A 17mer Peptide Interferes With Acidification-Induced Uncoupling of Connexin43

Guillermo Calero, Martha Kanemitsu, Steven M. Taffet, Alan F. Lau, Mario Delmar

Abstract—Structure/function analysis shows that the carboxyl terminal (CT) domain of connexin43 (Cx43) is essential for the chemical regulation of cell-cell communication. Of particular interest is the region between amino acids 260 and 300. Structural preservation of this region is essential for acidification-induced uncoupling (ie, pH gating). In this study, we report data showing that a 17mer peptide of the same sequence as amino acids 271 to 287 of Cx43 (CSSPTAPLSPMSPPGYK) can prevent pH gating of Cx43-expressing oocytes. Experiments were carried out in pairs of Xenopus oocytes previously injected with connexin38 antisense and expressing wild-type Cx43. Junctional conductance was measured electrophysiologically. pH was determined from the light emission of the proton-sensitive dye dextran-seminaphthodafiuor. Intracellular acidification was induced by superfusion with a bicarbonate-buffered solution gassed with a progressively increasing concentration of CO2. Injection of water alone into both oocytes of a Cx43-expressing pair or injection of a peptide from region 321 to 337 of Cx43 did not modify pH sensitivity. However, injection of a polypeptide corresponding to amino acids 241 to 382 of Cx43 interfered with the ability of gap junctions to close on acidification. Similar results were obtained when a 17mer peptide (region 271 to 287) was injected into both oocytes of the pair. Normal Cx43 pH gating was observed if (1) the amino acid sequence of the 17mer peptide was scrambled or (2) the N and the C ends of the 17mer peptide were not included in the sequence. This is the first demonstration of a molecule that can interfere with the chemical regulation of connexin channels in a cell pair. The data may lead to the development of small molecules that can be used in Cx43-expressing multicellular preparations to study the role of gap junction regulation in normal as well as diseased states. (Circ Res. 1998;82:929-935.)

Key Words: connexin ▪ gap junction ▪ peptide ▪ proline-rich peptide ▪ cardiac arrhythmia ▪ Xenopus oocyte

Connexins are integral membrane proteins that form intercellular channels called gap junctions. Cx43 is the most abundant gap junction protein in the mammalian heart. Its presence is essential for normal cardiac development as well as for the rapid propagation of the electrical wave front. The degree of communication between Cx43-expressing cells is regulated by pH as well as by various kinases. Acidification-induced closure of gap junctions after myocardial ischemia is considered a possible substrate for lethal cardiac ventricular arrhythmias.

Earlier studies from our laboratory showed that truncation of the CT region of Cx43 impaired pH gating. More recently, we have shown that acidification-induced closure of Cx43 results from an interaction between the CT domain and a separate region of connexin. The data have led us to a particle-receptor model, similar to the ball-and-chain model of voltage-dependent gating of nonjunctional channels. The interaction between regions of connexin may occur through intermediary molecules.

We have conducted a thorough survey of the regions of the CT domain that are necessary for pH gating. Of particular interest was the observation that amino acids within region 261 to 300 were essential in this process. This segment is rich in proline residues. A repeat (PXXP), is found between amino acids 274 and 283. Moreover, mutation of prolines 277 and 280 interfered with the normal acidification-induced uncoupling. Proline-rich sequences commonly form lefthanded α-helices and are often involved in protein-protein interactions. The ability of proline-rich regions to interact with other proteins is sequence specific, and the interaction involves not only the proline backbone but the flanking amino acids as well. We hypothesized that the proline-rich region of the Cx43 CT is implicated in a binding reaction that is critical for acidification-induced channel closure. Accordingly, pH gating should be blocked by a peptide of a sequence that is analogous to that of the proline-rich region. The experiments reported in the present study were designed to test the latter hypothesis. The data show that a synthetic 17mer peptide formed by amino acids 271 to 287 of Cx43 (CSSPTAPLSPMSPPGYK) interfered, in a sequence-specific manner, with acidification-induced uncoupling of Cx43. This is the first demonstration of a small molecule that can disrupt the chemical regulation of Cx43 channels. Our studies open the door for the development of other peptidic, or...
Peptide Block of Cx43 pH Gating

Selected Abbreviations and Acronyms

- CT = carboxyl terminal
- Cx43 = connexin43
- Gj = junctional conductance
- Gjmax = maximum Gj
- GST = glutathione S-transferase
- MAPK = mitogen-activated protein kinase
- SH3 = src homology 3
- SNARF-1 = seminaphthorhodafluor-1

Peptidomimetic, molecules that could selectively block the function of native gap junctions. Such a tool would be invaluable for studying the role of gap junction regulation in both health and disease.

Materials and Methods

Oocyte Preparation and Injection

Experiments were conducted on *Xenopus laevis* oocytes at stages V to VI of development. The procedures for oocyte preparation and injection have been detailed elsewhere. Briefly, cells were defolliculated and injected with antisense against connexin38. After 2 to 5 days of incubation, the vitelline layer was mechanically removed, and the cells were injected with mRNA for rat Cx43. Cells were paired 24 hours after mRNA injection and tested 12 to 24 hours after pairing.

Recording of Gj and pH

Gj was measured electrophysiologically using a conventional dual cell-to-cell voltage-clamp circuit. Only cells with resting potentials more negative than −40 mV were used for the study. pH was detected using the pH-sensitive fluorophore dextran-SNARF-1. The dye was injected into the cells simultaneously with the Cx43 mRNA. For recording, cells were placed on the stage of an inverted microscope. Microelectrode impalements were obtained from above the stage while pH measurements were recorded from below using a microscope. Microelectrode impalements were obtained from above the stage while pH measurements were recorded from below using a microscope.

Data Analysis

Between 6 and 11 oocyte pairs were used for each curve. Only oocyte pairs with Gj values below 10 mS/cm² were used for the study. For each pair, Gj values during acidification were normalized to the maximum value of Gj recorded. A curve was obtained from the best fit of all experimental data collected within a single experiment by using a Hill equation. The value of pH at which Gj decreased to 50% of its maximum was referred to as the pKa. With our experimental procedure, a value of pKa was collected from each individual experiment. An average pKa was estimated from all individual pKa values collected within an experimental group. In a few experiments, Gj decreased only to ~60% from maximum. In those cases, the data were fit with a Hill equation, and the curve was extrapolated to Gj/Gjmax of 0.5. To determine whether pH sensitivity had been altered after a particular treatment, statistical comparisons of the pKa and Hill coefficient values were conducted using ANOVA and Bonferroni tests.

Results

**pH Sensitivity of Cx43 Channels Expressed in Xenopus Oocytes: Effect of Water Injection**

The solid circles in Figure 1, and in all subsequent figures, show the average data relating pH (abscissa) and Gj (ordi-
The study of Ek-Vitorin et al.17 showed that 10 to 20 amino acid deletions from the region 300 to 374 did not significantly modify pH sensitivity. As a further control for our experiments, we tested whether a synthetic peptide of a sequence corresponding to region 321 to 337 of Cx43 (data labeled p321-337) could alter pH gating. Results are shown in Figure 1B. Open circles show data obtained from oocytes that were injected with 20 to 30 μmol/L of p321-337 (total injected volume was 25 nL; n=8). The injection was carried out 20 minutes before the onset of recording. Clearly, injection of p321-337 did not modify the pH sensitivity of Cx43 (pKa 6.67±0.08). These results demonstrate that the injection procedure and the addition of a nonspecific peptide molecule are not, by themselves, capable of altering pH gating.

Effect of Excess Cx43CT on the pH Gating of Cx43 Channels
As a first approach in studying the effect that an excess of relevant CT sequences can have on the pH gating of wild-type Cx43, both oocytes of a connexin-expressing pair were injected with a polypeptide corresponding to amino acids 241 to 382 of Cx43. This polypeptide (Cx43CT) was cleaved from the GST-Cx43CT fusion protein expressed in bacteria (see “Materials and Methods”). Figure 2 shows the results. Solid circles depict data from Cx43-expressing oocytes that were not injected; open circles show data from oocytes injected, 20 to 30 minutes before recording, with the CT fragment (30 μmol/L). Clearly, the presence of the CT domain interfered with the pH gating reaction. pKa was 6.43±0.05 in the presence of the CT fragment (n=6). This value was significantly different from that recorded from noninjected oocytes (P<0.001). The values of the Hill coefficient were not statistically different.

The 17mer Peptide Impairs pH Gating of Cx43
Previous studies have shown that amino acids within region 261 to 300 of Cx43 are involved in several regulatory functions, including pH gating.17 Therefore, we tested whether a 17mer peptide from this region (amino acids 271 to 287 of Cx43) could mimic the results obtained from injection of a polypeptide from the entire Cx43CT domain. As shown in Figure 3, injection of the 17mer peptide greatly impaired the ability of Cx43 channels to close on acidification. Panel A shows the simultaneous measurements of Gj (top) and pH1 (bottom) recorded from one experiment in which 30 μmol/L of the 17mer peptide had been injected 20 minutes before the onset of recording. Intracellular acidification progressed in a

![Figure 1. pH sensitivity curves for Cx43-expressing oocytes that were either not injected (●, A and B), injected with pure water (○, A), or injected with a peptide from region 321 to 337 of Cx43 (●, B). Ordinates represent Gj relative to the maximum value measured during the experiment (Gj max). Abscissas indicate the value of pH recorded simultaneously with Gj. Data points represent mean values; vertical bars are the standard error of the mean. Data for noninjected oocytes are reproduced from Ek-Vitorin et al.17 Clearly, acidification led to a decrease in intercellular communication. The pKa of the average curve (ie, the pH at which Gj decreased to 50% from maximum) was 6.73±0.07 (n=11). This curve was used as a standard for comparing the effect of peptide injections on the pH sensitivity of Cx43. The average Gj recorded in control experiments and the values of pKa and Hill coefficients from all experimental series reported in the present study are presented in Table 2.

The open circles in Figure 1A show data obtained from Cx43-expressing oocytes that were injected, 20 minutes before the onset of recording, with 50 nL of water. The results show that the injection procedure per se did not affect the susceptibility of Cx43 to acidification-induced uncoupling. The pKa of the average curve recorded from 8 oocyte pairs that were injected with water alone was 6.73±0.06. This value is within the variability that we have detected from noninjected oocytes.16,17

![Figure 2. Effect of the CT domain on pH gating reaction. ○ indicates pH sensitivity of Cx43 after injection of a polypeptide corresponding to amino acids 241 to 382 of Cx43 (labeled Cx43CT); ●, data from noninjected Cx43-expressing Xenopus oocytes. Injection of Cx43CT causes a significant change in pKa (P<0.005) and a not significant change in the Hill coefficient of the average curve. Absolute values are shown in Table 2.

Effect of a 17mer Peptide on pH Gating

**TABLE 2. Effect of Peptide Injection on pH Sensitivity of Cx43**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Gj Control, μS</th>
<th>pKa</th>
<th>Hill Coefficient</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninjected</td>
<td>7.06±2.6</td>
<td>6.73±0.07</td>
<td>4.9±0.3</td>
<td>11</td>
</tr>
<tr>
<td>H2O-injected</td>
<td>5.0±2.0</td>
<td>6.73±0.06</td>
<td>5.0±1.2</td>
<td>8</td>
</tr>
<tr>
<td>p321-337</td>
<td>5.9±2.2</td>
<td>6.67±0.08</td>
<td>4.4±0.6</td>
<td>8</td>
</tr>
<tr>
<td>Cx43 CT</td>
<td>6.0±1.9</td>
<td>6.43±0.05</td>
<td>2.8±0.5</td>
<td>6</td>
</tr>
<tr>
<td>17mer</td>
<td>7.8±1.9</td>
<td>6.39±0.08</td>
<td>2.7±0.4</td>
<td>7</td>
</tr>
<tr>
<td>Random</td>
<td>9.7±3.1</td>
<td>6.67±0.02</td>
<td>6.7±1.3</td>
<td>7</td>
</tr>
<tr>
<td>11mer</td>
<td>6.6±1.6</td>
<td>6.73±0.05</td>
<td>6.1±1.1</td>
<td>8</td>
</tr>
<tr>
<td>14mer</td>
<td>3.8±0.9</td>
<td>6.69±0.07</td>
<td>6.5±1.1</td>
<td>8</td>
</tr>
</tbody>
</table>

n indicates number of oocyte pairs.
ramplike fashion, going from a control value of 7.1 to 6.4 in 40 minutes. Gj showed an initial increase at the onset of acidification. This increase has been previously reported, and its possible nature has been extensively discussed in previous publications from our laboratory.13,14,26 Yet, contrary to what is observed in noninjected Cx43 channels (eg, see Reference 14), only a minimal decrease in Gj was observed consequent to acidification, even when the pHi dropped to 6.4. Panel B of Figure 3 shows the average data from noninjected oocytes (solid circles) and from Cx43-expressing oocytes that were injected with the 17mer peptide (open circles). The average pKa of Cx43 after injection of the peptide was 6.39 ± 0.08 (n = 7). This value was statistically different from the one recorded from noninjected oocytes (P < 0.001). Although the average curve obtained after the 17mer injection is clearly less steep than the one recorded in the control condition, the Hill coefficients were not statistically different, given the large scattering of the data within groups (see Table 2; see also “Discussion”). Interestingly, the peptide prevented major changes in Gj within the range of pHi values that can be observed in ischemic tissue.10,12 It should be noted that the data include one experiment in which there was normal uncoupling after peptide injection. The reason for this result is unclear. The possibility of error in the experimental procedure in that case, though unlikely, cannot be discarded (see “Discussion”).

The Ability of the 17mer Peptide to Prevent pH Gating Is Sequence Specific

Results from other laboratories have shown that the ability of proline-rich peptides to bind to their target molecules is highly sensitive to the sequence of the peptide.27 We determined whether the ability of the proline-rich peptide to block pH gating was sequence specific. The sequence of peptide 271 to 287 of Cx43 was randomized, although one restriction was maintained: no obvious consensus sites for phosphorylation were created (sequence shown in Table 1). Average results obtained from 7 cell pairs after injection of this randomized peptide are shown in Figure 4. The pKa of Cx43 in the presence of the random peptide was 6.67 ± 0.02. This value was not different from that obtained in the absence of the peptide or after water injection (Figure 1A). The results show that the amino acids contained within region 271 to 287 of Cx43 need to be in the appropriate sequence to prevent pH gating of wild-type Cx43 channels.

Effect of Shorter Peptides on pH Gating

To further evaluate the specificity of the 17mer peptide to alter Cx43 regulation, two shorter peptides were tested. In one case, the three amino acids at the N-terminal end of the 17mer peptide were deleted (14mer of sequence PATPLSPMSPPGYK). In another case, the 17mer peptide was shortened from both the N-terminal and the CT ends (11mer of sequence PTAPLSPMSPP). As shown in Figure 5, neither peptide modified the pH sensitivity of Cx43. This result is consistent with previous studies, using short peptides of analogous primary structures, showing that the sequences flanking proline-rich regions are essential for the ability of the peptide to interact with its target molecule.20,21,27

Discussion

Our results show that a polypeptide of the CT domain of Cx43 interfered with the pH gating process. Similar results

Figure 3. A, Time course of changes in Gj (top) and pHi (bottom) recorded from an oocyte pair injected with 30 μmol/L of the 17mer peptide 20 minutes before the onset of recording. In the presence of the peptide, acidification induced only a minor decrease in Gj, B, pH sensitivity of Xenopus oocytes expressing wild-type Cx43 and either not injected (●, same data as in Figure 1) or injected before recording with a peptide corresponding to amino acids 271 to 287 of Cx43 (○; see Table 1 for sequence). Average values of Gj control, pKa, and Hill coefficients are presented in Table 2. The presence of the 17mer peptide induced a significant shift in pKa (P < 0.001) and a not significant reduction in the Hill coefficient.

Figure 4. Average results obtained from 7 cell pairs after injection of randomized peptide (rndm). ● indicates pH sensitivity of Cx43-expressing Xenopus oocytes (data are the same as in Figure 1); ○, pH sensitivity of Cx43 after injection of a peptide for which the sequence 271 to 287 of Cx43 was randomized but putative consensus phosphorylation sites were avoided (sequence shown in Table 1). Average data for both groups are presented in Table 2.
The sequence of the 17mer peptide is the same as that of a proline-rich region of Cx43 that is important for pH regulation. We propose that this peptide competes against the equivalent region of Cx43 for binding to a target molecule that is required for acidification-induced uncoupling. We further propose that the peptide can bind the target molecule but fails to induce channel closure.

The average curves shown in Figure 3B indicate that both the Hill coefficient and the pKa were altered after injection of the 17mer peptide. In a purely competitive reaction, no change in the Hill coefficient would be expected. Our results may therefore suggest that the effect of the peptide involves a mechanism of action beyond that of pure competitive inhibition. However, the values of the Hill coefficient have to be interpreted with caution. Indeed, rigorous statistical analysis shows no significance (confidence level at <0.05) in the observed shift in Hill coefficient. The latter results from the scattering in Hill coefficient values within each group. It is our experience that data scattering can drastically alter the slope of the averaged curve. This is more pronounced when, as in this case, some experiments show no acidification-induced change in Gj, whereas another one shows a normal pH versus Gj curve. Our results show that the peptide prevents pH gating, but we cannot draw conclusions as to the type of inhibition (competitive, noncompetitive) that the peptide exerts on the particle-receptor reaction.

The proline-rich region is known to be involved in the interaction of Cx43CT with other molecules. Warn-Cramer et al have shown that the same 17mer peptide here shown to block pH gating also acts as a substrate for MAPK. It is not known whether the ability of the peptide to block pH gating is related to its ability to act as a MAPK substrate. Although the 11mer and 14mer peptides still contained the putative MAPK consensus sites, shortening of the peptide may have altered its conformation, thus disrupting its ability to interact with the kinase. Further studies would be necessary to determine whether the shorter peptides are still suitable MAPK substrates.

Additional experiments show that the same 17mer peptide can inhibit the in vitro interaction between the CT domain of Cx43 and the SH3 domain of v-src. Whether pH gating involves the interaction of the CT domain with an SH3 domain–containing protein is yet to be determined. The data obtained with the shorter peptides show that the ability of the peptide to block pH gating requires preservation of the amino acids that flank the proline-rich region. The latter is consistent with other results showing that small modifications in the sequence of proline-rich peptides can significantly alter their binding ability, despite preservation of the proline backbone.

In summary, the same primary structure (region 271 to 287 of Cx43) is involved in three seemingly independent regulatory mechanisms: MAPK-mediated phosphorylation, SH3 binding, and pH gating. We are not aware of biochemical evidence suggesting that MAPK is activated directly by acidification of the intracellular space, yet the regulation of this enzyme is highly complex, and acidification of the intracellular space causes significant changes in cell homeostasis. The possibility of indirect MAPK activation as a result of one of the many biochemical changes occurring in an oocyte after a drop in pH (eg, increase in intracellular calcium; see Reference 28) cannot be discarded. On the other hand, the pH (or calcium) dependence of the interactions between an SH3 domain and its ligands has seldom been studied. Future experiments will be directed at characterizing whether these different processes intersect into a common molecular path.

**Possible Mechanism of Action of the 17mer Peptide**

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**pH Gating of Cx43: Is There an Intermediary Step?**

Our laboratory has shown that truncation of the CT domain of Cx43 at amino acid 257 (mutant M257) interferes with pH gating. pH gating of M257 can be partly rescued by coexpression with mRNA coding exclusively for the CT domain. On the basis of these and other results, we have proposed that acidification-induced uncoupling of Cx43 channels follows a ball-and-chain–like model in which the CT domain acts as a gating particle (a “ball”) that, on acidification, binds to its receptor and closes the channel. A question remains as to whether the CT domain binds directly to another region of connexin (a purely intramolecular interaction like the voltage gating of Shaker channels) or whether intermediary molecules are involved. Evidence in favor of the latter has been recently reported in a book chapter. The results presented in Figure 2 further support the possible participation of an intermediary molecule in pH gating. We reason that if the interaction of the CT with the channel pore were to involve only two molecules (the CT acting as the particle and the pore region acting as a receptor), then adding an excess of the CT domain should enhance the closing of the wild-type channel. The data presented on Figure 2 indicate that this was not the case. Instead, partial inhibition of pH gating was observed. As shown in Figure
A simple bimolecular reaction between the CT domain and interacts (directly or indirectly) with another region of the membrane domains) cannot be discarded, yet we suggest that this in CT conformation that pushes/pulls pore-lining transmembrane gating particle.

The solid circle represents the CT domain; asterisk, an intermediary molecule required for acidification-induced uncoupling. Acidification leads to interaction of the CT domain with the intermediary, thus causing channel closure. B, According to this model, an excess of free CT in the cytoplasm could compete for the intermediary and inhibit gating. Two assumptions are as follows: (1) the intermediary is present in limited supply, and (2) the free CT, even when bound to the intermediary, cannot interact with the receptor in the presence of the wild-type CT.

6A, we suggest that pH gating requires of an intermediary (asterisk) that interacts with the CT domain (solid circle) to close the channel. An excess of free CT would compete for the intermediary, thus preventing channel closure (Figure 6B). This model assumes that (1) the intermediary is present only in limited supply and (2) even when bound to the intermediary, free CT cannot close the channel in the presence of wild-type CT. Further studies will be necessary to assess the validity of the proposed model.

The diagram in Figure 6 illustrates the gating particle plugging an open channel (analogous to the ShakerB ball-and-chain model15,16). This is only a simplified assumption. At this point, other mechanisms of channel closure (eg, a change in CT conformation that pushes/pulls pore-lining transmembrane domains) cannot be discarded, yet we suggest that this or any other alternative model should be consistent with the proposition that (1) the CT is an independent domain that interacts (directly or indirectly) with another region of the channel to achieve channel closure and (2) pH gating is not a simple bimolecular reaction between the CT domain and the pore.

**Limitations of the Study**

Although extreme care was applied to control the amount of peptide delivered into the oocyte, the actual final concentration inside the cells may vary. First, the estimate of intracellular volume was based on the assumption of a spherical oocyte of 1-mm diameter. However, minor variations in diameter or internal volume between cells are expected. Second, the estimation of injected volume is based on measurements of the diameter of a hanging drop in the absence of a cell, yet the actual volume delivered into the cell may change, given the resistance provided by the intracellular space as well as the possibility of minor backflow. Third, to ensure full recovery of membrane integrity after the injection, we waited for 20 to 30 minutes between the time of injection and that of the onset of recording; the possibility of some peptide digestion during this time cannot be discarded. Given these considerations, attempts at performing full dose-response curves were deemed subject to significant error. Thus, we chose to test a number of different peptides using an estimated concentration that we found effective for the 17mer peptide. Under these conditions, several peptides were found to be ineffective at modulating pH gating. However, the possibility that some effect may be obtained at larger concentrations cannot be discarded. Finally, we did not detect significant differences in the Gj values measured at normal pH between groups, yet minor effects could be masked by the normal variability of Gj observed between experiments within any given group. Given the demands of the experimental protocol, it was not possible to measure the Gj before and after peptide injection at normal pH for each individual experiment. Thus, the possibility that any of these peptides could have minor effects on Gj at normal pH values cannot be discarded.

**Small Peptides as Tools to Study Gap Junction Function and Regulation**

Gap junctions have, so far, evaded the control of pharmacologists. Molecules that target these channels with a reasonable degree of specificity are not available. Although a number of agents close gap junctions,30 several of them are known to be quite nonspecific (eg, see Reference 31). Recent reports have indicated that a small 10-amino-acid peptide can enhance cardiac gap junction conductance.32 Whether such a peptide acts by interfering with the regulation of Cx43 remains to be determined. Moreover, the above-mentioned study32 seems short in rigorous statistical analysis, in the sense that no direct comparison is presented to show that the average Gj values were different before and after peptide exposure. The data in that study may also be affected by the large Gj normally present between cardiac myocytes.33 To our knowledge, this is the first report of a small molecule that can interfere with the chemical gating of gap junctions. However, a 17mer such as the one used in the present study may not be directly permeated through the cell membrane. Future efforts should be directed to the production of a peptide that can be incorporated into cells without the need for injection.

Whether chemical gating of gap junctions has a beneficial or a deleterious effect on the susceptibility to ischemia-induced cardiac arrhythmias has puzzled cardiac electrophysiologists for years. Although the present study represents an early step, it does open a door for the development of a new generation of agents: agents that could modulate the ability of gap junctions to close in response to physiological stimuli. The potential relevance of these agents as tools for studying the pathophysiology of ischemia-induced arrhythmias can be readily appreciated.

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