be calculated by dividing k_{off} by k_{on} . These K_D values are independent of those derived from the steady-state data, which include the effects of Ca^{2^+} on the closed state.

The open-state Ca²⁺ apparent affinities thus computed for wild-type and K61R mutant channels (which retain the charge interaction between positions 50 and 61) were similar, with K_D values of 0.72 and 0.36 mM, respectively, which were close to the apparent K_D values derived from steady-state data (0.33 and 0.4 mM, respectively). Conversely, channels in which the interaction was disabled (D50N and D50N/K61R channels) showed substantially lower Ca²⁺ apparent affinity for open channels (42 mM for D50N and 17 and 15.7 mM for the slow and fast components of D50N/K61R channel deactivation, respectively). These results support the notion that Ca²⁺ sensitivity of the deactivation kinetics is predominantly accounted for by Ca²⁺ effects on the D50–K61 interaction that exists in open channels.

To gain information on the thermodynamic linkage between [Ca²+] and the D50–K61 interaction in the open state, we performed mutant cycle analysis based on the apparent rate constants derived from the above calculations. This analysis yielded a coupling energy ($\Delta\Delta G$) for the on-rate (k_{on}) and off-rate (k_{off}) of approximately -1.0 kcal/mol for the fast kinetic component of D50N/K61R deactivation but lower coupling energy for the on-rate (k_{on}) and off-rate (k_{off}) of -0.46 and -0.38 kcal/mol for the slow component, respectively

TABLE 1 Kinetics of Ca²⁺-dependent deactivations

| | Timeties of Ca | acpenaeni | acaciteations | |
|----------------|----------------|------------------|-----------------|-------|
| Hemichannel | | K _{off} | K _{on} | K_D |
| | | s | $M^{-1} s^{-1}$ | |
| Wild type | | 0.024 | 0.031 | 0.72 |
| D50N | | 0.084 | 0.002 | 42 |
| K61R | | 0.018 | 0.05 | 0.36 |
| D50N/K61R slo | v | 0.12 | 0.007 | 17 |
| D50N/K61R fast | | 0.338 | 0.021 | 15.7 |

(Table 2). These results further support a significant thermodynamic linkage between [Ca²⁺] and the D50–K61 interaction in the open state. Furthermore, the correspondence between the $\Delta\Delta$ Gs for this linkage derived from steady-state data (which cannot distinguish effects on open and closed channels) and from the kinetic data strongly suggests that disruption of the D50–K61 interaction occurs in open channels.

DISCUSSION

It is widely recognized that extracellular Ca²⁺ inhibits hemichannel activity. We show here that external Ca²⁺ also accelerates the deactivation of hCx26 tail currents, facilitating the closing of the channels. Mutation of D50 to uncharged residues (N/Y/C) results in rapid deactivation kinetics that are insensitive to Ca2+ and shifts rightward the steady-state effect of external Ca²⁺ to close the channels, resulting in more channels being open at physiological Ca²⁺ levels. Reintroduction of a negative charge at this position by modification with MTSES⁻ in the D50C mutant restores the Ca²⁺ dependence of the steady-state currents and wild-type Ca²⁺ dependence of deactivation kinetics. A simple interpretation is that a negative charge at position 50 plays a role in (a) stabilizing the open state, and (b) enabling Ca²⁺ to facilitate closing and destabilization of the open state. Based on these findings and the crystal structure, we speculated that in wild-type channels, D50 interacts electrostatically with K61 in the adjacent subunit, the only nearby positively charged residue. A mutant channel in which the charges at these two positions are swapped (the D50K/K61D mutant) displays Ca²⁺ sensitivity of steady-state currents, whereas channels with the corresponding single substitutions were nonfunctional. We therefore propose that the negative charge of D50 interacts with other residues, including K61 via a salt bridge, to stabilize the open state, and that these interactions are disrupted by Ca2+, resulting in destabilization of the open state and Ca²⁺-dependent closing kinetics. Double mutant cycle analyses support a thermodynamic linkage between Ca2+ and disruption of the D50-K61 interaction in open channels. Analysis of steady state and deactivation kinetics of channels in which the charge at position 50 is removed suggest that in addition to its action to disrupt the D50-K61 interaction

 ${\tt TABLE~2}$ Mutant cycle analysis of rates obtained from ${\it Ca^{2+}}$ -dependent deactivation

| Mulant cycle analysis of rates obtained from Ca -dependent dedictions | | |
|---|---------------------------|--|
| D50-K61 | $\Delta\Delta G$ coupling | |
| | kcal | |
| K _{off (K61RD50N; slow)} | -0.38 | |
| $K_{\rm off~(K61RD50N;~fast)}$ | -0.99 | |
| Kon (K61RD50N; slow) | -0.46 | |
| $K_{on~(K61RD50N;~fast)}$ | -1.1 | |
| | | |

in open channels, Ca²⁺ also acts on closed channels (in which there is no D50–K61 interaction) to stabilize the closed state.

In the absence of a validated kinetic model of connexin hemichannel gating that allows us to obtain reliable microscopic rate constants, mutant cycle analyses were performed using apparent affinity values for extracellular Ca2+ from wild-type and mutant channels, using both steady-state and kinetic data, with the latter focusing on Ca²⁺ effects on the open state only. These studies were constrained to reveal only part of the total coupling energies because we were unable to identify mutations at position 61 that both removed charge (to eliminate any direct D50-K61 charge interaction) and retained channel function. Instead, we used a charge-preserving mutation (K61R) that nonetheless perturbed Ca²⁺ sensitivity sufficiently to be useful. Because we had shown that the charges at both positions are involved in regulation by Ca²⁺, analyses using the K61R mutants could therefore reveal only a small portion of the total coupling energy between positions D51 and K61. The full magnitude of the coupling energy could only be revealed if charge was eliminated at position 61; most charge interactions with position 61 would be retained in the K61R mutant, with the substitution producing only small changes (reflected in the small change in K_D). In spite of this limitation, the coupling energies were significant, validating the view that there is interaction between D50 and K61 in the open state and that it is affected by Ca²⁺. In addition, using K_Ds derived from deactivation kinetics, we found that essentially all of the meaningful Ca²⁺ sensitivity of the current deactivation required the D50-K61 linkage.

Our data show that the presence of opposite charges at positions 50 and 61 is a key factor in regulation of the channels by Ca²⁺, and that there is a thermodynamic linkage between these charges. These results, and the indication of close proximity in the crystal structure, suggest the presence of a salt bridge between positions 50 and 61 (whether D50-K61 or D50K-K61D) that stabilizes the open state of the channel and is required for the ability of Ca²⁺ to destabilize the open state. The fact that the single mutations (D50K and K61D) do not yield functional channels, and the double mutant (D50K-K61D) does, confirms the specific importance of the interaction between the charges at these residues. However, the swapped charges do not fully restore the ability of Ca²⁺ to enhance the kinetics of deactivation (closing) of the channels. It appears that a negative charge at position 50 specifically is required for this.

Our experiments showing accessibility by MTS reagents indicate that position D50 is water accessible. Therefore, it is likely that the electrostatic interactions of D50, including that with K61 and linked interactions in this region of the protein, are also accessible by Ca²⁺. Our data do not bear directly on whether D50 itself is

a structural component of the Ca²⁺-binding site. We favor the view that intrasubunit and intersubunit electrostatic networks are involved in these gating reactions (Kwon et al., 2011, 2012). Thus, we cannot distinguish whether Ca²⁺ disrupts the D50–K61 interaction directly and/or by acting allosterically elsewhere in the network. There are undoubtedly other open-state stabilizing electrostatic interactions in these channels, some of which may be sensitive to Ca²⁺ and some not (Tong et al., 2013). The precise mechanism of pore occlusion to ion flux that is induced by interaction of Ca²⁺ with the channel open state remains elusive.

Our overall view is that in wild-type Cx26 hemichannels, salt bridge formation between positions D50 and K61 enhances the stability of the open state, and that Ca²⁺ disrupts this salt bridge. In this view, reducing Ca²⁺ favors formation of the salt bridge, increasing occupancy of open states as well as decreasing the deactivation time constant. Mutations at D50 that prevent formation of this salt bridge mimic the disruption of the salt bridge that occurs at high Ca²⁺, lowering open-state occupancy and mimicking the deactivation time constant observed at high Ca²⁺ concentration.

Given this view, what is the explanation for the significant but right-shifted Ca2+ dependence of the steadystate data observed in D50 mutants? We suspect and favor the idea that there are several closed and open states for hemichannels, as is the case for many other types of ion channels, with some of these transitions being Ca²⁺ sensitive and some Ca²⁺ insensitive. The persistence of Ca²⁺ sensitivity in the steady-state data in the face of its elimination in the deactivation kinetics in the charge-removing mutants at position 50 (D50N/Y/C mutations) points to Ca²⁺-dependent transitions among closed states and/or stabilization of closed states by Ca²⁺, independent of its disruption of the D50-K61 interaction. That is, in wild-type channels, the Ca²⁺-dependent disruption of the D50-K61 interaction is the rate-limiting step for deactivation. When it does not exist, as in the D50 mutants, Ca²⁺-independent transitions become rate limiting for deactivation, yet transitions out of closed states still have a Ca²⁺ dependence, producing a right-shift in steady-state current dependence on Ca²⁺. Kinetic analysis of the Ca²⁺-dependent deactivation also suggests that extracellular Ca²⁺ can affect both open- and closed-state transitions. The Ca²⁺ affinity derived from the deactivation data for the D50N mutant was much less than that derived from the steadystate data, compared with wild-type channels (1.5 mM from the steady-state analysis and 42 mM from the kinetic analysis). The kinetic analysis of deactivation excludes Ca²⁺ effects on closed channels, so the fact that there is a difference in the two apparent affinities points to an effect of Ca2+ on closed channels. Single-channel analysis will be necessary to reveal state-dependent binding/effects of Ca²⁺. Thus far, we have been unable to obtain single-channel recordings from hCx26 hemichannels under conditions that could provide a reliable kinetic understanding of this process.

Interpreting what happens, at the molecular level, in the closed state(s) is less clear, as we do not have closed-state structural data on which to base an explanation of the effects of mutation at position D50. However, the finding that MTSES modification of D50C mutants reduces the steady-state current at high Ca²⁺ (when a D50-K61 salt bridge should be disrupted; Fig. 3 C) supports the idea that in the presence of Ca²⁺, a negative charge at position 50 contributes to stabilization of the closed state, in the absence of the salt bridge. That is, in the closed salt bridge-disrupted state, D50 could contribute directly or indirectly to Ca²⁺ stabilization of the closed state. However, we have no experimental information regarding D50 interactions in the Ca²⁺-closed state that can further support this idea. Experiments to address this will be difficult to design in the absence of a Ca2+-bound closed structure. There is atomic force microscopy evidence that hemichannel gating by Ca²⁺ in several connexins, including Cx26, is accompanied by large structural changes near the extracellular end of the channel, where these residues are located (Müller et al., 2002; Thimm et al., 2005; Allen et al., 2011). These large conformational rearrangements may result in distinct electrostatic interactions of position D50 in the open and Ca²⁺-closed states.

Residues at the extracellular end of the pore, the region of transition between the first transmembrane segment (TM1) and the first extracellular loop (E1), where D50 is located, have been shown to undergo voltagedriven conformational changes that close the pore, a process referred to as "loop gating" (Kronengold et al., 2003; Tang et al., 2009; Verselis et al., 2009). It is unknown to what extent the conformational changes induced by voltage stimulation are similar to those triggered by extracellular Ca²⁺. A recent study suggests that the large conformational changes seen in atomic force microscopy with exposure to Ca²⁺ may not occur with voltage gating (Kwon et al., 2013). In calcium- and voltage-activated potassium channels, the voltage dependence shifts leftward with increased Ca²⁺ concentrations (Magleby, 2003; Latorre et al., 2010). In Cx46, the voltage dependence of loop gating shifts leftward with Ca²⁺ decrease (Ebihara et al., 2003). We do not know whether there is similar allosteric coupling between Ca²⁺ and voltage-driven loop gating in hCx26 hemichannels. It is possible that mutations at D50 alter voltage sensitivity, thereby indirectly affecting Ca²⁺ sensitivity. However, the deactivation kinetics of tail currents are only weakly affected by voltage using repolarizing pulses over the range of -120 to -20 mV (Fig. S5). Detailed biophysical analysis of Ca²⁺ gating, its interplay with voltage gating, and the role of D50 are best explored using single-channel recordings. An exploration of the interplay between Ca²⁺ and voltage regulation remains elusive in hCx26 hemichannels at this time.

The charges at positions 50 and 61, and the interaction between these positions, are likely to serve similar function in some other connexins, but not all. Human Cx30, which has identical residues at these positions, displays gating behavior similar to Cx26 hemichannels in response to changes in extracellular Ca²⁺ concentrations when expressed in Xenopus oocytes. Negative (D or E) and positive (R or K) charges at the analogous positions are found in Cx25, Cx30.2, and Cx31.9. Connexins with "swapped" charges at these positions include Cx45 and Cx47. However, residue pairs corresponding to positions D50 and K61 in other connexins strongly regulated by Ca²⁺ are not always charged or even polar. Lens connexins typically have negatively charged residues at both positions, and have a range of Ca²⁺ sensitivities (Paul et al., 1991; Zampighi et al., 1999; Beahm and Hall, 2002; Ebihara et al., 2003; Tong and Ebihara, 2006). The fact that these channels are functional and in hCx26 the K61D mutant is not suggests that other factors, such as compensatory sequence/structural changes or voltage sensitivities, or indeed completely distinct mechanisms, may contribute to the Ca²⁺ sensitivity in those connexins. It remains to be seen whether the general mechanism of Ca²⁺-mediated disruption of a salt bridge that stabilizes the open state, whether at these particular sites or not, operates in other connexin channels.

Physiological and pathophysiological implications

To date, more than 100 human mutations in hCx26 have been shown to cause sensorineural deafness and dermatological disorders. Many of these mutations result in defective channel biogenesis or trafficking. However, a significant number produce connexin channels that are functional at the plasma membrane yet cause pathology. These mutants likely induce altered gating and/or permeability. Indeed, the lack of associated skin disorders in cases of hCx26-null mutations shows that the epidermal dysfunction is not produced by the simple loss of Cx26 function. D50N/Y mutations are linked to keratitis-ichthyosis-deafness syndrome, which consists mainly of sensorineural hearing loss, keratitis, and severe skin lesions (Martínez et al., 2009; Xu and Nicholson, 2013). Our work suggests that the molecular basis of the pathology is elimination of the salt bridge between positions 50 and 61, with the consequence that the hemichannels are less sensitive to regulation by external Ca²⁺ and the elimination of the negative charge at position 50 that stabilizes the closed state.

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