Coexpression of Connexins 40 and 43 Enhances the pH Sensitivity of Gap Junctions

A Model for Synergistic Interactions Among Connexins

Hong Gu, Jose F. Ek-Vitorin, Steven M. Taffet, Mario Delmar

Abstract—Gap junctions are formed by oligomerization of a protein called connexin. Most cells express more than one connexin isotype. Atrial myocytes, for example, coexpress connexin (Cx) 40 and Cx43. The consequence of connexin coexpression on the regulation of gap junctions is not well understood. In the present study, we show that cells coexpressing Cx40 and Cx43 are more susceptible to acidification-induced uncoupling than those cells expressing only one connexin isotype. Xenopus oocytes were injected with mRNA for Cx40, Cx43, or a combination of both. Intracellular pH and junctional conductance were simultaneously measured while cells were progressively acidified by superfusion with a bicarbonate-buffered solution gassed with increasing concentrations of carbon dioxide. The data show that the pKa (ie, the pH at which junctional conductance decreased to 50% from maximum) shifted from ≈6.7 when cells expressed only Cx40 or only Cx43 to ≈7.0 when one of the oocytes was coexpressing both connexins. Truncation of the carboxyl terminal domains of the connexins caused the loss of pH sensitivity even after coexpression. The data are interpreted on the basis of previous studies from our laboratory that demonstrated heterodomain interactions in the regulation of Cx40 and Cx43 gap junctions. The possible implications of these findings on the regulation of native gap junctions that express both connexins remain to be determined. The full text of this article is available at http://www.circresaha.org.

Key Words: connexin • gap junctions • pH

Gap junctions are intercellular channels that allow for the passage of ions and small molecules between cells in a tissue. Their presence is critical for the coordination of cell function. In the heart, gap junctions play an essential role in cardiac development and in electrical synchrony.1

Gap junctions are formed by oligomerization of a protein called connexin. Connexins are rarely found alone. In most cases, more than one connexin is expressed in the same cell. For example, in atrial myocytes, both connexin (Cx) 40 and Cx43 are expressed.2–5 Recent evidence strongly suggests that these two connexins oligomerize within the same hemichannel, forming a heteromeric connexon. The unitary conductance and voltage dependence of the heteromeric connexons do not correspond to those found for a single isotype.5,6

Connexins are, for the most part, highly regulatable molecules. The ability of connexins to be regulated is probably central to their function.7 The response of a gap junction to a specific agonist varies depending on the connexin that forms the channel.8–10 Since a gap junction may contain more than one connexin isotype, the question arises as to the specific response of a heteromer to chemical regulators. The answer to this question may be relevant to the understanding of gap junction function in tissues for which more than one connexin is expressed.

We have previously demonstrated that the regulation of Cx43 by pHi follows a ball-and-chain model, whereby the channel is not sensitive to intracellular acidification if deprived of its carboxyl terminal (CT) domain, but the function is restored when the CT fragment is replaced as a separate molecule.11 (A similar model applies to the regulation of Cx43 by insulin or insulin-like growth factor12 as well as to the regulation of Cx43 by src.10) Recently, we demonstrated that Cx40 also follows the ball-and-chain model.9 We further demonstrated promiscuity in the interactions between Cx43 and Cx40. Indeed, a free Cx43CT domain can interact with a truncated Cx40 channel (and vice versa) in what we referred to as heterodomain interactions. Our data also showed that a heterodomain interaction between Cx43CT and a truncated Cx40 channel is more effective than the homodomain interaction at closing the channel. Consequently, we proposed that a heteromeric gap junction containing both Cx40 and Cx43 may be more sensitive to pHi than the homomeric channels.9 In the present study, we looked at the pH sensitivity of gap junctions formed in Xenopus oocytes coexpressing Cx40 and Cx43. The results show a heightened sensitivity to intracel-
cular acidification when compared with that of homomeric/homotypic gap junctions. This synergistic interaction of the two connexins likely results from heterodomain associations between the CT domain of one connexin (likely Cx43CT) and a receptor located in the other. Whether these data are relevant to the understanding of gap junction regulation in cells that endogenously coexpress Cx40 and Cx43 at different ratios remains to be determined.

Materials and Methods

Cell Preparation and Recording of Junctional Conductance and pH

Experiments were conducted in Xenopus oocytes, following previously described protocols. Briefly, stage V–VI oocytes were dissected, injected with Cx38 antisense, and stripped of their follicular layer 3 to 6 days before recording. Cells were then injected with the connexin cRNA of interest, the vitelline layer was mechanically removed, and the cells paired for a period of 20 to 48 hours before electrical recording. Cells were also injected with the dextran form of the fluorophore 2-4 seminaphthorhodafluor (SNARF). This dye was used to measure pH.

For recording, cells were placed on the stage of an inverted microscope. Junctional conductance (Gj) was measured using conventional dual two-electrode voltage clamp. Both cells were voltage-clamped to the same holding level. To create a transjunctional voltage difference (Vj), the voltage on one cell (cell 1) was stepped clamped to the same holding level. To create a transjunctional voltage difference (Vj), the voltage on one cell (cell 1) was stepped (20 mV) while the other one (cell 2) was held constant. The current required for the second cell to maintain its holding level was defined as the junctional current (Ij). Gj was defined as the ratio of Ij to Vj. pH was measured from the emitted fluorescence of SNARF, as detected by a pair of photomultipliers connected to a system to measure fluorescence ratios. Intracellular acidification was induced by superfusing the oocytes with a bicarbonate-buffered solution that had an effect on the functional properties of the recorded channels.

Experimental Design

The goal of the present study was to look at the regulation of gap junctions in cells expressing both Cx40 and Cx43. To reduce the probability of recording from homomeric channels, we took advantage of the fact that, in the oocyte system, homomeric Cx40 connexons do not form a heterotypic channel with homomeric Cx43. This property may not be universal, because heterotypic Cx40/Cx43 formation in mammalian cells transfected with these constructs has been shown recently. However, we and others have confirmed the absence of measurable junctional currents in oocytes expressing Cx40 and Cx43 in a heterotypic configuration. The experimental paradigm involved injecting one oocyte with both connexins and the other oocyte with only one connexin. An example is illustrated in Figure 1. Cell 1 was injected with cRNA for Cx40 and Cx43, and cell 2 with cRNA for Cx43. Cell 1 was also injected with cRNA for Cx43. Under this configuration, all connexins in cell 2 were fixed to be Cx43 homomers. On the other hand, three possible connexons could be formed in cell 1: homomeric Cx40, homomeric Cx43, or heteromeric Cx40-Cx43. Homomeric Cx40 connexons do not form with homomeric Cx43 to form a functional channel. Hence, the recorded current could be moving through two types of channels: homomorphic/homotypic Cx43 or heteromeric Cx40-Cx43 paired with Cx43. The properties of pH gating of Cx43 are well known. Thus, any departure from the pH sensitivity curve of Cx43 could be attributed to the coexistence of Cx40 subunits in cell 1.

To increase the likelihood of heteromerization in cell 1, cRNA concentrations were adjusted so that the amount of the second RNA injected was one tenth of that necessary for functional expression in the homomeric-homotypic configuration. Again, we use the case presented in Figure 1 as an example. The amounts of Cx40 cRNA injected in cell 1 and Cx43 cRNA injected in cell 2 were adjusted for each transcript to correspond to a level of expression of 1 to 10 µg in the homotypic-homomeric configuration (between 1 and 5 ng for Cx40 and between 1 and 5 ng for Cx43). Only 0.1 to 0.5 ng (one tenth of the amount used in cell 2) of Cx38 cRNA was injected in cell 1. Our hypothesis was that given the larger amount of Cx40 transcript in the cell, if heteromerization occurred, Cx43 subunits would have a higher probability of combining with Cx40 subunits than of forming homomorphic connexons. Given that the assumptions for binomial distribution of subunits are unproven in our system, we make no predictions as to the likely ratios of heteromerization. Our experiments only intended to test whether coexpression of constructs had an effect on the functional properties of the recorded channels.

Results

Cx40-Cx43 Coexpression Leads to Enhanced pH Sensitivity

Figure 2 shows the pH sensitivity curve for oocyte pairs expressing Cx40 and Cx43 in cell 1 and only Cx43 in cell 2 (see diagram on the left in Figure 2 and open triangles on the right).
Shift in pH Sensitivity Is a Function of the Amount of Cx43 cRNA in Cell 1

One possible interpretation of the data presented in Figure 2 is that Cx40 and Cx43 heteromerize in cell 1. If so, increasing the proportion of Cx43 in that cell would increase the probability of finding Cx43 homomers, thus shifting the pH sensitivity curve closer to that of homologous Cx43. To test for this possibility, we increased the amount of Cx43 cRNA injected in cell 1 by a factor of 5. Results are presented in Figure 3. The cRNA used for both series of experiments was obtained from the same in vitro transcription, to avoid variability resulting from transcription efficiencies. Once again, closed circles depict the data for homologous Cx43, and open triangles show the results from the Cx40-Cx43 combination (as shown in Figure 2). The closed triangles show results obtained from oocytes for which a larger amount of Cx43 cRNA was injected in cell 1. Experiments with the different cRNA ratios were run in parallel. Clearly, an increase in the Cx43 cRNA injected led to a shift in the pH sensitivity curve closer to that of homologous Cx43 channels, supporting the hypothesis that heteromerization was involved in the enhancement of pH sensitivity observed with the combination of these two connexins.

pH Sensitivity Is Enhanced Regardless of the Connexin Expressed in Cell 2

Results similar to those presented in Figure 2 were obtained when the cell coexpressing Cx40 and Cx43 was paired against one expressing only Cx40 (see diagram on the left in Figure 4). As in the case presented above, the concentrations of cRNA were adjusted according to the efficiency of functional expression, and the amount of Cx40 cRNA in cell 1 was one tenth of that injected in cell 2. Results from these experiments are presented in Figure 4 (open triangles); quantitative parameters are summarized in the Table. The figure also depicts the pH sensitivity curve of homomeric/homotypic Cx40 channels (open circles) and homomeric/homotypic Cx43 channels (closed circles) for comparison (data previously published by our laboratory). A significant enhancement of pH sensitivity was observed when Cx43 and Cx40 were coexpressed in cell 1 ($P<0.001; \text{ANOVA}$). Taken together, the data show that coexpression of Cx43 and Cx40 in one cell led to an increased susceptibility to acidification-induced uncoupling, regardless of whether the coexpressing cell was paired against one expressing homomeric Cx43 (Figure 2) or homomeric Cx40 (Figure 4) connexon.
Role of CT Domains

Previous studies from our laboratory showed that the pH sensitivity of Cx43 and Cx40 is highly related to the presence and integrity of the CT domain. We therefore tested whether truncated forms of Cx40 and Cx43 still lacked pH sensitivity even if both connexins were coexpressed. Separate controls showed that the truncated forms of Cx40 and Cx43 were still unable to form heterotypic channels. Results are presented in Figure 5. Open triangles reproduce the data obtained from oocyte pairs expressing Cx40 and Cx43 in cell 1 and Cx43 in cell 2 (Figure 2, open triangles). Closed circles in Figure 5 correspond to data recorded from cells expressing the same connexins but after truncation of their CT domains. Clearly, the absence of the CT domains caused a loss of pH sensitivity, indicating that both connexins, when combined, still rely on their CT domains for pH gating. It also shows that the enhancement of pH sensitivity is related to an interaction between the connexins that involves their CT domains and not to a different mechanism involving other regions of the proteins.

Coexpression of Full-Length Connexins Yields a Similar Result From Coexpression of Connexin Fragments

One possible explanation for the enhanced pH sensitivity after Cx40-Cx43 coexpression is that heteromerization of connexins allowed for heterodomain interactions. If that is the case, we would expect the pH sensitivity of the coexpressed channels to match that observed after either coexpression of the separate heterologous fragments or expression of a chimeric construct where the CT domain of one connexin and a receptor provided by the other isotype.

Coexpression of full-length connexins is similar to that observed after coexpression of heterologous connexin fragments. Top and bottom, ○, Data collected from cell pairs expressing Cx43 and Cx40 in cell 1 and Cx40 in cell 2 (same data as in Figure 4, △). Top, •, Data resulting from coexpression of a truncated Cx40 channel (amino acids 1 to 248) and mRNA for the Cx43CT (amino acids 255 to 382). Bottom, ◼, Data obtained from expression of a chimeric construct formed by amino acids 1 to 244 of Cx40 in tandem with amino acids 255 to 382 of Cx43. Data shown by • (top and bottom) are reproduced from Stergiopoulos et al. pKa values were 6.99±0.02 (mean±SEM) for the coexpressed full-length connexins (top and bottom), 6.84±0.06 for the Cx40 truncated channels coexpressed with the Cx43CT fragment (top), and 6.96±0.03 for the gap junctions formed by the chimeric construct (bottom). These values were not statistically different (ANOVA and Bonferroni test).

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Discussion
Our laboratory has extensively characterized the pH sensitivity of homologous8,9,11 as well as heterotypic gap junctions.8 In the present study, we demonstrate that the coexistence of two connexins in the same cell yields gap junctions with sensitivity to regulation that is unexpected from the properties of the individual subunits. We focused our attention on Cx43 and Cx40. Homologous channels formed by either of these two connexins are pH-sensitive and show a pKa of ≈6.7.9 However, the coexpression of both connexins in one cell yields gap junctions that are much more sensitive to pH (pKa ≈7.0; see Table). This result can best be explained by considering that both connexins heteromerize; we further propose that the subunits interact within the heteromeric connexon in a synergistic manner. This synergism may involve heterodomain interactions between the CT domain of one connexin and the pore-forming region of another.

Heteromerization of Cx40 and Cx43 Subunits
Previous studies have shown, functionally and by biochemistry, that Cx40 and Cx43 heteromerize when expressed in the same cell.5,6 Although no biochemical evidence is available from the oocyte system, it seems logical to propose that the enhanced pH sensitivity observed after coexpression is a result of interactions occurring within heteromeric channels. An alternative possibility would involve one isotype, physically separate from the actual pore-forming connexon, modulating the function of a homomeric connexon of a different isotype. No evidence is available in support of this hypothesis. In fact, no catalytic function has been ascribed to connexins whereby a given molecule would be modified after interaction with a connexin, nor is it known that such a modification would then lead to regulation of a functional channel. Thus, although this possibility cannot be completely discarded, it seems unlikely. A more direct explanation for our results is that Cx40 and Cx43 heteromerize in the oocytes (as they do in other cells5,6), forming channels with a unique regulatory behavior.

Heterodomain Interactions as a Mechanism for Enhanced pH Sensitivity
The question arises as to the intra- and intermolecular interactions that may lead to the regulatory synergism described. We propose that the synergism results from the interaction between the CT domain of one connexin and the pore-forming region of the other. Indeed, our laboratory has previously demonstrated that the pH gating of both Cx43 and Cx40 follows a ball-and-chain model.9 Furthermore, we have shown promiscuity in the interaction between the gating particle of one connexin (ie, its CT domain) and the receptor of another (purportedly a region affiliated with the pore). Our data further show that these heterodomain interactions are actually more efficient than homodomain interactions at closing the channel (see Figure 6). Accordingly, we proposed that a heteromeric channel would be more susceptible to acidification-induced uncoupling given the increased likelihood of heterodomain interactions within the connexon.9 As expected (Figure 6), the pH sensitivity curves obtained from the fragmented, or the chimerized, Cx40-Cx43CT combina-

Synergistic Interactions Among Connexins: Possible Biological Relevance
The biological relevance of connexin multiplicity, as well as coexpression, has often been questioned. This is the first demonstration that connexin coexpression causes drastic changes to the regulation of a gap junction by a factor of potential physiopathological relevance.20 A report, presented only in abstract form, has also suggested that heteromeric Cx40-Cx43 channels may be more susceptible to closure by halothane.21 Yet, it is important to emphasize that our results apply only, so far, to the regulation of an exogenously expressed heteromeric channel that is paired against a homomeric connexon. The extrapolation of our data to the regulation of cell-cell communication in native tissues is still premature. Whether synergistic interactions among connexins could participate, for example, in the closure of Cx40-Cx43 gap junctions in the atria or in the specialized conduction system under ischemic conditions20,22 remains a subject of further study.

In summary, we have shown that a combination of two connexins (Cx40 and Cx43) leads to increased susceptibility to acidification-induced uncoupling. We have also shown that this phenomenon requires the presence of the CT domains of both connexins. Moreover, we have proposed that the synergism results from heterodomain interactions within heteromeric channels, and we have speculated that this kind of synergism may be present in cardiac cells that coexpress Cx40 and Cx43. Whether these interactions participate in the electrophysiological behavior of normal and ischemic cardiac myocytes remains to be determined.

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References


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