Identification of a Novel Peptide That Interferes With the Chemical Regulation of Connexin43

Junko Shibayama, Rebecca Lewandowski, Fabien Kieken, Wanda Coombs, Sejal Shah, Paul L. Sorgen, Steven M. Taffet,* Mario Delmar*

Abstract—The carboxyl-terminal domain of connexin43 (Cx43CT) is involved in various intra- and intermolecular interactions that regulate gap junctions. Here, we used phage display to identify novel peptidic sequences that bind Cx43CT and modify Cx43 regulation. We found that Cx43CT binds preferentially to peptides containing a sequence RXP, where X represents any amino acid and R and P correspond to the amino acids arginine and proline, respectively. A biased “RXP library” led to the identification of a peptide (dubbed “RXP-E”) that bound Cx43CT with high affinity. Nuclear magnetic resonance data showed RXP-E–induced shifts in the resonance peaks of residues 343 to 346 and 376 to 379 of Cx43CT. Patch-clamp studies revealed that RXP-E partially prevented octanol-induced and acidification-induced uncoupling in Cx43-expressing cells. Moreover, RXP-E increased mean open time of Cx43 channels. The full effect of RXP-E was dependent on the integrity of the CT domain. These data suggest that RXP-based peptides could serve as tools to help determine the role of Cx43 as a regulator of function in conditions such as ischemia-induced arrhythmias. (Circ Res. 2006;98:1365-1372.)

Key Words: Cx43CT ■ connexin ■ particle-receptor interaction ■ gap junctions

Connexins are integral membrane proteins that oligomerize to form intercellular channels called gap junctions. The most abundant gap junction protein in a number of mammalian systems is connexin43 (Cx43). Our previous work has suggested that regulation of Cx43 channels results from the association of the carboxyl-terminal (CT) domain, acting as a gating particle, and a separate region of the connexin molecule, acting as a receptor for the gating particle.1,2 Additional studies have shown that this intramolecular interaction can be modulated by other intermolecular interactions in the microenvironment of the gap junction plaque.3 Thus, the emerging picture of a gap junction plaque is that of a macromolecular complex in which proteins act in concert to modulate intercellular communication. At the center of these interactions is the CT domain, which acts as a substrate for a number of kinases,4 a ligand for noncatalytic proteins, and a gating particle to modify coupling between cells.5

As a key player in the regulation of gap junctions, CT presents itself as a target of chemical or genetic manipulation intended to modify function.6 Here, we sought to disrupt the regulation of Cx43 by chemical means. Our rationale was based on the knowledge that Cx43CT is capable of interacting with other proteins. We reasoned that this “stickiness” of Cx43CT can be used to “adhere” peptidic sequences to it. We further speculated that the interaction of Cx43CT with small peptides can modify the behavior of the gap junction channel. This rationale was supported by previous work showing that peptides can modify both the chemical and voltage-gating behavior of Cx43.6,7 In the present study, we used a high-throughput phage display screening to find peptidic sequences that bind Cx43CT. Further analysis using a combination of surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), and dual patch clamp led to the identification of a specific peptide that binds to Cx43CT with high affinity, affects residues 343 to 346 and 376 to 379 of Cx43, and partially prevents octanol-induced and acidification-induced uncoupling. These results support the feasibility of a peptide-based strategy to manipulate Cx43 regulation in native tissues. These peptides can be used as tools to characterize the specific role of gap junction regulation in health and disease.

Materials and Methods

Phage Display
Recombinant Cx43CT (amino acids 255 to 382 of rCx43) was produced as previously described1 and used as “bait” for peptide screening. For peptide presentation, we used a “library” of bacteriophage, displaying ~55 copies of 2.7×10^12 random 12-mer peptides (Ph.D.-12 Phage display peptide library kit; New England BioLabs Inc). Details of the protein production and screening procedures are

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From the Departments of Pharmacology (J.S., R.L., W.C., S.S., M.D.) and Microbiology/Immunology (S.M.T.), State University of New York, Upstate Medical University, Syracuse; and Department of Biochemistry and Molecular Biology (F.K., P.L.S.), University of Nebraska Medical Center, Omaha.
*Both authors contributed equally to this study.
Correspondence to Dr Mario Delmar, SUNY Upstate Medical University, Department of Pharmacology, 766 Irving Ave, Syracuse, NY 13210. E-mail delmarm@upstate.edu
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Biased Phage Display
We used the Ph.D. Peptide Display Cloning System (New England BioLabs Inc) to create a biased phage display library of sequence XXXXRXPXXXX, where X is any amino acid flanking an arginine and a proline with an additional random residue at the center. The randomized peptides were followed by a GGGS spacer. These phages were analyzed for titer and sequence before screening for Cx43CT.

Surface Plasmon Resonance
SPR is a spectroscopic method to determine binding amplitude and kinetics in real time.9,10 Recombinant Cx43CT was covalently bound to a carboxymethyl dextran matrix.10 Dissociation constants (Kd) were calculated from the time course of binding and unbinding of the ligand, using a 1:1 (Langmuir) association and dissociation kinetic model (Biacore software package). In both phases (association and dissociation), the first 5 to 8 seconds of recording were not included in the fit, as to avoid artifacts resulting from peptide distribution within the flow cells.10

Nuclear Magnetic Resonance
All NMR data were acquired on a Varian INOVA 600-MHz NMR spectrometer using a cryoprobe;11 the sample temperature was maintained at 7°C. Gradient-enhanced 2D 1H-15N HSQC experiments12 were used to observe all backbone amide resonances in 15N-labeled Cx43CT. Methodological details are presented in the online data supplement.

Electrophysiological Analysis
Experiments were conducted on N2a (neuroblastoma) cells. Cx43 was expressed in a lac-switch stable system (induced by 0.1 to 1.0 mmol/L IPTG; see Zhong et al13) or transiently using an IRES plasmid. Synthetic peptides were diluted in the pipette solution to a final concentration of 0.1 mmol/L as in previous work.7 For some experiments, octanol (2.0 mmol/L) was superfused during recording. Methods for single-channel recordings and criteria for inclusion and analysis were as reported before,7,14 and as outlined in online data supplement.

Results
Nonbiased Phage Display
Initial control experiments were conducted to standardize the phage display assay. The library was purified to streptavidin, a protein known to bind preferentially to peptides containing an HPQ consensus motif.15,16 After 3 rounds of enrichment, 14 phage plaques were selected for sequencing. All 14 showed preservation of the HPQ motif, indicating that the experimental conditions were adequate for appropriate peptide recognition by the target.

The library was presented to Cx43CT. After 3 rounds of selection and amplification, DNA of 156 phage plaques was purified and sequenced. Of the estimated 2.7×109 different sequences in the library, 48 unique sequences were recovered. One particular sequence (PRPTMGNLPDVL), recovered from 45 different plaques, showed homology with a 10 amino acid segment (RATLLNVPDL) of the second PDZ domain of zonula oculudens-1 (ZO-1). When aligned, 5 amino acids were identical and 4 were conserved; binding between the second PDZ domain of ZO-1 and Cx43CT in vivo and in vitro has been well documented.17,18 The results supported the notion that the phage display method can be helpful at recognizing Cx43CT binding sequences.

The “RXP” Motif and Biased Phage Display
Further analysis of the 12-mer peptides revealed that 16 of 48 unique sequences shared a motif RXP (where X represents any amino acid). The RXP motif was not detected in phage that bound to streptavidin. The motif was shown in 11 peptides in the N- to C-terminal orientation and 5 of them in the reverse direction. Specific sequences are presented in Table 1. The RXP sequence occurred in different positions within the 12-mer peptide, thus preventing proper alignments to determine the frequency of other amino acids at specific positions relative to RXP. To overcome this limitation and search for peptides with higher binding affinity (see section on SPR, below), we generated a biased phage display library where the RXP motif was forced to the center of the sequence, flanked at each side by 4 randomized amino acids. Sixty clones containing an insert (of an estimated 3 to 4×105 total clones in the library) were chosen at random for sequencing before exposure of the library to Cx43CT. All inserts coded for an 11-mer peptide with the RXP motif in the appropriate location. In 3 independent runs, the same library was presented to Cx43CT. The binding step was performed at pH 6.5 in 2 of the runs (runs 1 and 2) and at pH 7.4 in 1 additional run (run 3). The total number of phage plaques containing a full-coding insert that were recovered after the binding step were 120, 119, and 163 for runs 1, 2, and 3, respectively. Surprisingly, 89% of all sequences corresponded to “doublets,” that is, peptides where 2 RXP 11-mers had been inserted in tandem (see supplemental Table I and Discussion). Although we did not detect a clear preference for specific amino acids in a given position relative to the RXP core, we did observe a preponderance of basic residues in sequences recovered from all screenings (see supplemental Figure I and related text). Furthermore, 5 specific peptides were recovered from all 3 runs and represented a large

### Table 1. Alignments of 16 RXP-Containing Sequences Derived From the Phage Display Screen.

| RXP-1     | N-GHLHLRVRPTLKM-C          |
| RXP-2     | N-EFRPSHSDVWL-C            |
| RXP-3     | N-SOSNPPMPRPR-C            |
| RXP-4     | N-RBPYPPVPKL-F             |
| RXP-5     | N-SLYERPASTYP-C            |
| RXP-6     | N-HTVSPRPPLPSS-G-C         |
| N-RTHINGNLLKPP-P-C                  |
| C-RREPTYTONLNR-N                      |
| C-TLAVPRPPLSY-N                  |
| N-RKPTQSLPRTL-V-C               |
| N-TRAPKMRSDPL-C                  |
| C-PRVPTKTHWLT-N                  |
| N-SROFLHSDLRP-C                |
| C-ANRPHDHLLHL-H-N                |
| N-QTQYQAPLAVA-C             |
| C-DQWLLHHRHPW-N               |
fraction of the repeats (Table 2). The absence of doublets in the initial library before screening and their high frequency after the binding step strongly suggest that these peptides, although rare, were highly selected for Cx43CT binding.

In Vitro Binding Detected by SPR
Phage display allows for the rapid screening of thousands of peptides, but the characteristics of binding cannot be properly defined. Furthermore, the peptides are part of a capsid protein; the latter may affect the ability of the peptide to properly interact with the target protein. We, therefore, selected some of the peptides identified by phage screening to characterize their ability to bind Cx43CT using SPR. Recombinant Cx43CT was covalently bound to a carboxymethyl dextran matrix and synthetic peptides presented for binding. A total of six 12-mer peptides from the nonbiased screening (labeled RXP-A to RXP-E; Table 1) and 5 peptides (peptides labeled RXP-1 to RXP-6; Table 1) were tested. RXP-2, RXP-3, RXP-5, and RXP-6 showed negligible signal was obtained from exposure of Cx43CT to the largest deflection in SPR signal, and a small, near-negligible signal was obtained from exposure of Cx43CT to RXP-1. Examples are shown in Figure 1A. RXP-2, RXP-3, RXP-5, and RXP-6 showed no significant binding (<100 resonance units of maximal response at peptide concentration of 1 mM/L). Among the peptides obtained from the nonbiased search, RXP-4 caused the largest deflection in SPR signal, and a small, near-negligible signal was obtained from exposure of Cx43CT to RXP-1. Experiments are shown in Figure 1A. RXP-4 (blue line) of RXP-1 (red line) was added at time 0 (peptide concentration was 250 mM/L). Washout was initiated after 2 minutes of exposure. The peptide dissociated rapidly and completely, suggesting poor binding affinity to Cx43CT. We tested higher concentrations of the peptide but could not detect an asymptotic maximum response. In addition, dissociation rates were too fast to reliably use them to calculate the kinetics of binding. As such, these results do show Cx43CT–RXP-4 binding, but the interaction between the 2 molecules was too weak to allow for proper calculation of kinetic values. A different result emerged from the testing of the doublets. RXP-A and RXP-E showed significant binding to Cx43CT. In particular, peptide RXP-E generated a large, concentration-dependent deflection followed by a slow dissociation on washout (Figure 1B). The transitions were well fit by exponential functions and the rates of association and dissociation were used to estimate kinetic parameters (see Materials and Methods). A full range of concentrations was tested in 3 separate occasions and at 2 different pH values of the solvent (6.5 and 7.4). No differences were observed as a function of pH. From these studies, we calculated a dissociation constant \( (K_d) \) of 3.9 mM/L for the Cx43CT–RXP-E interaction. This value is similar to that measured for the association of Cx43CT to binding partners such as the SH3 domain of c-src,\(^3\),\(^17\) the second PDZ domain of ZO-1,\(^3\),\(^17\) or the binding of Aquaporin-0 to Cx45.\(^6\),\(^19\) Overall, these results show that different RXP peptides are able to interact with Cx43CT in vitro with various degrees of affinity. The possibility of structural modifications caused on Cx43CT as a result of RXP interactions (and hence an initial approach to the possible location of the binding site) was assessed next.

Nuclear Magnetic Resonance
Peptides RXP-1, RXP-4, and RXP-E were tested for their ability to modify the structure of Cx43CT. The peptides were diluted in PBS (pH 5.8) containing \(^{15}\)N-Cx43CT, and \(^{15}\)N-HSQC spectra were acquired. An example of the resonance shifts caused by the presence of RXP-E is shown in Figure 2. Contours obtained in the presence of RXP-E are labeled red. Contours obtained in the presence of RXP-E are labeled red. In previous studies, we have assigned the specific resonance peaks that correspond to each amino acid in the Cx43CT sequence.\(^20\) Accordingly, shifts in the resonance assignments directly reveal the identity of the amino acids whose position in space is modified by the presence of the peptide. Addition of RXP-E peptide strongly affected the resonance peaks of residues R376, D378, and D379 of Cx43CT (bottom panels). In addition, there was a minor shift in amino acids 343 to 346 (middle). These residues are part of the \( \alpha \)-helical domains of Cx43CT and may be involved in intramolecular interactions relevant for Cx43 regulation\(^17\) (see also Discussion). Similar results were obtained from the exposure of Cx43CT to RXP-4.

TABLE 2. Most Commonly Identified Peptides

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence</th>
<th>Frequency Run 1 (%)</th>
<th>Frequency Run 2 (%)</th>
<th>Frequency Run 3 (%)</th>
<th>Total Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXP-A</td>
<td>DVPGRDPGYKGGGS(1)LNRRKPSLYD</td>
<td>20.8</td>
<td>64.7</td>
<td>36.2</td>
<td>40</td>
</tr>
<tr>
<td>RXP-B</td>
<td>EIQPRSPLMFSGGGS(1)AKEARWPRAH</td>
<td>6.7</td>
<td>2.5</td>
<td>13.5</td>
<td>8.2</td>
</tr>
<tr>
<td>RXP-C</td>
<td>GIAAREPNSHDGGGS(3)1RLWPRKAKSL</td>
<td>12.5</td>
<td>11.8</td>
<td>19.6</td>
<td>15.2</td>
</tr>
<tr>
<td>RXP-D</td>
<td>WEEPRPFTMSGGGS(3)1PMRRHLPGVNH</td>
<td>16.7</td>
<td>0.8</td>
<td>1.8</td>
<td>6</td>
</tr>
<tr>
<td>RXP-E</td>
<td>SDDLSPOLIHHGGGS(3)1HMVRKPRNPR</td>
<td>0.8</td>
<td>4.2</td>
<td>3.7</td>
<td>3</td>
</tr>
</tbody>
</table>

(1) indicates linker ‘AHARVPFYHS’; (2), linker ‘AETHARVPFYHS’; (3), linker ‘AVPFYHS’.
(supplemental Figure II). Resonance peaks for G291, A323, and I358 are presented as examples of residues whose position was unaffected by the peptides. It is important to note that residues P375 and P377 do not provide an identifiable resonance peak because they do not contain an amide bond; yet these residues may also be part of the structural modification caused by the presence of the peptide. In contrast, no resonance shifts were observed when Cx43CT was exposed to RXP-1 (data not shown). This is consistent with SPR experiments showing very weak (almost undetectable) interaction between RXP-1 and Cx43CT (Figure 1A). Overall, the data show that RXP-4 and RXP-E alter the conformation of Cx43 residues 343 to 346 and 376 to 379.

**Effect of RXP-E on Cx43 Channels**

The ability of RXP-E to bind Cx43CT led us to propose that this peptide may also alter the behavior of Cx43 channels. Gap junction currents were recorded from N2a cells transfected with Cx43. To reduce macroscopic currents, cell pairs were superfused with 2 mmol/L octanol. To our surprise, the presence of RXP-E in the internal pipette solution prevented octanol-induced uncoupling (ie, reduction of $G_j$ to 0) in all 8 pairs tested. Results are presented in Figure 3A. Data were obtained from cell pairs recorded in control conditions (closed circles; $N=7$) or when the internal pipette solution contained either RXP-E (solid triangles; $N=8$), RXP-1 (open squares; $N=6$) or a peptide containing the same amino acids as RXP-E but in a randomized sequence (open triangles; $N=6$). Peptide concentration in all cases was 0.1 mmol/L. The plot correlates percentage of junctional conductance (relative to control) as a function of time after onset of octanol superfusion. Clearly, octanol exposure led to a rapid drop in electrical coupling either in control or in the presence of RXP-1 or scrambled RXP-E peptides. However, all cell pairs
Figure 3. Effect of RXP-E on octanol-induced and acidification-induced uncoupling of Cx43-expressing N2a cells. Peptides were diluted in internal pipette solution (0.1 mmol/L). Junctional conductance (Gj) was measured using dual patch clamp (see Seki et al12 and online data supplement). Transjunctional voltage (Vj), 60 mV; pulse duration, 10 seconds; interpulse interval, 10 seconds. Bars represent SEM. A, Time course of octanol superfusion. The average conductance (Gj) was measured immediately after patch break and every 20 seconds thereafter. Figure 3C shows these results. In the absence of RXP-E, Gj decreased progressively, reaching 12.08 ± 3.48% of control within 15 minutes after patch break (solid circles; N=5). In the presence of RXP-E (solid triangles; N=5), a decrease in Gj was also observed, but it was significantly dampened; after 15 minutes, average Gj decreased only to 45.28 ± 10.89% of the initial value. This value was significantly different from that recorded in control (P<0.05). Interestingly, the presence of the scrambled RXP-E did not disrupt acidification-induced uncoupling (open triangles; N=5; P>0.05 when compared with control). Overall, the data show that RXP-E partially prevented the closure of Cx43 channels consequent to a reduction in pHj.

Effect of RXP-E on Single-Channel Activity
We were able to record spontaneous single-channel activity from 4 Cx43-expressing cell pairs exposed to RXP-E. Results were compared with those obtained in control. Examples of single-channel traces are shown in Figure 4A (Vj=60 mV). Top trace corresponds to control (pipettes filled with normal internal pipette solution); bottom trace was recorded from a different pair, using patch pipettes containing RXP-E (0.1 mmol/L). The control recording shows the characteristic properties of Cx43 channels: 3 states (open, closed, and residual), fast transitions between open and residual, and a unitary conductance of ~100 pS in the open state. In contrast, in the presence of RXP-E, the events recorded showed a
unitary conductance similar to that of the wild-type channel, but open times were greatly prolonged and the residual state was absent. All-events histograms of unitary conductance are shown in Figure 4B. The data collected from cell pairs in the absence of RXP peptides was best described by 2 Gaussian functions, centered at 80.7 pS and 104.5 pS (left panel labeled “control”); N = 5; n = 383; and in the presence of RXP-E (right); N = 4; n = 110. Histograms were fit by a Gaussian function (Origin 7.0; OriginLab Corp, Northampton, Mass). C, Frequency histogram of open time in control (left) and in the presence of RXP-E (right). Data were best fit by monoexponential functions. Control: N = 3, t = 0.12 seconds; RXP-E: N = 3, n = 368, t = 1.27 second. V = 60 mV. Open times were measured from single-channel events recorded in the absence of uncouplers (only 1 or 2 channels active).

Discussion

We have conducted a high-throughput assay to identify peptidic sequences that bind Cx43CT. We identified a group of peptides sharing an RXP motif and an excess of basic residues within the sequence. The binding kinetics and structural modification of CT were studied in 3 of these peptides (RXP-1, RXP-4, and RXP-E); 1 peptide (RXP-E) showed a clear effect on Cx43 channel function. Before discussing the implications of our study, some technical issues need to be addressed.

Technical Considerations

Phage display is a powerful screening technique; yet, like any high-throughput method, it is prone to both false-positive and false-negative results. It is possible that, because of experimental conditions, we failed to identify sequences that would be of relevance. Similarly, it is possible that some of the sequences identified do not represent good Cx43CT binders when isolated from the phage. Despite this limitation, the system did provide us with the ability to recognize a group of molecules with the potential to significantly modify channel behavior (Figures 3 and 4).

Our screening of the biased prebound library failed to identify any double sequences. Yet, the majority of bound RXP peptides corresponded to doublets, which suggests that Cx43CT had a strong preference for longer sequences. Whether this is consequent to increased number of RXP motifs, increased number of basic residues (note that at least some of the linkers were also rich on basic amino acids), increased size of the peptide, or any combination of the above remains to be determined. What was clear is that among the “double RXP” series, we found 1 peptide with a high-binding affinity and significant effect on channel function.

The formation of doublets can be explained based on the analysis of the sequences of the linkers. To produce phage with random inserts, a single-stranded oligonucleotide containing a randomized sequence must be replicated using the Klenow fragment of DNA polymerase. This forms a double-stranded DNA fragment with blunt ends. Restriction digestion puts “sticky ends” on this fragment for insertion into phage DNA. In rare cases, 1 of these sticky ends was either not produced because of incomplete digestion or filled after digestion because of continued activity of the DNA polymerase. This led to blunt ends capable of ligation, rather than mismatched sticky ends. A more detailed explanation of this mechanism can be found in supplemental Figure IV. These events were rare enough as to not be detected in the sampling of the library sequences performed before screening.

It is worth noting that the peptides most frequently repeated in the phage display screening were not the ones with the highest binding affinity when tested by SPR. This apparent discrepancy may result from the fact that codon distribution (and consequently, transfer RNA [tRNA] availability) is not equal in the bacteria used to amplify the phage. Accordingly, even if a peptide binds with high affinity, its
amplification may be limited by the presence of rare codons in the phage. Consistent with this hypothesis, RXP-E contained the least represented codon in E. coli, likely acting as a limiting factor in its production. The latter emphasizes not only this specific limitation of the phage display method but also the importance of using alternative in vitro techniques to assess binding by the peptides identified through the screening process.

**Structural Modifications in Cx43CT**

Structural analysis of Cx43CT revealed that both RXP-4 and RXP-E caused a shift in the resonance peaks of amino acids within the 375 to 379 region. Interestingly, this region is within the PDZ binding domain and near areas relevant for Cx43 phosphorylation. Moreover, both peptides also modified the position of residues within the second α-helical domain of Cx43CT, which is involved in pH-dependent dimerization of the protein. By modifying the position of relevant amino acids in space, RXP peptides may interfere with both intra- and intermolecular interactions that could regulate the function of gap junctions. Yet, we are still short of demonstrating a causal link between the specific resonance shifts and the functional effects. Moreover, which residues were modified as a result of a direct physical interaction with the peptide (ie, a binding site) and which ones shifted as a result of a distant secondary effect remain to be determined.

**Octanol-Induced Uncoupling and the Effect of RXP-E**

RXP-E prevented octanol-induced uncoupling, and the full effect required the integrity of the CT domain. The molecular mechanism by which octanol causes gap junction closure is not completely understood. It has been proposed that volatile anesthetics and long-chain alcohols exert their effects on membrane channels (including gap junctions) via nonspecific actions on the lipid bilayers. However, recent studies on ligand-gated channels show that n-alkanols exert their functional effect by interacting directly with specific binding pockets in the channel proteins. Our results suggest a direct interaction between octanol and Cx43. However, the structural involvement of the CT in this process is unclear, because this domain is not part of the pore-forming region of the channel. Yet, it is important to note that Cx43CT interacts with regions of the channel affiliated with the pore. It is therefore possible that RXP-E may use the CT as a “scaffolding,” from which it interacts with pore-forming or pore-vestibular regions (including the CL domain), thereby holding the channel in its open state. An RXP-E-dependent delay in octanol-induced uncoupling observed in M257 channels (Figure 3B) suggests a possible interaction of RXP-E with a domain of Cx43 different from the CT.

Previous studies have shown that unsaturated free fatty acids (FFAs) are generated during ischemia and can have an arrhythmogenic effect. Other authors have shown that FFAs can cause gap junction channel closure, perhaps by a mechanism similar to that of octanol-induced uncoupling. Whether RXP peptides can interfere with the arrhythmogenic effects of FFAs remains to be determined.

**Effect of RXP-E on pH Gating of Cx43**

Also relevant to pathophysiology is the observation that RXP-E partially prevented acidification-induced uncoupling; the latter is considered 1 of the possible substrates for ventricular arrhythmias following myocardial ischemia or infarction. Yet, the extent to which pH gating of Cx43 is beneficial or deleterious to function in the ischemic heart remains undetermined. By interfering with gap junction regulation, RXP-E (or future derivatives of it) may serve as a tool to dissect the specific role that gap junction regulation plays in the electrophysiology of the ischemic heart.

**Future Implications**

Peptides or peptide-derived molecules have been used in the past in an attempt to regulate Cx43. Sequence analysis shows that none of the Cx-modifying peptides previously described carries an RXP motif. A number of those sequences have been derived from Cx43 itself. Others, although apparently capable of preserving intercellular coupling under certain conditions, seem to act indirectly via modulation of molecules (such as kinases) that in turn regulate Cx43. The latter carries a high risk of connexin-unrelated effects on cell function, as kinases are likely to interact with a variety of molecules, not only Cx43. Here we present the first demonstration of a peptidic molecule, exogenous to the cell, that can prevent a form of chemically induced uncoupling likely by direct interaction with Cx43CT. Insofar, RXP-E delivery has required the use of patch pipettes. Yet, our preliminary studies show that RXP-E can be introduced into intact cells by use of peptide transfer domains. These sequences are now used widely in the development of peptide-based pharmacophores targeted to intracellular proteins. Much remains to be learned about the effects of RXP peptides on Cx43, other connexins or other channels. The present description opens the door for future development of chemical tools to regulate the function of Cx43-containing gap junctions both in health and in disease.

**Acknowledgments**

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Online Methods

Phage Display. We utilized a ‘library’ of bacteriophage, displaying ~55 copies of $2.7 \times 10^9$ random 12-mer peptides (Ph.D.-12™ Phage display peptide library kit; New England BioLabs Inc.). Wells in 24-well plates were coated with $15 \mu g$ of recombinant CT and blocked with $5mg/ml$ BSA. Phage were prebound to uncoated wells and then allowed to bind to the CT-coated wells. After removing the unbound viruses by washing with Tris buffered saline containing 0.5% tween-20 (TBS-T), low affinity binders were eluted with a solution of TBS-containing Cx43CT at a concentration of 100 $\mu g/ml$. High affinity binders were then recovered by overlaying the well with a culture of E. coli, to allow the tightly bound phage to infect the bacteria. This culture was amplified and the virus precipitated with PEG. The amplified product was used for the subsequent round of panning. All steps were conducted at pH 6.5 unless otherwise indicated.

After three rounds of selection and amplification, the phage recovered from the last round of binding were grown on a lawn of E. coli for plaque purification. Each plaque, representing a single clone, was picked and amplified. The phage were isolated and analyzed by DNA sequencing. The peptide sequences displayed were deduced from these sequences.

NMR: Data were acquired with 1024 complex points in t2 and 128 complex points in t1. Sweep widths were 10,000 Hz in the proton dimension and 2,500 Hz in the nitrogen dimension. The concentration of RXP peptide to Cx43CT was approximately 2.4 mmol/L to 0.8 mmol/L, respectively (3:1 ratio). All NMR data were processed using NMRPipe ¹ and analyzed using NMRView ².

Patch Clamp and Data Analysis: Studies on the octanol and pH sensitivities of Cx43, as well as on the properties of Cx43 channels, were carried out in N2a cells stably transfected with Cx43 DNA under the control of a LacSwitch system ³. Cells were pre-treated with 0.1 mmol/L IPTG 7-15 hours before recording. Studies on mutant M257 (a mutant lacking amino acids 258 to 382 of rat Cx43; ⁴) were conducted in transiently transfected N2a cells. In all cases, the dual-whole-cell voltage clamp technique was used to record gap junction currents. Both cells in the pair (cell1 and cell2) were independently voltage
clamped at the same holding potential (-40 mV). The prejunctional cell (cell1) was stepped to +20 mV, creating a potential difference across the junction (Vj) of +60 mV during repetitive 10-30 sec steps. The current injected by the amplifier in cell 2 to maintain the holding potential of that cell (-40 mV) during the voltage step in cell 1 was considered to be equal and opposite to the current flowing through the gap junctions (Ij)\(^5,6\). Junctional conductance (Gj) was calculated from Ohm’s law (Gj = Ij/Vj). Patch pipettes were filled with a solution containing cesium (in mmol/L: 130 CsCl; 0.5 CaCl\(_2\); 10 HEPES; 10 EGTA; 2.0 Na\(_2\)ATP; 3.0 MgATP; pH 7.2). Pipette resistance was 4.0-6.0M\(\Omega\). Synthetic peptides were diluted in the pipette solution to a final concentration of 0.1 mmol/L. During recording, cells were kept at room temperature in a cesium-containing solution (in mmol/L: 160 NaCl; 10 CsCl; 2.0 CaCl\(_2\); 0.6 MgCl\(_2\); 10 HEPES; pH 7.4). Macroscopic junctional currents were filtered at a cutoff frequency of 0.1 kHz and digitally sampled at 0.5 kHz. Single channel currents were sampled at 2.0 kHz (-3 db, four-pole Bessel filter, LPF202; Warner Instrument Corp., Hamden, CT). Traces of junctional currents were acquired and analyzed using the Clampex software (pClamp version 9.0, Axon Instruments, Union City, CA). Cell pairs showing a leak current larger than 0.1 nA were excluded from the analysis.

**Octanol experiments**

Octanol superfusion was initiated 5 minutes after patch break and continued for a maximum of 15 minutes. The concentration of octanol was 2 mmol/L in all experiments. Gj was measured in control and every minute after the onset of octanol superfusion. All cell pairs showed an initial Gj value of <35 nS.

**Intracellular acidification**

To acidify the intracellular space, the pipette solution was prepared as described above but HEPES was replaced by MES (10 mM). The final pH of the internal pipette solution was 6.2. Gj was measured immediately after patch break and for the following 15 minutes. All cell pair showed an initial Gj value of <25 nS.

**Unitary conductance**

Single channel activity was recorded either from cell pairs that were poorly coupled (data on RXP-E; see Figure 4 in paper) or cell pairs that were uncoupled by means of octanol superfusion (control in Figure 4 in
Results obtained from octanol-uncoupled pairs were not different from those previously observed in the absence of the uncoupler (see 4). Single channel currents were recorded at a sampling rate of 2 kHz and filtered at a cut-off frequency of 0.1 kHz. Single channel events were selected for analysis based on the following criteria: events needed to appear in both recordings (I1 and I2), thus reflecting movement of current through the gap junctions (see 5). Events needed to last more than 20 ms (to avoid filtering artifacts) and events obtained from recordings were the baseline drift was larger than 25 pA were discarded. For measurement of open times, events where the channels were opened at the end of the voltage pulse (i.e., where the open-closed or open-residual transition was not recorded) were excluded from analysis. Open times were measured from recordings where only one channel was active at a time. Histograms were constructed using Origin (version 7.0; Microcal, Northampton, MA) 5 .

**Statistical analysis**

When appropriate, data were statistically compared by Student’s t test. Only one comparison was allowed for each data set. Differences yielding p values <0.05 were regarded as significant.

**Additional Results**

**Online Figure 1.** Balance of basic (B) and acidic (A) residues in peptides. Although we did not detect a clear preference for specific amino acids in a given position relative to the RXP core, we did observe a preponderance of basic residues in sequences recovered from all screenings. To quantitate this observation, the frequency of each group of amino acids was determined (for simplicity, the linker sequence in the doublets was not taken into account). Results were compared to those obtained from peptides in the pre-bound library. As all sequences detected from the control library corresponded to single 11-mer peptides, we randomly paired them, forming theoretical “22-mer” peptides that compared in length to the doublets. To confirm that no bias occurred in the pairing, we characterized the amino acid frequency in “pairs” conformed in three different random assignments. The frequency distributions are shown in this figure. Ordinates indicate the number of basic (H, K or R) minus acidic (D or E) residues. The arginine residue in
the center of the sequence (part of the “RXP” motif) was included in the count. Sixty control peptides (thirty “pairs”) were compared to the 30 doublets recovered from all screenings. Each unique doublet was counted as one, regardless of the frequency with which it appeared after Cx43CT binding. Regardless of the randomization, control peptides had a balance of two basic residues (Panels A-C). This is consistent with the fact that the library was biased to express one arginine in each 11-mer. In contrast, the frequency histogram obtained from the doublet peptides that bound Cx43CT had an abundance of 4 basic residues (Panel D). These results indicate that Cx43CT preferentially bound peptides with a higher number of basic residues, perhaps consequent to the positive balance of charge within the peptide.

**Online Figure 2.** Resonance peaks corresponding to Cx43CT residues as recorded by an NMR $^{15}$N-HSQC protocol. The peak shift was compared in the absence (black lines) or the presence (red lines) of RXP4. The top inserts are examples of amino acids whose position in the HSQC map was unaffected by the peptides. As RXP-E (see Figure 2), RXP-4 also caused a shift in the both position of residues 376, 378 and 379, and 343-346. In contrast (data not shown), RXP-1 did not modify the position of any of the resonance peaks, consistent with its very poor binding to Cx43CT.

**Online Figure 3.** Percent of pairs that remained coupled (i.e., $G_j > 0$) at the end of each minute after onset of octanol. Time zero corresponds to the onset of octanol superfusion (Solid line: absence of RXP-E; dotted line: presence of RXP-E). **A:** Effect of RXP-E on octanol (2 mmol/L)-induced uncoupling in Cx43-expressing N2a cells. In the absence of RXP-E, all 7 cell pairs were completely uncoupled ($G_j = 0$) whereas octanol failed to uncouple when RXP-E was present in the pipette solution (0.1 mmol/L; N=8). **B:** Effect of RXP-E on octanol (2 mmol/L)-induced uncoupling in N2a cells expressing M257 (a truncation mutant of Cx43 lacking amino acids 258-382). Regardless of RXP-E, the cell pairs were uncoupled by octanol. (N=13 in control; N=8 in the presence of RXP-E).

**Online Figure 4.** Mechanisms responsible for the generation of doublets in the biased RXP phage display library. Details are outlined in the figure.
Reference List


## Online Table 1. Frequency of occurrence of doublet peptides in biased phage display screening.

<table>
<thead>
<tr>
<th>Run</th>
<th>pH</th>
<th>Total inserts sequenced</th>
<th>Total Doublets</th>
<th>Total Unique Doublets</th>
<th>Total Singlets</th>
<th>Total Unique Singlets</th>
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<td>102</td>
<td>18</td>
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<tr>
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<td>10</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
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<td>150</td>
<td>19</td>
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<tr>
<td>Totals</td>
<td></td>
<td>402</td>
<td>359</td>
<td>47</td>
<td>43</td>
<td>41</td>
</tr>
</tbody>
</table>
Online Figure 2

Cx43CT with RXP-4
online figure 3

A  Cx43-WT  B  Cx43-M257

% of Coupled Pairs

Control  RXP-E

Time (min)

% of Coupled Pairs

Control  RXP-E

Time (min)
**POSSIBLE MECHANISMS OF DOUBLET FORMATION:**

In the production of the phage display library, a series of randomized (nonbiased) or partly randomized (biased) bases are inserted between an Acc65I restriction site, and an EagI site. Prior to the EagI site, a GGG linker (three glycines that will act as a spacer between the 12-mer peptide and the phage capsid protein) is cloned.

The DNA sequence flanking the region of the insert (X) is:

```
Acc65I                             EagI
CATGCCGGGGTACCTTTCTATTCTACTCTXXGGCGCGGCTCGGCCGAAACATG
1 ---------+---------+---------+---------+------- 47
GTACGGGCGCCATGGAAAGATAAGAGTGAGANNNCCGCCGCGGAGCGGGCTTTGTAC
```

The amino acid sequence encoded by the DNA that flanks the insert is:

```
H A R V P F Y S H S X X X X R X P X X X X G G G S A E T
```

(using the RXP biased library as the example)

The sequence inserted into the phage after restriction cutting (Acc65I and EagI) is:

```
VPFYSHSSXXXPXXGGG
```

In other words, every peptide in either the biased or the nonbiased phage display libraries is flanked by sequence VPFYSHS (at the start) and GGGS (at the end).

The following linker sequences were identified in the doublets after screening of the biased library:

1) GGGSAETHARVPFYSHS
2) GGGSAHARVPFYSHS
3) GGGSAVPFYSHSH

**Possible mechanism for formation of linker 1:**

Both restriction enzymes (EagI and Acc65I) fail to cut, leaving two blunt sites: the end of one insert, coding for GGGSAET, and the beginning of a second one, coding for HAR... Both blunt ends ligate, thus concatenating two inserts through the linker:

```
GGGSAET     HARVPFYSHS
end of insert   start of insert
one                  two
```

**Possible mechanism for formation of linker 2:**

Acc65I fails to cut. EagI does cut, leaving the coding sequence for GGGS in the insert. The overhang fills, forming the coding sequence for A. The two blunt ends ligate.

```
GGGSA     HARVPFYSHS
```
Possible mechanism for formation of linker 3:
Both restriction enzymes cut and both overhangs are filled, thus creating two blunt ends that ligate.

<table>
<thead>
<tr>
<th>GGGSA</th>
<th>VPFYSHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>From insert</td>
<td>from insert</td>
</tr>
<tr>
<td>one</td>
<td>two</td>
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