RXP-E
A Connexin43-Binding Peptide That Prevents Action Potential Propagation Block

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Abstract—Gap junctions provide a low-resistance pathway for cardiac electric propagation. The role of GJ regulation in arrhythmia is unclear, partly because of limited availability of pharmacological tools. Recently, we showed that a peptide called “RXP-E” binds to the carboxyl terminal of connexin43 and prevents chemically induced uncoupling in connexin43-expressing N2a cells. Here, pull-down experiments show RXP-E binding to adult cardiac connexin43. Patch-clamp studies revealed that RXP-E prevented heptanol-induced and acidification-induced uncoupling in pairs of neonatal rat ventricular myocytes. Separately, RXP-E was concatenated to a cytoplasmic transduction peptide (CTP) for cytoplasmic translocation (CTP–RXP-E). The effect of RXP-E on action potential propagation was assessed by high-resolution optical mapping in monolayers of neonatal rat ventricular myocytes, containing ~20% of randomly distributed myofibroblasts. In contrast to control experiments, when heptanol (2 mmol/L) was added to the superfusate of monolayers loaded with CTP–RXP-E, action potential propagation was maintained, albeit at a slower velocity. Similarly, intracellular acidification (pH 6.2) caused a loss of action potential propagation in control monolayers; however, propagation was maintained in CTP–RXP-E-treated cells, although at a slower rate. Patch-clamp experiments revealed that RXP-E did not prevent heptanol-induced block of sodium currents, nor did it alter voltage dependence or amplitude of Kir2.1/Kir2.3 currents. RXP-E is the first synthetic molecule known to: (1) bind cardiac connexin43; (2) prevent heptanol and acidification-induced uncoupling of cardiac gap junctions; and (3) preserve action potential propagation among cardiac myocytes. RXP-E can be used to characterize the role of gap junctions in the function of multicellular systems, including the heart. (Circ Res. 2008;103:519-526.)

Key Words: Cx43CT | particle–receptor interaction | gap junctions | connexin43 | rotigaptide

Connexins (Cxs) are integral membrane proteins that oligomerize to form intercellular channels called gap junctions (GJs). The most abundant GJ protein in a number of mammalian systems is Cx43. GJs allow passage of ions and small molecules between cells and are regulated by a variety of chemical interactions between the Cx molecule and the microenvironment. As such, GJs act as active filters to control passage of intercellular messages and modulate function.

Our previous work has suggested that regulation of Cx43 results from the association of the carboxyl-terminal (CT) domain, acting as a gating particle, and a separate region of the Cx molecule acting as a receptor for the gating particle.1,2 Additional studies have shown that this intramolecular particle–receptor interaction can be modulated by other intermolecular interactions in the microenvironment of the GJ plaque.3–5 Based on the particle–receptor model, we reasoned that regulation of Cx43 could be disrupted by the binding of exogenous molecules to regions of the gating particle required for its interaction with the receptor. The latter rationale led us to the identification of a 34-aa peptide dubbed “RXP-E.”6 This peptide bound in vitro to Cx43CT with an apparent K_D of 3.9 μmol/L, modified the structure of Cx43CT, partially prevented octanol-induced and acidification-induced uncoupling of N2a cells transfected with exogenous Cx43, and caused a significant prolongation of the single channel open time.6 Accordingly, we proposed that RXP-E could be developed as a tool to address the relevance of preserved intercellular communication in the maintenance of electric synchrony in the heart. Here, we show that RXP-E binds to native cardiac Cx43, prevents the closure of cardiac GJs, can be introduced into multicellular preparations, and can prevent heptanol- and acidification-induced action potential propagation block in monolayers of
neonatal cardiac myocytes. Our results suggest that RXP-E can be used as a platform for development of a pharmacophore that could interfere with Cx43 regulation. The potential of GJ modifiers as antiarrhythmic agents has been described before. However, the identity of the molecular target for existing compounds is unknown, thus making them unsuitable for optimization by targeted drug design. Our results identify RXP-E as the first molecule known to bind Cx43 and, in doing so, preserve action potential propagation in cardiac myocytes under conditions otherwise expected to induce propagation block.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. Brief descriptions are provided below.

Recombinant Protein Production and Immunochemical Assays
All immunochemical protocols, as well as production of recombinant glutathione S-transferase (GST) fusion proteins and the GST pull-down assays, followed standard techniques. Details are provided in the online data supplement.

Myocyte Isolation and Culture
Primary cultures of neonatal rat ventricular myocytes (NRVMs) for patch-clamping, immunofluorescence, and monolayers were obtained using established procedures. After dissociation, cells were resuspended in supplemented medium 199 and preplated for 2 hours to reduce the presence of noncardiomyocytes. Cells were plated on 35-mm tissue culture dishes at a density of 1.2×10⁶ cells per dish for monolayers and at low density on 22-mm coverslips for patch-clamp experiments. For immunofluorescence, cells were seeded onto 22-mm coverslips at a density of 5.0×10⁵ cells per coverslip.

Translocation of RXP-E Into NRVMs
Peptides were concatenated with a cytoplasmic transfer peptide (CTP) sequence for translocation into the cytoplasm of NRVMs. Peptide synthesis was carried out by a commercial supplier (Anaspec Inc). Purity of peptide products was >85%.

Electrophysiological Analysis

Determination of GJ Currents
Experiments were performed in NRVM pairs endogenously expressing Cx43. The dual whole-cell voltage clamp technique was used to record GJ currents, as previously described.

Determination of Nav1.5 Currents
Voltage clamp experiments were conducted in HEK293 cells (ATCC no. CRL1573) transfected (Effectene Transfection Kit, Qiagen) with human SCN5A cDNA subcloned in a pcDNA3.1 vector. The protocol and solutions used for recording sodium current from human SCN5A with established procedures. The presence or absence of RXP-E in the internal pipette solution using methods previously described.

Effect of Heptanol Superfusion on Ionic Currents
The effect of heptanol on cardiac GJ currents, as well as in the amplitude of Nav1.5 and Kir2.1 to 2.3 currents, was assessed in the presence and absence of RXP-E in the internal pipette solution using methods previously described.

Results
We have previously shown RXP-E binding to recombinant Cx43CT. Here, we explored whether RXP-E is able to induce propagation block.
appropriate size was recovered from the precipitate of beads coated with GST–RXP-E and exposed to heart lysate (fourth lane from the left). On the other hand, no signal was obtained from samples obtained after exposure of heart lysate to either GST, or GST-Scr, or from coated beads that were not exposed to heart lysate. Additionally, the density of the Cx43-immunoreactive band was found to be dependent on the concentration of the heart lysate, as demonstrated in Figure 1B. Overall, the results indicate that RXP-E can interact with native cardiac Cx43.

Effect of RXP-E on Heptanol-Induced Uncoupling of NRVMs
The ability of RXP-E to bind cardiac Cx43 led us to propose that this peptide may also alter the behavior of cardiac GJs. Whole cell GJ currents were recorded from NRVMs expressing neonatal myocytes. Peptides were diluted in internal pipette solution (0.05 mmol/L). Gj was measured using dual-patch clamp (see elsewhere and the online data supplement). Bars represent SEM. A, Time course of heptanol-induced uncoupling in NRVMs. Time 0 indicates onset of heptanol superfusion. Gj in cells exposed to RXP-E decreased to 71.8% of control at 10 minutes after onset of superfusion (P<0.001 when compared to control). Data were compared with those obtained in the presence of scrambled RXP-E (green triangles). B, Time course of acidification-induced uncoupling. Ordinates represent time elapsed after patch break. Internal pipette solution was buffered to pH 6.2. Ten minutes after patch break, Gj in control decreased to 1.3% of the initial value. Scrambled RXP-E peptide did not disrupt acidification-induced uncoupling (green triangles; n=10; P>0.05 when compared to control). In contrast, in the presence of RXP-E, Gj decreased only to 71.2% of maximum (P<0.05).

RXP-E Partially Prevented Acidification-Induced Uncoupling in NRVMs
Next, we assessed whether RXP-E can interfere with the extent and time course of uncoupling induced by reduced intracellular pH (pH). Patch pipettes were filled with a 2(N-morpholino) ethanesulfonic acid (MES)-containing solution, buffered to a pH of 6.2. Junctional current was measured immediately after patch break and every 20 seconds thereafter. Figure 2B shows the results. In the absence of RXP-E, Gj decreased progressively, reaching 1.3% of control within 10 minutes after patch break (black squares; n=10). In the presence of RXP-E (red circles; n=10), a decrease in Gj was also observed, but it was significantly dampened; after 10 minutes, average Gj decreased only to 71.2% of the initial value. This value was significantly different from that recorded in control (P<0.05). Interestingly, scrambled RXP-E did not disrupt acidification-induced uncoupling (green triangles; n=10; P>0.05 when compared to control). Overall, the data show that RXP-E partially prevented closure of Cx43 channels consequent to a reduction in pH.

Does RXP-E Prevent the Effect of Heptanol on Nav1.5 Currents?
Our results show that RXP-E prevents heptanol-induced closure of GJ channels. Heptanol is known to affect sodium currents as well. Here, we asked whether RXP-E can interfere with the effect of heptanol on the ionic current obtained after expression of the SCN5A gene in transfected HEK293 cells. Figure 3A shows the results. Peak current amplitude was plotted as a function of the test voltage (see Materials and Methods for details). From each cell, a complete current–voltage relation was obtained before and 10 minutes after onset of superfusion with heptanol (2 mmol/L). To minimize variability between experiments, the amplitude of the peak current recorded at each voltage step was normalized to maximum peak current amplitude obtained from the same cell before heptanol superfusion. As expected, heptanol caused a drastic reduction in the amplitude of the
Nav1.5 current. Yet, as opposed to what we observed in the case of GJs, the effect of heptanol was the same regardless of whether or not RXP-E was present in the pipette solution. Peak current amplitude was observed at a command potential of $-35\pm5$ mV for control (black symbols; $n=12$), and $-30\pm5$ mV in the presence of RXP-E (red symbols; $n=12$; $P>0.05$). After heptanol, the command voltage that elicited maximum peak current amplitude was $-30\pm5$ mV for control and $-30\pm5$ mV in the presence of RXP-E ($P>0.05$ when values before and after heptanol were compared). Moreover, the relative reduction of maximum peak current amplitude was 36.6$\pm$4.8% for control and 30.5$\pm$5.1% in the presence of RXP-E (green and blue symbols, respectively; $P>0.05$). Overall, the data show that RXP-E does not interfere with heptanol-induced reduction of sodium current amplitude.

**RXP-E and Inward Rectifier Currents**

Further assessment of RXP-E specificity involved measurements of Kir2.1 and Kir2.3 currents recorded from double stably transfected HEK293 cells. Figure 3B shows the results. Current–voltage relations were generated as detailed in Materials and Methods. In this case, currents were normalized to the amplitude measured at $-100$ mV. Kir2.1 and Kir2.3 currents were not affected by heptanol, and no significant shifts in the current–voltage relation were recorded in the presence of RXP-E.

**From Cell Pairs to Multicellular Preparations: Transfer of RXP-E to the Intracellular Space in Multicellular Preparations**

Our results show that RXP-E can prevent closure of GJs in cardiac cell pairs. As a next step, we developed an assay for introducing RXP-E into multicellular preparations. The latter was necessary for assessment of the effect of RXP-E on action potential propagation in cardiac cells. Monolayers of NRVMs were exposed to synthetic fusion peptides containing a cytoplasmic transduction peptide (CTP512 domain; sequence, YGRRARRRRRR15). A cysteine residue at the C-terminus allowed us to conjugate fluorescein isothiocyanate (FITC) to the peptides for detection by fluorescence microscopy. Synthetic peptides were created for both RXP-E and its scrambled version. Figure 4A shows an example of peptide transfer into NRVMs. Monolayers were prepared from the hearts of neonates (1-day old); cells were cultured for 6 days. The top image was obtained from cells maintained in control conditions (no CTP–RXP-E). The image in the bottom was obtained from cells previously incubated with CTP–RXP-E (0.1 mmol/L) for 2 hours. Cx43 was immunolabeled (red) and nuclei were stained (Hoechst33528; blue) for easier identification of individual cells. No FITC signal was present in control; however, intense signal was recorded from all cells that had been preincubated with CTP–RXP-E. These experiments were repeated, this time with the scrambled CTP–RXP-E, as shown in Figure 4B. Top images correspond to cells not presented with the peptide. The bottom images were recorded after cells were incubated for 30 minutes with a solution containing 0.1 mmol/L of scrambled CTP–RXP-E. Right images show the overlay of the blue (nuclei) green (fluorescein-labeled RXP-E) and red (Cx43) signals. Left images show the DIC image of the myocyte monolayers. Clearly, a bright green fluorescein signal was detected from all cells within the field, indicating successful peptide translocation. Signal was detected up to 18 hours after peptide incubation (not shown). Further confirmation of successful transfer into almost 100% of the cells was obtained by flow-cytometric detection of the FITC signal from NRK cells (not shown). Similarly timed transduction experiments were repeated in NRK cells (supplemental Figure II). Overall, the data show that CTP allows for efficient transfer of FITC-labeled peptides into the cytoplasm of rat neonatal cardiac myocytes. We used this translocation protocol to test for the effect of RXP-E on action potential propagation in monolayers of NRVMs.

**Effect of RXP-E on Action Potential Propagation**

The effect of RXP-E on action potential propagation was assessed by high-resolution optical mapping. Monolayers of...
NRVMs were labeled with a voltage-sensitive dye (di-8-ANEPPS; 40 μmol/L) and a high-resolution, high-speed camera was used to record the electric activity in the 35-mm monolayer (see elsewhere; see also Materials and Methods for details). A stimulating electrode was placed in the center of the preparation (see the left images in Figure 5). The electric activity propagated from the center toward the periphery of the preparation, as shown by the activation map displayed in the right images of Figure 5A. (Notice, however, the “shadow” of the stimulus electrode, preventing view of activity at the site of stimulation.) Overall, conduction velocity in normal saline solution was unaffected by the presence of heptanol-induced uncoupling.

Figure 5. Effect of RXP-E on action potential propagation in heptanol-induced uncoupling. A stimulating electrode was placed in the center of the preparation (left images). Activation maps are shown on the right (calibration bar represents activation times, in milliseconds). Exposure of the monolayer to heptanol caused total loss of propagated activity (B). Propagation in a monolayer loaded with CTP–RXP-E is shown in C. In this case, action potential propagation was maintained in the presence of heptanol, albeit at a slower velocity (D).
or absence of the RXP-E peptide. Indeed, the average conduction velocity of all experiments in monolayers not treated with CTP peptides was 164±8 mm/sec (n=12). This value was not statistically different from those obtained from monolayers in which either CTP–Scr–RXP-E or CTP–RXP-E had been translocated into cells (conduction velocity, 158±10 mm/sec, n=6, and 180±7 mm/sec, n=10, respectively). Exposure of nontreated monolayers to 2 mmol/L (n=4) or even 1 mmol/L (n=4) heptanol, caused a total loss of propagated activity (see Figure 5B) that was restored on washout. Similar results were obtained from monolayers treated with CTP–Scr–RXP-E (2 experiments at each heptanol concentration; complete conduction block in all 4 cases). However, a different result was obtained from CTP–RXP-E–treated monolayers. In that case, the propagated activity observed in normal saline solution (Figure 5C) was not interrupted by exposure to the uncoupler (Figure 5D). Indeed, when the higher dose of heptanol (2 mmol/L) was added to the superfusate, action potential propagation was maintained, albeit at a slower velocity. In average, conduction velocity in CTP–RXP-E–treated monolayers exposed to 2 mmol/L of heptanol was 87±5 mm/sec (n=4; P<0.001 by paired t test when compared to conduction velocity before heptanol superfusion in the same preparation). The result was consistent with the ability of RXP-E to preserve GJ communication but not to prevent the effect of heptanol on sodium currents.

RXP-E also prevented action potential propagation failure caused by acidification of the intracellular space. The recordings shown in Figure 6A were obtained from a preparation not treated with peptide. When the bathing solution was kept at normal pH (7.4), action potentials propagated through the preparation with a conduction velocity of 180±18 mm/sec. When the same preparation was exposed to a low pH solution (intracellular pH 6.2, as estimated by the protocol described in Materials and Methods), propagation failure was observed (n=6; see Figure 6B). Propagation failure was also observed in 2 monolayers pretreated with CTP–Scr–RXP-E. Yet, in preparations pretreated with CTP–RXP-E, propagation was maintained despite acidification of the intracellular space (see Figure 6C), although conduction velocity was significantly decreased (average conduction velocity, 93±28 mm/sec; n=4, P<0.05 when compared by paired t test with values at normal pH measured from the same preparation). Overall, these results demonstrate that RXP-E can preserve action potential propagation under conditions which consistently induce propagation block.

**Discussion**

Previous work from our laboratory, using high-throughput phage display analysis, led to the identification of the “RXP” sequence as a Cx43CT binding motif. Of the sequences identified, a particular 34-aa peptide (RXP-E) was shown to prevent heptanol- and acidification-induced uncoupling of N2a cells exogenously expressing Cx43. Here, we show that RXP-E: (1) binds to endogenous cardiac Cx43; (2) prevents heptanol and acidification-induced uncoupling of cardiac GJs; (3) can be introduced into monolayers of cardiac myocytes using a peptide translocator; and (4) preserves action potential propagation among cardiac myocytes. These results strongly suggest that RXP-E can be used as a platform for the development of a pharmacophore that could interfere with Cx43 regulation. Overall, the results identify RXP-E as the first Cx43-binding molecule capable of preserving action potential propagation in cardiac myocytes under conditions otherwise expected to induce propagation block.

**Cellular Delivery**

The utility of peptide molecules targeting intracellular domains is limited by the fact that, in general, peptides are not membrane-permeable molecules. Recent advances, however, have made it possible to translocate peptides into the intracellular space. A particular strategy consists of fusing the sequence of interest with a “cell-penetrating peptide” (CPP). For our studies, we concatenated RXP-E with a cytoplasmic transduction peptide (CTP), as described by Kim et al. Our results show that a fluorescein-labeled molecule abundantly localized to the cytoplasm, though, showed no preference for junctional membranes. Whether the CTP–RXP-E–FITC complex remained as concatenated inside the cells remains to be determined. If peptide cleavage occurred, the FITC signal would not pinpoint the exact location of RXP-E but, rather, of the cleaved FITC molecule. If, on the other hand, the complex remained intact, our results suggest that RXP-E distributes diffusely within the cell. This may be
a reflection of the limited concentration capacity of the peptide at the site of cell–cell apposition, consistent with its low binding affinity to Cx43.6 What is evident from our data are that the peptide modified GJ function and prevented propagation block, suggesting that, although its affinity for Cx43 may be low (see Shibayama et al6), its efficacy to modify GJ function was preserved. Overall, the CTP system allowed for pharmacological manipulation of a molecular target previously “protected” by the cell membrane. These and other techniques will be useful to determine whether modulation of the pH regulatory mechanism of Cx43 can modify the likelihood of cardiac arrhythmias under conditions that cause acidification of the intracellular space.

Conduction Velocity and Effect of RXP-E

It is worth noting that the propagation velocities measured in our experiments were lower than those reported by other authors.20,25 A likely explanation is that our preplating and culture methods did not completely remove fibroblasts from the cell preparation.26 In fact, ancillary experiments in which we immunolocalized nonmyocyte cells using a α-smooth muscle actin antibody (Anaspec Inc; data not shown) indicated that ≈25% of the cells were nonmyocytes. Our conduction velocities, therefore, correspond well with those measured by Miragoli et al.25 The presence of fibroblasts is unlikely to affect the outcome of our results. In fact, it is interesting to note that a number of cardiac pathologies associate with deposition of fibroid tissue and, likely, fibroblasts electrically coupled to the cardiac myocytes.26,27 It is also of interest that RXP-E by itself did not modify propagation. Conduction velocities measured from samples kept in control conditions were not different from those preloaded with RXP-E. This is similar to results obtained with other GJ openers28,29 and supports the notion that, in a well-coupled system of cells, GJ modification minimally affects propagation.30,31 Yet, once the system was challenged, RXP-E was capable of preserving action potential propagation, albeit at a slower velocity than in control. The latter is consistent with the observation that RXP-E did not prevent heptanol-induced decrease in sodium current amplitude (see Figure 3). In addition, because other Cx isoforms also express in NRVMs, it is possible that the observed decreases in $G_i$ (and conduction velocity) could reflect, at least in part, closure of those channels by heptanol or acidification. The effect (or lack thereof) of RXP-E on other Cxs remains to be determined. Or data do show that RXP-E preserves propagation albeit with a slower conduction velocity. Whether preservation of conduction at a slower velocity is an effective antiarrhythmic strategy, or instead, a proarrhythmic event, remains to be determined.

Pharmacology of GJs

It is unclear whether holding GJs open under pathological conditions such as ischemia would be beneficial or deleterious to the heart, although initial data seem to indicate that preservation of GJ communication may have a powerful antiarrhythmic effect (see studies with rotigaptide, previously known as ZP1238). However, among the reasons why such a gap in knowledge exists is that we lack pharmacological agents that can, with at least some partial selectivity, interfere with GJ regulation. (Much has been learned about the functional role of sodium channels, for example, from results obtained in the presence and absence of tetrodotoxin.) Our previous studies show that RXP-E can interfere with chemically induced closure of GJs. In the present study, we sought to determine the extent of selectivity of RXP-E action and its effect on propagation velocity and susceptibility to block.

Although other molecules (eg, cAMP) have been described that increase GJ coupling,28,32–36 their target remains elusive, and studies have not identified the Cx molecule itself as the target.37 Previous studies have determined that a small peptide (AAP10) can facilitate intercellular communication in heart preparations.38 Additional chemical modifications on AAP10 led to a compound initially identified as ZP123 (now rotigaptide). Experiments have suggested a potential therapeutic value to this molecule39 and, in general, to the approach of modifying GJ conductance to prevent or treat cardiac arrhythmias.8 Yet, although rotigaptide-based experiments suggest that GJ openers represent a promising strategy for antiarrhythmic therapy, this particular molecule lacks an identifiable target. (The molecule[s] acting as receptor for rotigaptide is not yet known.)9 The latter limits the possibility of conducting structure-activity studies to optimize the mechanisms of action of rotigaptide through target-based drug design. Moreover, recent studies suggest that rotigaptide may modify the catalytic activity of some kinases.39 Given the broad range of substrates and intracellular signaling pathways that can be affected by kinase modification, secondary effects on other cellular functions are likely. Here, we have used a different approach, that is, to select the target molecule (Cx43CT) as the bait for identification of the ligand (RXP-E). With this knowledge, we now demonstrate that RXP-E acts as a ligand for cardiac Cx43 and prevents the action of at least 2 well-known chemical uncouplers.

In conclusion, we have presented evidence indicating that a molecule derived from the RXP series of Cx43-binding molecules5 is capable of binding to cardiac Cx43, modifying cardiac GJs and preventing action potential propagation block. This is the first Cx43-targetted molecule capable of preserving electric conduction in a preparation of cardiac cells. Much remains to be done to determine the arrhythmogenic versus antiarrhythmic effect of GJ closure/opening under conditions of metabolic stress. Although pharmacological optimization and minimization is necessary, the results presented here suggest that RXP-E can serve as a starting point for the development of a target-based class of drugs centered on the objective of modifying cardiac GJ function.

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Disclosures

None.

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MATERIALS AND METHODS

GST-Pulldown Experiments

Production of GST fusion protein

A GST-RXP-E and GST-Scr-RXP-E fusion proteins were produced as previously described.1 Briefly, synthetic oligonucleotides from RXP-E’s and Scr-RXP-E’s full-length sequences (SDDLRPQLHNGGSAVPFYSHSHMVRRKPRNPR and SPVGYRMDLRPAHNRQLGFHSNGKRSSVPRHSDP, respectively) were inserted into pGEX-6P-2 (Amersham) and expressed in BL-21-competent bacterial cells. The resultant GST-fusion proteins were purified using a glutathione column. Protein concentration was measured using the Bio-Rad DC Protein Assay. Protein purity was assessed by SDS-PAGE.

Preparation of heart lysates

Fresh mouse or rat heart lysates were prepared by homogenizing tissue in heart lysis buffer (containing in mmol/L: 50 Tris/HCl pH7.5, 50 NaCl, 2 MgCl₂, 1 NaVO₄, 50 NaF, 2 EDTA, 1% β-mercaptoethanol) on ice. The components of the lysate were separated by centrifugation at 12,000 rpm for 25 minutes. For pre-clearing, one ml of supernatant was exposed to 250 µl of glutathione sepharose beads for 30 minutes at 4°C. After centrifugation, the pre-cleared supernatant was used for the pulldown assays described below. Total protein content of heart lysate was determined by DC protein assay (Bio-Rad) with bovine serum albumin as a standard.

GST-Pulldown assay
Bound GST fusion proteins were incubated with approximately 15 mg of pre-cleared rat or mouse heart lysate in 1ml of lysis buffer for 90 minutes, rocking at 4°C. Additional concentration-dependent experiments varied the concentration of heart lysate from 0mg/ml-5mg/ml. A separate sample was incubated with lysis buffer only as a control. Unbound proteins were removed by washing 2 times with lysis buffer. The final pellet was resuspended in Laemmli sample buffer and probed by western blotting as described below.

*Western blots for Cx43*

The pulldown pellets from the GST-fusion protein assay described above are kept at 55°C in Laemmli buffer (10 μl Laemmli buffer per 50μl of sample) for 10 minutes. Samples were run on an 8-16% tris-glycine gradient gel, transferred to nitrocellulose membranes and blocked for 1 hour at room temperature (blocking buffer consisted of 1% non-fat milk and 0.05% Tween in PBS). Membranes were then incubated in primary antibodies overnight at 4°C followed by incubation in secondary antibody (anti-mouse HRP, Sigma) for 45 minutes at room temperature. Signal was detected by chemiluminescence (Pierce, SuperSignal West Pico, Chemiluminescent Substrate). The primary antibody used was a monoclonal mouse Cx43 (diluted 1:100 in 0.05% Tween in PBS) that recognizes the amino terminal (NT) domain of Cx43 (Fred Hutchinson Cancer Research Center).

*Translocation of RXP-E into neonatal rat ventricular myocytes*

*Peptide constructs*
Peptides were concatenated with a cytoplasmic transfer peptide (CTP\(^2\)) sequence for translocation into the intracellular space of rat neonatal ventricular myocytes. Peptide sequences utilized were: CTP-RXP-E (using the one letter amino acid code): YGRRARRRRRRR-SDDLRLSPQLHNGGGSAVPFYSYSHSHMVRRKPRNPR-GC and CTP-Scrambled RXP-E: YGRRARRRRRRR-SPVGYRMRLPAHNRQLGFHSNGKRSSVPRHSDP-GC. Peptide synthesis was carried out by a commercial supplier (Anaspec, Inc). Purity of the peptide products was >85%.

*Labeling of synthetic peptides with Fluorescein-5-Maleimide*

Demonstration of peptide translocation required fluorescent labeling of synthetic peptides. The synthesized peptides with the available sulfhydryl end (CTP-RXP-E-GC, and CTP-Scr-RXP-E-GC) were dissolved in PBS pH 7.4. To prevent the formation of disulfide bonds while the peptide was in storage, 0.2mmol/L DTT was added. Coupling of Fluorescein-5-Maleimide (18mmol/L; 25-fold molar excess; Molecular Probes) to the peptides was conducted in PBS solution (pH 7.4). To protect the sample from light, the solution was prepared in an amber vial, and incubated at room temperature for 2 hours. The sample was then run over a gel filtration column to remove the unreacted Fluorescein-5-Maleimide. The column was washed several times with PBS pH 7.4, and the fractions containing fluoresceinated peptide were kept for RXP-E identification.

*CTP fusion peptide delivery*
CTP-mediated translocation of peptide sequences is known to occur independently of the lipid raft-dependent macropinocytosis pathway.²,³ Peptides (18-100µmol/L) were diluted in M-199 media (pH=7.4) and presented to monolayers of rat neonatal ventricular myocytes for a period of 30-120 minutes (incubation at 37°C; incubation time was 30 minutes for all optical mapping experiments). Cytoplasmic delivery was confirmed by the presence of fluoresceinated peptides, both by fluorescence microscopy (see Figure 4, and also Online Figure II) and by flow cytometry (data not shown).

*Fluorescence microscopy*

**Intracellular localization of CTP-RXP-E (NRVM’s):** Neonatal rat ventricular myocytes (NRVM’s) were plated to ~ 70% confluency on coverslips in 12-well dishes and treated with or without fluorescein-concatenated CTP-peptides as described above. In selected experiments, CTP peptides (0.1mmol/L) were kept in the media for 5 minutes, 30 minutes, 2 hours, or four hours, to determine the time course of peptide translocation. After incubation, coverslips were washed with PBS and fixed with ethanol (room temp) for 10 minutes. Following this incubation, 50% of the volume was exchanged for PBS in 3 separate washes. Images were taken and processed using an Axioplan 2 bright field microscope (Zeiss) equipped with 63X Plan Apo lens, and Axioplan 2E imaging with structural illumination (apotome).

**Immunolocalization of Cx43:** In some cases, NRVM’s were co-stained for Cx43 and CTP-RXP-E. In those cases, after 3 washes in PBS and fixation with ethanol (room temp) for 10 minutes, coverslips were exposed for 2 hours to a blocking buffer consisting
of 4% BSA and 0.1% Triton X-100 in PBS, followed by an overnight incubation with primary antibody against Cx43 at 4°C in a humid box. Coverslips were subsequently rinsed 3 times with PBS, and incubated with secondary antibody for 45 minutes at room temperature in a dark, humid box. Following 3 PBS washes, coverslips were mounted using Permafluor mounting media (THERMO electron corporation), and kept at 4°C for 24 hours. Preparations were examined at 63X magnification. Antibodies used were: a monoclonal mouse Cx43 antibody that recognizes the carboxy terminal (CT) domain of Cx43 (Fred Hutchinson Cancer Research Center) diluted 1:100 in blocking buffer (0.05% Tween in PBS), and a secondary antibody (Alexa fluor 594 donkey anti-mouse (Molecular Probes), diluted 1:1000 in blocking buffer). Hoescht dye for nuclear staining was diluted 1:1000 in blocking buffer. Images were taken and processed using an Axioplan 2 bright field microscope (Zeiss) equipped with 63X Plan Apo lens, and Axioplan 2E imaging with structural illumination (apotome).

**Intracellular localization of CTP-RXP-E (NRK’s):** Normal Rat Kidney cells (NRK’s) were plated to ~ 70% confluence on coverslips in 12-well dishes and treated with or without fluorescein-concatenated CTP-peptides as described above. In selected experiments, CTP peptides (0.1mmol/L) were kept in the media for 5 minutes, 30 minutes, 2 hours, or four hours, to determine the time course of peptide translocation. After incubation, coverslips were washed with PBS and fixed with ethanol (room temp) for 10 minutes. Following this incubation, 50% of the volume was exchanged for PBS in 3 separate washes. Images were taken and processed using an Axioplan 2 bright field
microscope (Zeiss) equipped with 63X Plan Apo lens, and Axioplan 2E imaging with structural illumination (apotome).

**Myocyte Isolation and Culture**

All experiments involving animals conformed to the protocols in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, Revised 1996), and the animal protocol was approved by the Department of Laboratory Animal Resources and the Committee for the Humane Use of Animals (CHUA) of the SUNY Upstate Medical University. Primary cultures of NRVM’s for patch clamping, immunofluorescence, and monolayers were obtained using established procedures. Ventricular myocytes from neonatal Sprague-Dawley rats (Charles River, Mass) were isolated and cultured according to Rohr et al. Briefly, the hearts from 1- and 2- day-old rats were aseptically removed and collected in calcium- and magnesium-free Hanks’Balanced Salt Solution (HBSS; without Ca$^{2+}$ and Mg$^{2+}$; Sigma). The ventricles were minced and incubated in a solution containing 0.125% trypsin (Roche Applied Science) and 0.15% pancreatin (Sigma). Digestion took place at 36°C in consecutive steps. Two hour periods of differential preplating were used to reduce the presence of noncardiomyocytes. Cells were then suspended in medium M199 (Cambrex) containing 10% fetal bovine serum (FBS) (Cellgro), 20 U/mL penicillin, 20 mg/mL streptomycin, and 100 μmol/L bromodeoxyuridine (Sigma) to inhibit fibroblast proliferation. Cells were plated on 35-mm tissue culture dishes at a density of 1.2x10$^6$ cells/dish for monolayers and at low density in 22-mm coverslips for patch clamp experiments. For immunofluorescent experiments, cells were seeded onto 22-mm coverslips at a density of 5.0x10$^5$
cells/coverslip. Media changes were performed after 24 hours and every 48 hours thereafter with 5% FBS medium. RXP-E incubation was performed prior to optical mapping and immunofluorescent experiments, as described below in CTP-RXP-E transfer.

**Patch Clamp experiments**

*Determination of gap junction currents*

Experiments were performed in neonatal rat ventricular myocyte pairs endogenously expressing Cx43. 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 (-10 mV). The prejunctional cell (cell1) was stepped to +50 mV, creating a potential difference across the junction (Vj) of + 60 mV during repetitive 10 sec steps. The current injected by the amplifier in cell 2 to maintain the holding potential of that cell (-10 mV) during the voltage step in cell 1 was considered to be equal and opposite to the current flowing through the gap junctions (Ij).\(^8\)

\(^9\) 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 CaCl2; 10 HEPES; 10 EGTA; 2.0 Na2ATP; 3.0 MgATP; pH 7.2). Pipette resistance was 4.0–6.0MΩ. Synthetic peptides were diluted in the pipette solution to a final concentration of 0.05 mmol/L. During recording, cells were kept at room temperature in a cesium-containing solution (in mmol/L: 160 NaCl; 10 CsCl; 2.0 CaCl2; 0.6 MgCl2; 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. 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 0.1nA were excluded from the analysis.

*Determinable of Nav1.5 currents*

Voltage clamp experiments were conducted in HEK293 cells (ATCC, CRL1573) using an Axopatch 200B amplifier (Axon Instruments). HEK293 cells were transfected (Effectene Transfection Kit, Qiagen) with human SCN5A cDNA subcloned in a pcDNA3.1 vector. The protocol for recording sodium current from HEK293 cells stably expressing the SCN5A gene involved holding the cell at a potential of -120 mV and applying 30 msec steps from -115 mV to +80mV in increments of 5 mV in a bath solution containing (in mmol/L): 137 NaCl, 5.4 KCl, 1.3 CaCl2, 0.8 MgSO4, 4.2 NaHCO3, 0.5 KH2PO4, 0.3 NaH2PO4, and 10 HEPES, which was titrated to pH 7.40 with 1 mol/L NaOH. Additional experiments were conducted in a bath solution containing (in mmol/L): 5 NaCl, 1 MgCl2, 1 CaCl, 0.1 CdCl2, 20 HEPES, 11 Glucose, 132.5 CsCl, (pH = 7.4, CsOH). In both cases, the pipette solution consisted of (in mmol/L): 5 NaCl, 135 CsF, 10 EGTA, 5 MgATP, 5 HEPES; pH 7.2 (CsOH). The HEK cells do not have any other major cardiac current, so no TTX was used. All sodium current recordings were performed at room temperature. Pipette DC resistances for experiments ranged between 4 and 5MΩ.

*Determinable of Kir2.1-Kir2.3 currents*

Kir2.1 and Kir2.3 currents were recorded from HEK293 cells (ATCC, CRL1573) using an Axopatch 200B amplifier (Axon Instruments). HEK293 cells were doubly transfected
(Effectene Transfection Kit, Qiagen) with guinea pig cDNA for Kir2.1 and Kir2.3 subcloned in a pECEP4 and pcDNA3.1 vector respectively.\(^{10}\) The protocol for recording inward rectifying potassium current from HEK293 cells stables expressing both Kir2.1 and Kir2.3 involved holding the cell at a potential of -50 mV and applying a ramp protocol at 25mV/sec from -100 mV to +50mV for 6 seconds in a bath solution containing (in mmol/L): 140 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 5.0 Hepes; (pH = 7.4, NaOH). The pipette solution consisted of (in mmol/L): 20 KCl, 90 K-aspartate, 10 KH\(_2\)PO\(_4\), 5.0 EDTA, 1.9 K\(_2\)ATP, 5 HEPES and 7.9 Mg\(_2^+\); pH 7.2 (KOH).\(^{11}\) The HEK cells do not have any other major cardiac current. Bath temperature was held at 37ºC. Pipette DC resistances for experiments ranged between 4 and 5M\(\Omega\).

Peptides used for patch clamp experiments

RXP-E (SDDLRSPLHNGGSAVPFYSHSHMVRKPRNPR) and Scrambled RXP-E (SPVGYRMDLRPAHNRQLGFHSNGKRSSVPRHSDP) used for patch clamping experiments were synthesized by Anaspec, Inc and diluted in the internal pipette solution at a final concentration of 0.5-1.0mmol/L.

Effect of heptanol superfusion on ionic currents

The effect of heptanol on cardiac gap junction currents, as well as in the amplitude of Nav1.5 and Kir2.1-2.3 currents was assessed in the presence and absence of RXP-E in the internal pipette solution. For gap junction channels, heptanol superfusion was initiated 10 minutes after patch break and continued for a maximum of 10 minutes. The concentration of heptanol was 1-2 mmol/L in all experiments.
**Effect of intracellular acidification on junctional currents**

Experiments were conducted to assess the effect of RXP-E on acidification-induced uncoupling of NRVM’s. To acidify the intracellular space, the pipette solution was prepared as described above but HEPES was replaced by MES (10 mmol/L). The final pH of the internal pipette solution was 6.2. Gj was measured immediately after patch break and for the following 10 minutes. All cell pair showed an initial Gj value of <25 nS.

**Optical Mapping Experiments**

*Optical recording of electrical activation*

Optical mapping was used to study action potential propagation in monolayers of rat neonatal ventricular myocytes. Measurements were performed in cells kept for 3-4 days in culture. The preparations were stained for 15 minutes with the voltage sensitive dye (di-8-ANEPPS (40μmol/L; Molecular Probes) in order to optically determine the characteristics of impulse propagation. A custom-made recording setup was used to assess changes in fluorescence that correspond to transmembrane voltage changes. Details have been previously described. Culture dishes were placed on a heating chamber and continuously superfused with HBSS without bicarbonate (Sigma) containing: (mmol/L) CaCl₂ 1.6, KCl 5.4, MgSO₄ 0.8, KH₂PO₄ 0.4, NaHCO₃ 4.2, NaCl 136.9, NaHPO₄ 0.3, D-Glucose 5.5 and HEPES 10; pH 7.4 (NaOH). All experiments were carried out at 35° C. Repetitive stimuli (duration: 5 ms; strength: twice diastolic threshold) were applied at 1 Hz. The changes in fluorescence corresponding to transmembrane voltage were recorded by an 80x80 pixel CCD camera (SciMeasure
Analytical Systems, Inc., Decatur, GA) in 2-second movies at 500 frames per second (LabWindows Acquisition). Illumination was restricted to the time of recording to minimize the effects of phototoxicity limiting the exposure times to a range of 10 - 50 seconds. The signals were amplified, filtered and digitized for off-line analysis. No electromechanical uncouplers were used for motion suppression. Recordings were made with a 20x, 0.75 numerical aperture objective, which permitted the measurement of impulse propagation characteristics over a distance of 750 µm with a spatial resolution of 50 µm.

After staining, preparations were superfused with HBSS (Sigma) containing (mmol/L) 137 NaCl, 5.4 KCl, 1.3 CaCl₂, 0.8 MgSO₄, 4.2 NaHCO₃, 0.5 KH₂PO₄, 0.3 NaH₂PO₄, and 10 HEPES, which was titrated to pH 7.40 with 1 mol/L NaOH and contained 1% serum. Preparations were stimulated at 2 Hz with rectangular pulses (duration 1 ms; suprathreshold intensity) for ≥10 s before a given optical recording. All experiments were performed at 36°C.

Cells were loaded with CTP-RXP-E or CTP-Scr-RXP-E following the protocol previously described. These experiments allowed us to define quantitatively the ability of RXP-E to preserve action potential propagation under conditions likely to induce, among other effects, the loss of connexin-mediated electrical coupling.

*Heptanol experiments*
Heptanol superfusion was initiated 3 minutes after successful pacing of preparations was established. The concentration of heptanol was either 1 or 2mmol/L in all experiments, and was prepared in HBSS solution described above. Heptanol perfusion was continued for 5 minutes, followed by washout. All monolayers demonstrated complete washout of heptanol, as established by return to paced propagation.

**Intracellular acidification in monolayers of NRVM’s**

Intracellular acidification was induced by modifying the proton concentration of a Na-acetate superfusing solution. The pH-adjusted sodium acetate solutions contained (in mmol/L) 72 NaCl, 5.4 KCl, 1.3 CaCl2, 0.8 MgSO4, 4.2 NaHCO3, 0.5 KH2PO4, 0.3 NaH2PO4, 65 NaAcetate and 10 HEPES (pH=7.4). For the low pH sodium acetate solution (pH=6.2), HEPES was replaced by MES (10 mmol/L). The pH of the control sodium acetate solution was checked prior to use and was 7.4.

**Determination of intracellular pH**

The proton-sensitive fluorophore BCECF was used to estimate the value of intracellular pH in our recording conditions, as shown in Online Figure I. First, ratiometric measurements were obtained from BCECF in solution. The fluorophore was diluted in an HBSS solution containing (in mmol/L) 137 NaCl, 5.4 KCl, 1.3 CaCl2, 0.8 MgSO4, 4.2 NaHCO3, 0.5 KH2PO4, 0.3 NaH2PO4, and 10 HEPES. The pH of the solution was adjusted to various values, as measured by a pH-meter. The emission ratios obtained from BCECF in the solution of known pH were then recorded using a Synergy HT Multi-Detection Microplate Reader (Synergy HT, Bio-Tek, Winooski, VT). The fluorophore
was excited at 440/30 and 485/20nm and emission recorded at 528/20nm using a Synergy HT Multi-Detection Microplate Reader (Synergy HT, Bio-Tek, Winooski, VT). In parallel, NRVM’s were treated with BCECF-AM (30 minutes of incubation at 37ºC in HBSS solution kept at pH 7.4). The fluorescent properties of BCECF in the intracellular space of NRVM’s were recorded by a Synergy HT Multi-Detection Microplate Reader (Synergy HT, Bio-Tek, Winooski, VT).

Data analysis of optical mapping recordings

Optical raw data were digitally low-pass filtered at corner frequencies (f₀) ranging from 0.1 to 0.5 kHz, and action potential amplitudes (APA) were set to 100%. Assuming an average APA of 100 mV, the scaled values given as %APA translate directly into millivolts. Local activation times for each recording site were determined as described before and conduction velocities (v; mm/s) were calculated from the slope of a linear least square fit of activation times along the preparation. Values for maximal upstroke velocities (dV/dt_max) were calculated in relation to %APA and are given as %APA/ms. Under the assumption of an average APA of 100 mV, %APA/ms corresponds to V/s.

Statistical analysis

All experimental results were presented as mean ± SEM. Data were compared using ANOVA or student’s t-test to determine the significance of differences between the means, as appropriate. A value of P ≤ 0.05 was used as the criterion for significance.

RESULTS
Intracellular acidification in monolayers of NRVM’s

A calibration curve of dye emission (ratio of emission intensities obtained at the two different excitation wavelengths) as a function of pH was generated (see Online Figure I). In parallel, NRVM’s were treated with BCECF-AM. Excitation and emission wavelengths were the same as those used for the calibration curves in solution. pH of the external solution was modified using a NaAcetate-based buffer, as described above in Materials and Methods. A calibration curve of the emission ratios obtained for various pH values is shown as black triangles in Online Figure I. Based on these results, the composition of the bathing solution was adjusted for acidification-induced uncoupling experiments to cause a drop in intracellular pH to 6.2 (see above).

Timed Transduction Transfer of RXP-E to the Intracellular Space in Multicellular Preparations (NRK’s)

Monolayers of NRK’s were exposed to synthetic fusion peptides containing a cytoplasmic transduction peptide (CTP512 domain; sequence: YGRRARRRRRR\(^2\)). A cysteine residue at the C-terminus allowed us to conjugate FITC to the peptides for detection by fluorescence microscopy. Synthetic peptides were thus created for both RXP-E, and its scrambled version. Online Figure II shows an example of peptide transfer into neonatal NRK’s. The image in the top panels 2A were obtained from cells maintained in control conditions (no CTP-RXP-E). The images in the middle panels were obtained from cells incubated with CTP-RXP-E (0.1mmol/L) for 2 hours. The images in the bottom panels was obtained from cells previously incubated with CTP-RXP-E (0.1 mmol/L) for 4 hours. For these images, nuclei were stained with Hoechst33528 (blue) for
easier identification of the individual cells. There was a clear absence of FITC signal in the control; however, intense signal was recorded from all cells that had been pre-incubated with CTP-RXP-E. These experiments were repeated, this time with the scrambled CTP-RXP-E, as shown in Online Figure II-B. Top images correspond to cells that were not presented with the peptide. Middle panels were recorded after cells were incubated for 2 hours with a solution containing 0.1 mmol/L of scrambled CTP-RXP-E, and bottom panels were recorded after the cells were incubated for 4 hours with a solution containing 0.1 mmol/L of scrambled CTP-RXP-E. Left panels show the overlay of the blue (nuclei) and green (fluorescein-labelled RXP-E) signals. Middle panels show the DIC image of the NRK monolayers. Right panels show the fluorescein peptide signal alone. Clearly, a bright green fluorescein signal was detected from all cells within the field, indicating successful translocation of the peptide. Signal was detected up to 18 hours after peptide incubation (not shown). Further confirmation of successful transfer into near-100% of the cells was obtained by flow-cytometric detection of the FITC signal from NRK cells (not shown). Overall, the data show that CTP allows for efficient transfer of FITC-labeled peptides into the cytoplasm of NRK’s, which endogenously express Cx43. We used this translocation protocol to test for the effect of RXP-E on action potential propagation in monolayers of NRVM’s.
REFERENCES


ONLINE FIGURE LEGENDS

Online Figure I. Intracellular pH Calibration Curve. The spectral ratio (excitation intensity @ 440/30 and 485/20nm, emission at 528/20nm) is plotted against pH. In parallel, NRVM’s were treated with BCECF-AM. Excitation and emission wavelengths were the same as those used for the calibration curves in solution. Two calibration curves were generated: one for BCECF dissolved in solutions buffered at various pHs (red triangles), and another one for BCECF-AM in NRVM’s (black triangles). Given the pH dependence of the emission ratios, measurements of pH below 5.8 were not taken into account.

Online Figure II. Timed Transduction and Immunolocalization of RXP-E in Normal Rat Kidney Cells (NRK’s) that were either kept in control conditions (top panels), treated with fluoresceinated CTP-RXP-E (green in bottom panels A) or with a “scrambled” construct (bottom panels B). Columns on the left display merged images of nuclei-labeled cells with fluorescein-labeled peptides; columns in the middle display DIC images. Intracellular distribution of fluorescein-labeled constructs after CTP-mediated transfer into NRK’s is shown in green in the third columns. Nuclei were labeled with Hoechst33528.
ONLINE FIGURE I

- ▲ 485:440 ratio in myocytes
- ▼ BCECF acid alone

Normalized fluorescence intensity vs. pH

5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5
ONLINE FIGURE II

A) RXP-E

Untreated

2 hour treatment

4 hour treatment

B) Scr- RXP-E

Untreated

2 hour treatment

4 hour treatment