The Carboxyl Terminal Domain Regulates the Unitary Conductance and Voltage Dependence of Connexin40 Gap Junction Channels

Justus M.B. Anumonwo, Steven M. Taffet, Hong Gu, Marc Chanson, Alonso P. Moreno, Mario Delmar

Abstract—Chemical regulation of connexin (Cx) 40 and Cx43 follows a ball-and-chain model, in which the carboxyl terminal (CT) domain acts as a gating particle that binds to a receptor affiliated with the pore. Moreover, Cx40 channels can be closed by a heterodomain interaction with the CT domain of Cx43 and vice versa. Here, we report similar interactions in the establishment of the unitary conductance and voltage-dependent profile of Cx40 in N2A cells. Two mean unitary conductance values ("lower conductance" and "main") were detected in wild-type Cx40. Truncation of the CT domain at amino acid 248 (Cx40tr248) caused the disappearance of the lower-conductance state. Coexpression of Cx40tr248 with the CT fragment of either Cx40 (homodomain interactions) or Cx43 (heterodomain interactions) rescued the unitary conductance profile of Cx40. In the N2A cells, the time course of macroscopic junctional current relaxation was best described by a biexponential function in the wild-type Cx40 channels, but it was reduced to a single-exponential function after truncation. However, macroscopic junctional currents recorded in the oocyte expression system were not significantly different between the wild-type and mutant channels. Concatenation of the CT domain of Cx43 to amino acids 1 to 248 of Cx40 yielded a chimeric channel with unitary conductance and voltage-gating profile indistinguishable from that of wild-type Cx40. We conclude that residence of Cx40 channels in the lower-conductance state involves a ball-and-chain type of interaction between the CT domain and the pore-forming region. This interaction can be either homologous (Cx40 truncation with Cx40CT) or heterologous (with the Cx43CT).

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Key Words: Cx40 ■ connexin ■ carboxyl terminal domain ■ unitary conductance

Gap junctions are essential for direct communication between neighboring cells. These channels mediate vitally important processes such as impulse propagation,1–3 regulation of cell growth,4 and organ development.5,6 Moreover, several hereditary human diseases are linked to mutations in a gap junction protein.7 Clearly, a better understanding of the molecular mechanisms controlling channel function is warranted.

In vertebrates, gap junctions are formed by oligomerization of a protein called connexin (Cx). Most cells express more than one connxin isotype.7 In particular, Cx40 and Cx43 are both expressed in the atrium,8,9 endothelium,10 and smooth muscle cells,10,11 and there is evidence that they heteromerize.12–15 The various connexins also have a similar putative membrane topology, as follows: 4 membrane-spanning domains linked by 1 cytoplasmic and 2 extracellular loops and a cytoplasmic N- and C-terminus.7 The cytoplasmic N- and C-termini of connexins differ in primary sequence and length, whereas the transmembrane and extracellular domains are highly conserved.7

Connexins are highly regulatable molecules, susceptible to association with a number of kinases and with other junctional and nonjunctional proteins.17 We have previously proposed a “ball-and-chain–like” model18 for the chemical regulation of Cx40 and Cx43. According to this model, the carboxyl terminal (CT) domain acts as a gating particle that, under the appropriate conditions (eg, intracellular acidification or phosphorylation), binds to a receptor affiliated with the pore and closes the channel. Consistent with this model, we showed that truncation of the CT domain of Cx40 and of Cx43 resulted in a diminished pH sensitivity of the channels, whereas coexpression of the truncated connexin with mRNA coding for the CT domain (as a separate fragment) restored normal function.18 A similar ball-and-chain model applies to the regulation of Cx43 by insulin or insulin-like growth factor,19,20 as well as by v-src.21 Moreover, we have found that the chemical sensitivity of one truncated connexin channel can be restored by coexpression of the CT domain of a different connexin.20 This promiscuity in the particle-receptor association led to the concept of “heterodomain
interactions,” in which the CT domain of one connexin can interact with the pore-forming region of another. Heterodomain interactions are likely to occur in heteromeric Cx40-Cx43 channels, and they may be responsible for the enhanced sensitivity of these gap junctions to pH. Given the presence of heteromeric gap junctions in native cells, it is relevant to understand the structural bases of their function and regulation.

In this study, we pursue the question of whether interactions between the CT domain and the pore-forming region of connexins are also involved in the control of the biophysical properties of the channel in N2A cells as well as in Xenopus oocytes. Previous data show that truncation of the CT domain of Cx43 alters its voltage dependence. There is evidence that substates as well as the “residual state” of Cx43 results from an interaction between the CT domain and the channel-forming part of the protein (Elenes et al, unpublished data, 2001). In this study, we show that in the N2A cells, (1) the lower-conductance state of Cx40 and a component of its voltage-dependent relaxation are eliminated by truncation of the CT domain, (2) the lower-conductance state is rescued by coexpression of the CT domain of either Cx40 or Cx43, and (3) a chimeric construct formed by Cx40 with its CT replaced by that of Cx43 shows a unitary conductance and voltage-gating profile indistinguishable from that of wild-type Cx40. Overall, our results indicate that the lower-conductance state of Cx40 can be modeled as a ball-and-chain type of interaction and suggest that heterodomain interactions may participate in the multiple conductance states observed in Cx40-Cx43 heteromers. Whether these interactions also modulate the permselectivity of the channels and their ability to couple metabolic functions remains to be determined.

Materials and Methods

Experiments were carried out in N2A cells as well as in Xenopus oocytes using conventional voltage-clamp techniques in cell pairs. Procedures for oocyte preparation and cell culture, mRNA preparation, cDNA transfection, and electrophysiological recordings have been previously described, and an expanded Materials and Methods section is available in an online data supplement at http://www.circresaha.org.

Results

Cx40 single-channel properties have been previously described. As an initial step in this project, we characterized the behavior of wild-type Cx40 channels for later comparison with that of cell pairs expressing the mutant channels. Figure 1A shows junctional current traces obtained from N2A cells stably transfected with wild-type Cx40.
2 traces on the top correspond to the activity simultaneously recorded from cells 1 and 2, in which cell 1 was subjected to a pulse ($V_j = +60$ mV). Thus, the current in cell 2 is equal and opposite to junctional current trace ($I_j$) (see Materials and Methods). The cell pair was uncoupled using octanol. This uncoupler has been shown to modify channel open probability and to have no effect on the unitary conductance value.28

A section of the $I_j$ trace (arrows) is expanded to show the transitions between the closed state and 2 conductive states of different magnitudes. In this article, we will refer to the transitions as the "main open state" and the "lower-conductance state" of the channel. An all-points histogram is shown to the right of the magnified trace (Figure 1). The peaks were $\pm 3.4$ and $10$ pA away from the baseline and represent unitary conductances ($\gamma$) of 56 and 168 pS for the lower-conductance state and main state, respectively.

Figure 1B shows an all-transitions histogram of events as a function of unitary conductance from 5 cell pairs (n = 204). In agreement with previous studies,14,26,27 we found a 2-gaussian distribution, with peaks centered at 59 and 172 pS.

A very different single-channel profile was recorded from cells expressing the truncation mutant. The sets of traces in Figure 2A depict $\approx 7$ seconds of recording from a cell pair stably transfected with Cx40tr248. Junctional currents are the bottom traces in each set. The $V_j$ was $+60$ mV. The all-events histogram (Figure 2B) contains the pooled data from 9 cell pairs with a total of 368 channel events. The data were pooled from cell pairs expressing the truncated channel alone (n = 158) or the truncated channel plus enhanced green fluorescent protein (EGFP) (n = 210; see Materials and Methods). Fitting the data to a single gaussian yielded a peak of 168 pS.

We have previously demonstrated that pH sensitivity of Cx40 is decreased after CT truncation and restored by coexpression of the CT fragment.20 Here, we determined whether the presence of the lower-conductance state could also be restored by separate expression of the homologous CT domain. Figure 3A shows a recording from a cell pair stably transfected with Cx40tr248 and in which the pIRES plasmid containing EGFP and Cx40CT was transiently transfected.

Figure 3B depicts the composed histogram of events (number of events, n = 312, number of cell pairs, N = 4). Fitting the data to a multipeak gaussian yielded 2 peaks centered at 53 and 172 pS. Clearly, although expressed as a completely separate fragment, the CT domain was able to interact with the pore-forming region of the protein to rescue the lower-conductance state.

Our previous studies also showed promiscuity in the particle receptor interaction during chemical gating.20 We therefore ascertained whether heterodomain interactions could occur during single-channel gating and modulate unitary conductance. As shown in Figure 4A, coexpression of the CT domain of Cx43 restored the lower-conductance state of the Cx40 channel. A, Currents recorded in a cell pair transfected with Cx40tr248 (stably) and with Cx43 CT domain (transiently). This pair was naturally poorly coupled, and recording was done in the absence of octanol. An expanded segment of the junctional current trace ($I_j$) and corresponding all-points histogram are also shown. c, 01, and 02 represent, respectively, closed, open1, and open2 states. B, Frequency-distribution histogram of events. Gaussian fits have peaks centered at 57 and 173 pS (n = 401, N = 3).
Heterodomain interactions may be limited by the physical separation of the fragments, as well as by the relative concentrations of the 2 proteins. To assess the ability of the Cx43CT domain to interact with the Cx40 channel under conditions similar to those of the wild-type construct, we tested the single-channel behavior of a Cx40tr-43CT chimera. Figure 5A shows currents recorded from an N2A cell pair stably transfected with chimera. Recording was done in the presence of octanol ($V_j = +60$ mV). Trace magnification of junctional current (at arrows) demonstrates channel openings to 2 different levels. c, o1, and o2 represent, respectively, closed, open1, and open2 states. B, Frequency-distribution histogram of events pooled from 4 cell pairs ($n = 265$). The 2 peaks in the gaussian distribution are centered at 57 and 174 pS.

Figure 5. Single-channel properties of Cx40tr-43CT chimera. A, Currents recorded from a cell pair stably transfected with chimera. Recording was done in the presence of octanol ($V_j = +60$ mV). Trace magnification of junctional current (at arrows) demonstrates channel openings to 2 different levels. c, o1, and o2 represent, respectively, closed, open1, and open2 states. B, Frequency-distribution histogram of events pooled from 4 cell pairs ($n = 265$). The 2 peaks in the gaussian distribution are centered at 57 and 174 pS.

However, frequency of events at the lower-conductance state was less than for the wild-type channels.

Voltage Dependence and Current Kinetics

Previous studies have characterized the effect of CT truncation on the voltage-gating properties of Cx32 and Cx43. There was a change in the gating kinetics with the disappearance or decrease (Elenes et al, unpublished data, 2001) in the magnitude of the fast component, and the concurrent decrease in the minimum conductance ($G_{min}$). Figure 6 illustrates the time course of wild-type (panel A) and truncated (panel B) Cx40 channel currents ($V_j = -100$ mV). Current decline in the cell pair expressing the wild-type channels is best described by a double-exponential function (see superimposed fit). In contrast, current decline in Cx40tr248 channels relaxed monoexponentially (a single-exponential fit is superimposed). The exponential fits in panels A and B are shown in panel C. Thus, truncation of Cx40 channels resulted in a faster relaxation rate and the elimination of one of the exponential components. Figure 6D is a comparison of the voltage dependence of macroscopic junctional conductance in wild-type ($n = 7$) and mutant Cx40 channels ($n = 5$). The continuous lines are the Boltzmann fits to mean values of conductance (see Table for fitting parameters). We observed a lower $G_{min}$ after truncation, consistent with the elimination of the lower-conductance state. No significant changes on other voltage-gating parameters were detected. These data are similar to those obtained after truncation of Cx32 and Cx43 channels.

We have shown that coexpression of Cx43CT rescues the lower-conductance state of the truncated Cx40 (Figure 4). We therefore determined whether the CT of Cx43 could restore the voltage-gating behavior of Cx40. Figure 7A compares voltage dependence of macroscopic junctional conductance in wild-type and chimeric Cx40 channels in N2A cells. $G_j/V_j$ plots for the wild-type ($n = 7$) and chimeric channels ($n = 5$) are shown superimposed in this panel for the N2A cells. The wild-type channel data are the same as shown in Figure 6. The continuous lines on the data are the fits to mean values of conductance. Other parameters returned from the fitting of individual data sets are presented in the Table. A comparison of voltage-gating variables recorded from wild-type and chimeric channels revealed no significant differences, suggesting that the CT of Cx43 is capable of functional interaction with the pore-forming domains of Cx40, modifying gating kinetics in a voltage-dependent manner.

Similar experiments were carried out in oocytes; results of these are illustrated in Figure 7B and in the Table. Unlike in the N2A cells, the voltage-gating variables as well as the kinetics ($V_j = -100$ mV) recorded from wild-type channels in oocytes were not significantly different (except for the slow component in the chimeric channel) from those recorded in the truncated or in the chimeric channels expressed in the oocytes (Table).

Discussion

We have examined the role of the CT domain in determining the unitary conductance and voltage-gating behavior of homomeric Cx40 channels. Our data show that the presence of
the lower-conductance state of Cx40 depends on the existence of its CT domain (Figures 1 and 2); the CT is able to interact with the channel even when coexpressed as a separate fragment (Figure 3). Moreover, replacing Cx40CT with the CT domain of Cx43 rescues the behavior of the homologous channel, both when the fragments (ie, the Cx40 truncated channel and the Cx43 CT domain) are separately coexpressed and when both domains are covalently attached. This heterodomain interaction (a gating particle from one connexin and a pore-forming region from another) is similar to the heterodomain interactions previously demonstrated for chemical gating.20 Finally, we show that truncation of the CT changes the relaxation kinetics of the Cx40 current in the N2A cells in a manner similar to that observed for other connexins24 and demonstrate that the voltage-gating behavior is restored by concatenation of the CT domain of Cx43.

Unitary Conductance of Wild-Type and Truncated Cx40 Channels

The basic biophysical properties of Cx40 channels are well characterized.13,14,26,27 The mean unitary conductance values observed in our experiments are consistent with those previously obtained27 using recording conditions similar to those described here. Furthermore, we show that truncation of the CT domain causes the disappearance of the lower-conductance state without affecting the mean unitary conductance of the main open state. These data suggest that the main open state of the channel is structurally

Overall, our results provide a ball-and-chain type of model in which the CT domain of Cx40 or of Cx43 can act as a voltage-dependent effector of gating, probably by interaction with amino acids in or near the conductive path.

Analysis of Kinetics and the Boltzmann Function Parameters of Activation

<table>
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<tr>
<th>Connexin</th>
<th>G_{in}±V_{i}</th>
<th>V_{0}±V_{j}</th>
<th>A</th>
<th>Time Constant, ms</th>
<th>G_{in}</th>
<th>V_{i}</th>
<th>A</th>
<th>Time Constant, ms</th>
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<tr>
<td>Cx40 WT</td>
<td>0.33±0.01</td>
<td>+53±1.1</td>
<td>7.2±1.2</td>
<td>94±9</td>
<td>0.24±0.02</td>
<td>36.1±1.0</td>
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<td>40±5</td>
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<td>(n=7/7)</td>
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<td>−56±1.3</td>
<td>5.8±0.6</td>
<td>1009±73</td>
<td>0.17±0.02</td>
<td>37.5±0.9</td>
<td>3.4±1.0</td>
<td>119±18</td>
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<td>Cx40tr248</td>
<td>0.20±0.03</td>
<td>+54±4.0</td>
<td>6.0±1.0</td>
<td>100±28</td>
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<td>37.2±1.2</td>
<td>1.7±0.7</td>
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<td>(n=5/5)</td>
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<td>−58±5.0</td>
<td>6.4±1.1</td>
<td>...</td>
<td>0.19±0.01</td>
<td>38.4±0.4</td>
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<td>Cx40-43CT</td>
<td>0.35±0.01</td>
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<td>6.0±0.3</td>
<td>81±11</td>
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<td>34.1±0.7</td>
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<td>42±2</td>
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<td>(chimera)</td>
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<td>345±85</td>
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Values are mean±SE (n=number of cell pairs/oocytes). A indicates slope factor; WT, wild type. Cell pair data were individually fitted and then pooled. For kinetic analysis (±100 mV, 2 seconds), Cx40 wild-type and chimeric channel currents were fitted with a double-exponential function. In N2A cells, the truncated channel current was fitted using a single-exponential function.
independent from the CT region and that transitions to the lower-conductance state result from separate interactions that involve the CT domain.

Ball-and-Chain Model for Gating to the Lower-Conductance State

The data show that separate coexpression of either the homologous (Cx40) or the heterologous (Cx43) CT fragments with the truncated channel restored the lower-conductance state (see Figures 3 and 4). This result implies that the CT fragment behaves like an independent domain. It also indicates that the lower-conductance state involves the interaction between (at least) 2 separate domains of the protein, as follows: the CT domain on one hand and a region that is part of the truncated Cx40 sequence on the other. By analogy with other biological reactions involving 2 separate molecules (eg, hormone receptor), we describe this intramolecular interaction by a generic model in which the CT acts as a ligand that binds to a receptor. Because of the applicability of the ball-and-chain model to the description of chemical gating in Cx40 and Cx43,18,20 we incorporate the same term here and propose the ball and chain as a model for the gating of Cx40 to its lower-conductance state.

It should be noted that the frequency of transitions to the lower-conductance state was lower when the CT fragment was expressed separately from the channel-forming protein than when both elements were covalently attached (compare the data on Figures 3 and 4 with those on Figure 1). It can be speculated that residence in the lower-conductance state is limited by the steric constraints imposed on the CT fragment when it is physically separated from the rest of the molecule. Additionally, it is possible that the concentration of the CT fragment in the coexpression experiments is such that the appropriate stoichiometric ratio for full functionality is not achieved. Finally, we speculate that the ability of the CT fragment to hold the channel in a residual state may be related to its ability to associate to a partner molecule. Indeed, recent data show that Cx43 (and likely other α-connexins that share the ability to bind to PDZ domains) do not seat alone in the membrane but associate with at least one scaffolding protein (ZO-1; see Toyofuku et al17). A recent study by Toyofuku et al29 further shows that this association can be disrupted by c-src binding. The interaction of Cx43 with v-src is known to follow a ball-and-chain form of gating.21 It is therefore tempting to propose that voltage gating may modify the conformation of the connexon to the point where other molecular associations are affected, and this association may be compromised when the CT fragment is not an integral part of the connexin structure.

Kinetics and Voltage-Dependent Gating

Available experimental data show that mutations in various connexin regions influence the voltage-gating mechanism, suggesting that this process results from a complex molecular rearrangement of the protein30,31 (reviewed by Skerret et al32). Our data in the N2A cells and those of others24 indicate that the CT domain forms a part of that complex process. Moreover, the role of the CT on voltage gating seems conserved among at least some connexins. Indeed, as in the case of Cx40 (see Figure 6 and the Table), truncation of the CT has little effect on the \( V_0 \) (voltage value at half channel relaxation of junctional conductance) and slope factor of gating but causes a small reduction in the \( G_{\text{min}} \) of both Cx32 and Cx43.24 Relaxation kinetics are best described by a biexponential function for the wild-type channels but become mostly monoexponential after truncation of the CT domain.24 It is tempting to speculate (see Revilla et al24) that the separate time courses reflect 2 independent voltage-gating mechanisms, 1 of which is directly mediated by the CT domain. It is noteworthy, however, that in that study, the overall time course of current relaxation after CT truncation was slower for Cx43 and faster for Cx32. Our data also show an acceleration of the inactivation rate after CT truncation. Given that the Cx40 wild-type phenotype is recovered after chimerization of the Cx43CT, these results suggest that the
amino acid sequence or length of the CT domain is not the factor responsible for the rate-limiting step of inactivation. Rather, we propose that the Cx40CT or the Cx43CT acts as a rather nonspecific effector of gating.

In the oocyte experiments, we did not find any significant differences between the wild-type, truncated, and chimeric channels with respect to junctional current kinetics and the voltage dependence of channel gating (Figure 7, Table). However, compared with the wild-type channel, there was an increase in the time constant of the slow kinetic component of the chimeric channel. The differences in the macroscopic behavior of the channels between the N2A cells and the oocytes cannot be precisely determined from this study, but we speculate that they may be attributable to the different expression systems (oocytes versus N2A cells). In this regard, it is noteworthy that the voltage-dependent gating parameters of the wild-type Cx40 channels are different in the 2 expression systems (compare, eg, Beblo et al27 with Bruzzone et al23): gating may be affected by association of the channel protein with other molecules, the nature of which may depend on the cell expression system. Furthermore, differences in the lipid environment of expression systems may also affect channel gating. Consequently, the expression system used to study an ion channel may affect the basic as well as the regulatory properties of the channel.

Previous studies have associated the existence of a lower conductance (or “residual”) state in the single-channel behavior of connexins with the prevalence of a residual conductance during macroscopic voltage gating.34 The elimination of the lower conductive state after truncation would lead to the prediction that macroscopic $G_{\text{max}}$ should decrease to 0 during a voltage step. Yet, our experiments show a decrease in $G_{\text{max}}$ min but not its complete disappearance. We lack a clear mechanistic explanation as to why $G_{\text{max}}$ min is preserved even though low-conductance events are not detected. At least 4 possibilities can be considered, as follows: (1) the amplitude of the low-conductance events decreased to undetectable levels; (2) the dwell time became too short (although the transitions could be frequent); (3) channels with prominent low-conductance states were more susceptible to uncouplers (selective block by uncouplers has been shown by He and Burt23); or (4) after truncation, the channels resided permanently in a voltage-independent, open, low-conductance state. Currently, none of these possibilities can be discarded. Nevertheless, our data show that under our recording conditions truncation of the CT domain leads to the disappearance of a particular range of conductances that are otherwise present in wild-type, chimeric, or coexpressed configurations. This is a novel observation, relevant to our understanding of the molecular/structural mechanisms controlling gap junction channel conductance, particularly in cell systems in which Cx40 and Cx43 are coexpressed.

Coexpression of the CT fragment restores the lower-conductance state of a truncated Cx43, but not its voltage-gating behavior (Elenes et al, unpublished data, 2001). On the other hand, our results show that chimerization of the Cx43CT on the Cx40 truncated channel restores the voltage-gating kinetics of Cx40. This suggests that transjunctional voltage can drive the CT domain into an interaction with the pore-forming region but only if the CT is covalently attached to the rest of the molecule. This is consistent with a model in which the CT domain acts as an effector of voltage gating (Elenes et al, unpublished data, 2001). Whether the voltage sensor and/or the transducer of gating is structurally related to the CT domain remains to be determined.

Heterodomain Interactions and Channel Gating

Our data demonstrate that a homologous Cx40 channel can enter the lower-conductance state as a result of its interaction with the CT domain of either Cx43 or Cx40. These results suggest that despite their differences in primary sequence, the CT domains of Cx40 and of Cx43 can reach a similar conformation, thus allowing for interaction with a common binding domain. The way in which this function is integrated in a heteromeric connexin is unknown. It is possible that, in a heteromer, gating results not only from interactions within a connexin but across heterologous connexin domains within the connexon. A similar model has been proposed for pH gating of Cx40-Cx43 heteromers.20 In that case, heterodomain interactions are actually more efficient than homodomain interactions at closing the channels.22 Future studies will determine whether heterodomain interactions have a synergistic effect, thus enhancing the ability of the channel to reside in the lower-conductance state.

Chemical and Voltage Gating: Two Steps of the Same Process?

Previous studies have suggested that, whereas voltage gating causes a reduction of conductance from open to residual, chemical gating drives the channel into the closed state.35 It is thus interesting to note that an intramolecular particle-receptor interaction seems responsible for both chemical gating and residence in the lower-conductance state. We speculate that the lower-conductance state constitutes a required conformation for the chemically induced closure of the channel. A “linear” model in which the channel transits into the lower-conductance state before reaching complete closure during chemical gating can be used as a first approach to understand the relation between these two processes.

In summary, we have shown that the presence of the CT domain is required for the Cx40 channel to reside in the lower-conductance state. Coexpression experiments indicate that the CT of either Cx40 or Cx43 can act as an independent domain that interacts with the Cx40 channel to limit conductance. We propose that the lower-conductance state is consequent to a particle-receptor interaction, of the type described for the ball-and-chain model of gating, and that this process allows for heterodomain interactions in heterologous channels. Whether the lower-conductance state is a necessary prelude to the closed state that is brought about by chemical gating remains to be determined.

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References


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