Loss of Plakophilin-2 Expression Leads to Decreased Sodium Current and Slower Conduction Velocity in Cultured Cardiac Myocytes

Priscila Y. Sato, Hassan Musa, Wanda Coombs, Guadalupe Guerrero-Serna, Gustavo A. Patiño, Steven M. Taffet, Lori L. Isom, Mario Delmar

Rationale: Plakophilin-2 (PKP2) is an essential component of the cardiac desmosome. Recent data show that it interacts with other molecules of the intercalated disc. Separate studies show preferential localization of the voltage-gated sodium channel (Na\textsubscript{v}1.5) to this region.

Objective: To establish the association of PKP2 with sodium channels and its role on action potential propagation.

Methods and Results: Biochemical, patch clamp, and optical mapping experiments demonstrate that PKP2 associates with Na\textsubscript{v}1.5, and that knockdown of PKP2 expression alters the properties of the sodium current, and the velocity of action potential propagation in cultured cardiomyocytes.

Conclusions: These results emphasize the importance of intermolecular interactions between proteins relevant to mechanical junctions, and those involved in electric synchrony. Possible relevance to the pathogenesis of arrhythmogenic right ventricular cardiomyopathy is discussed. (Circ Res. 2009;105:523-526.)

Key Words: plakophilin-2  ■ intercalated disc  ■ arrhythmogenic right ventricular cardiomyopathy  ■ cardiac desmosomes

A high-resolution image of the site of end–end contact between cardiomyocytes reveals an electron-dense organization called “the intercalated disc.” Its classic definition involves 3 structures: desmosomes and adherens junctions, providing mechanical coupling; and gap junctions, allowing electric/metabolic synchronization between cells. Recent studies show that other molecules, not directly involved in intercellular coupling, also reside preferentially at the intercalated disc. Among them is Na\textsubscript{v}1.5, the major α subunit of the cardiac sodium channel. Here, we ask whether Na\textsubscript{v}1.5 and the desmosomal protein plakophilin-2 (PKP2) coexist in the same molecular complex and whether loss of PKP2 expression affects (1) the amplitude and kinetics of the sodium current and (2) action potential propagation in a monolayer of cardiomyocytes. Our data demonstrate a functional crosstalk between a protein defined in the context of intercellular junctions (PKP2) and another protein that is fundamental to the electrical behavior of the single myocyte.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results and Discussion
Initial experiments aimed at whether Na\textsubscript{v}1.5 and PKP2 are present in the same molecular complex. A recombinant protein formed by glutathione S-transferase (GST) concatenated to the head domain of PKP2 (GST-PKP2-H) was bound to glutathione-Sepharose beads, and presented to an adult heart lysate. The precipitate was immunoblotted for Na\textsubscript{v}1.5 (Figure 1A). GST, bound to beads but not PKP2-concatenated, was used as control (first 2 lanes). PKP2-H pulled down Na\textsubscript{v}1.5 from heart lysate, suggesting a physical interaction, direct or indirect, between the 2 proteins. Interaction between Na\textsubscript{v}1.5 and native PKP2 was tested by coimmunoprecipitation. Na\textsubscript{v}1.5 was precipitated by antibody-coated beads (Figure 1B, top). Western blots demonstrated PKP2 in the precipitate (bottom). Immunofluorescence studies in freshly dissociated myocytes showed colocalization of Na\textsubscript{v}1.5 with PKP2 at the site originally occupied by the intercalated disc (Figure 1C and 1E). This subcellular distribution shifted with time in culture. After 6 days (time frame required for silencing experiments; see below), the density of immunoreactive signal at the cell ends decreased, coincident with the appearance of punctate immunoreactive spots on the lateral membranes, better resolved for PKP2 staining (Figure
1D and 1F). Na\textsubscript{1.5} staining was less sharply defined, making it difficult to establish the extent of colocalization, though a coincidence of signals could be found (Figure 1F). Protein relocalization did not affect the kinetics of $I_{\text{Na}}$ (Online Figure 1). Next, we assessed the properties of $I_{\text{Na}}$ as a function of PKP2 expression.

PKP2 level was decreased (80% from control) by use of short hairpin (sh)RNA. In a first group, a mixture of 4 separate oligonucleotides, designed for selective PKP2 mRNA knockdown (KD) was introduced into myocytes using a transfection reagent (Dharmafect-1). Alternative nontargeted siRNA was used as control (KD; details online). For every experiment, PKP2 silencing was confirmed by immunoblot (Online Figure II). Sodium currents were recorded by voltage clamp (Online Data Supplement). Figure 2A shows an example of currents obtained from cells either untreated (UNT) or treated with an oligonucleotide mixture. Composite data are displayed in Figure 2B through 2D. Treatment with control KD constructs did not affect current parameters. However, a decrease in peak current density (2B), a shift in voltage dependence of steady-state inactivation (2C), and a prolongation of time-dependence of recovery from inactivation (2D) were observed in PKP2-silenced cells.

To reduce the possibility of off-target effects, additional experiments were conducted where a single silencing construct, of different sequence than those in the KD mixture and yet selective for PKP2, was transferred into myocytes via adenoviral infection (shRNA). Results were compared with

Non-standard Abbreviations and Acronyms

<table>
<thead>
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<tr>
<td>Cx</td>
<td>connexin</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>KD</td>
<td>plakophilin-2 knockdown</td>
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<tr>
<td>\text{\textit{\textsc{\textsuperscript{\textdagger}}}KD}</td>
<td>nontargeted knockdown</td>
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<td>Na\textsubscript{1.5}</td>
<td>sodium \textalpha subunit 1.5</td>
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<td>plakophilin 2</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>untreated</td>
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Figure 1. A, Head domain of PKP2 (PKP2-H; amino acids 1 to 335) concatenated to GST pulled down Na\textsubscript{1.5} from adult heart lysate. Top, Western blot for Na\textsubscript{1.5}. Bottom, Coomassie blue staining of parallel gel from same samples demonstrating abundance of GST (first 2 lanes) or GST-PKP2-H (GST concatenated to the head domain of PKP2) (lanes 3 and 4). Symbols + and − indicate exposure or not, respectively, of heart lysate to GST proteins. B, Coimmunoprecipitation of PKP2 with Na\textsubscript{1.5}. Immunoblots (IB) for Na\textsubscript{1.5} (top) and PKP2 (bottom) from samples exposed to protein A/G beads coated with rabbit IgG (negative control) or Na\textsubscript{1.5} antibody. Sample not exposed to heart lysate was used as control. Heart lysate (1:12 dilution, top; 1:6 dilution, bottom) ran as positive control. Numbers at right, molecular weights (kDa). C and E, Immunolocalization of Na\textsubscript{1.5} (red) and PKP2 (green) in isolated adult cardiocytes within 24 hours after dissociation. D and F, Day 6 after dissociation. Calibration bars: 20 μm.
those obtained from cells untreated (UNT), or treated with a nonsilencing construct (ΦshRNA). These results (Online Figures III and IV), consistently demonstrated that loss of PKP2 expression associated with reduced peak current density, negatively shifted voltage dependence of inactivation, and prolonged recovery from inactivation of sodium currents. Overall, we show that loss of PKP2 expression affects sodium current properties, regardless of the experimental procedure to achieve PKP2 knockdown.

Previously, we demonstrated that loss of PKP2 expression in neonatal rat ventricular myocytes causes connexin (Cx)43 remodeling and an $\approx 60\%$ decrease in cell–cell dye coupling. We predicted that, combined with the change in sodium current function reported here, loss of PKP2 expression would significantly affect propagation properties in cardiomyocytes. Optical mapping experiments in monolayers of neonatal rat ventricular myocytes revealed that loss of PKP2 expression caused slowing of action potential propagation, rate-dependent activation failure, and arrhythmic behavior. Results are shown in Figure 3. Cells were treated with adenovirus containing either the ΦshRNA or the PKP2-shRNA constructs. We chose this method of silencing for consistency with our previous studies. Cells were paced at constant frequency from a stimulating electrode in the center of the dish. Only quiescent preparations were used for the study. Care was taken to minimize the presence of fibroblasts. Average conduction velocity in control monolayers paced at 1 Hz was 24.55 cm/sec ($n=9$). Examples of isochrone maps from preparations treated with either ΦshRNA or PKP2-shRNA are shown in Figure 3A and 3B, respectively. A Western blot demonstrating loss of PKP2 is shown in Online Figure V. Loss of PKP2 expression resulted in significant decrease in conduction velocity, as shown by the crowding of isochrone lines (Figure 3B). A plot of average conduction velocity as a function of pacing frequency under control conditions (black) or after treatment with either nonsilencing (ΦshRNA; red) or silencing PKP2-shRNA (blue), is shown in Figure 3C. Consistent with the increase in sodium current amplitude in single ventricular myocytes after adenoviral treatment (Online Figure IV), we observed an increase in average conduction velocity in monolayers treated with ΦshRNA virus. In contrast, we observed a large decrease in conduction velocity in preparations treated with PKP2-shRNA. In addition, a 1:1 stimulus:response capture at pacing frequencies higher than 8 Hz was possible in a fraction of preparations either untreated, or treated with ΦshRNA, whereas we failed to obtain 1:1 capture in all PKP2 knockdown monolayers. Instead, we observed nonpaced sustained reentrant activity within the preparation (see phase map in Figure 3D). This is the first report demonstrating a link between loss of PKP2 expression, impaired cardiac propagation, and loss of electric synchrony.

The relative contributions of decreased junctional conductance versus sodium current on the observed changes in conduction velocity remain undefined. Cable equations predict a decrease in conduction velocity for an increase in axial resistivity. Yet, studies in genetically modified animals indicate that a 50% decrease in Cx43 content, and a concurrent decrease in electrical coupling, are not enough to significantly decrease conduction velocity, perhaps because of the relatively large contribution of myoplasmic resistivity to the total internal resistance (see Beauchamp et al and references within). Likely, the observed decrease in conduction velocity resulting from loss of PKP2 expression is consequent to both decreased electrical coupling and decreased sodium current, although dissecting the relative contributions will require further experimentation. In addition, our present results do not discard a possible effect of PKP2 silencing.
on membrane potential; membrane depolarization could be another factor, leading to slow conduction velocity in these preparations. Future experiments will be necessary to address this possibility.

Our data suggest a link between three components of the intercalated disc: desmosomes, gap junctions, and the voltage-gated sodium channel Na\textsubscript{v}1.5 complex. Crosstalk between PKP2 and Cx43 and coprecipitation of Cx43 and Na\textsubscript{v}1.5 have been demonstrated.\textsuperscript{2,3} Yet, this is the first evidence of association between PKP2 expression and function of the sodium channel complex and the first demonstration that molecular integrity of mechanical junctions can be relevant to ion channel function. We further demonstrate that loss of PKP2 expression leads to slow conduction velocity and propensity for frequency-dependent arrhythmias. The mechanisms by which this triad (desmosomes, gap junctions, sodium channels) is linked remain unknown. We emphasize that, because of the time required for PKP2 silencing, patch-clamp experiments were carried out within a time frame that overlaps with intercalated disc remodeling.\textsuperscript{6} As such, the PKP2-Na\textsubscript{v}1.5 interaction did not occur within the confines of the intercalated disc. Yet, it was only after PKP2 knockdown that the change in \(I_{\text{Na}}\) properties became apparent. The latter indicates that during intercalated disc remodeling, PKP2 retains a physical interaction, direct or indirect, with Na\textsubscript{v}1.5, which carries a functional effect. We speculate that PKP2 may be important in the association of Na\textsubscript{v}1.5 with its noncovalently linked \(\beta\) subunit(s), and/or the cytoskeletal adaptor protein, ankyrin-G; alternatively (or in addition), a direct PKP2-Na\textsubscript{v}1.5 association with impact on the biophysical properties of the channel cannot be discarded. Furthermore, whether these interactions occur in the heart in vivo, and/or in the confines of the intercalated disc of an intact heart, remains to be demonstrated. Of relevance, mutations in PKP2 are linked to arrhythmogenic right ventricular cardiomyopathy, an inherited disease associated with ventricular arrhythmias and sudden death in the young. The mechanisms by which mutations in mechanical junction proteins affect electric synchrony remain undefined. Whether the results presented here, in an in vitro system, bear relevance to the pathogenesis of arrhythmogenic right ventricular cardiomyopathy is a tantalizing hypothesis for future investigation.

Sources of Funding

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Disclosures

None.

References

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Preparation of recombinant GST-fusion proteins

Recombinant GST fusion proteins were generated in BL-21 E. Coli. Production of a protein fragment corresponding to the head domain of PKP2 (GST-PKP2H; amino acids 1-335 of PKP2a) followed the methods described elsewhere. Briefly, crude lysates were prepared by sonication of one liter bacterial pellets in 20 ml of PBS protein purification buffer containing (in mM): NaCl, 138; KH₂PO₄, 1.5; Na₂HPO₄, 8; KCl, 2.7 (pH 7.4); DTT, 1.0; PMSF, 1.0 as well as NP-40 (1%), followed by centrifugation at 12,000 rpm for 25 minutes. A total of 200 µl of crude bacterial lysate was mixed with 50 µl of Glutathione Sepharose 4B (GE Healthcare) in 1 ml of lysis buffer overnight at 4° C. Unbound proteins were removed by washing three times with heart lysis buffer (in mM): Tris-HCl, 50; NaCl, 50; MgCl₂, 2.0; EDTA, 2.0; NaF, 50; NaVO₃, 1.0; as well as 1% β-mercaptoethanol, 1% TritonX-100, and 1X Complete protease inhibitor (Roche Diagnostics). The solution was buffered to pH 7.4.

GST pulldown assay

Fresh rat heart lysates were prepared by homogenizing tissue on ice in TE solution (50mM Tris-HCl pH 7.4, 10mM EGTA with 1X Complete Protease inhibitors from Roche Diagnostics). The crude heart lysate was centrifuged at 3,500 rpm for 10 minutes. The remaining pellet was resuspended with TE, homogenized and centrifuged again as above. The supernatants were pooled and centrifuged at 40,000g for 30 minutes at 4° C. The pellet was resuspended with a lysis buffer containing 150mM NaCl, 50mM Tris-HCl pH 7.4, 1mM EDTA, 1% TritonX-100, 0.2mM DTT, 1mM PMSF, 50mM NaF, 1mM NaVO₃ and 1X Complete Protease Inhibitor. One ml of supernatant was exposed to 250 µl of glutathione sepharose beads for 30 minutes at 4° C. After centrifugation at 2,500 rpm for 5 minutes, approximately 15 mg precleared heart lysate (+) was incubated with bound GST fusion proteins in 1 ml of lysis buffer for 90 minutes, rocking at 4° C. 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, heated at 37° C for 10 minutes and probed by standard western blot protocol. The same samples were run on a separate gel and stained (Coomassie) to assess total protein content.

Co-immunoprecipitation assay

Fresh rat heart lysates were prepared by homogenizing tissue on ice in a lysis buffer containing 50mM NaCl, 20mM Hepes, 1% TritonX-100, 0.5% NP-40, 1mM EDTA, 1mM PMSF, 1mM NaVO₃, 50mM NaF and 1X Complete Protease Inhibitor. The crude heart lysate was centrifuged at 3,500 rpm for 15 minutes. To preclear the lysate, one ml of supernatant was exposed to 30 µl of Protein A/G Agarose beads (Santa Cruz Biotechnology) for 30 minutes at 4° C. After centrifugation at 3500 for 5 minutes, a protein concentration assay (Biorad) was performed. Immunoprecipitations were carried out in a volume of 1ml for 400 µg of total protein. A total of 15 µg of rabbit anti-NaV1.5 (Sigma #S0819) were added to the precleared lysates and the samples were incubated for 1 hour, rocking gently at 4° C. A total of 25 µl of protein A/G agarose beads were added, and the samples were incubated on a rocker for 1 hour at 4° C. The antibody-protein A/G agarose complex was spun down at 2500 rpm for 5 minutes and washed with lysis buffer three times. After the final spin, 100ul of lysis buffer and 20 µl of 6X
Laemmli buffer were added. 60 µl of each sample were transferred to a new tube. One set of tubes, to be probed for PKP2, was boiled for 10 minutes. The other set of tubes, to be probed for NaV1.5, was heated at 37° C for 10 minutes. Fifteen µl of samples were subjected to SDS-PAGE and co-immunoprecipitation was tested by western blot analysis using a rabbit Nav1.5 antibody (Alomone #ASC-005 ) As negative controls, two additional reactions were carried out in parallel: one with the same amount of pre-cleared homogenate using an equivalent amount of Rabbit IgG bound to the protein A/G agarose beads and the other one omitting the precleared heart lysate. As a positive control, a diluted sample of the heart lysate was run on the same gel.

**Cell culture**

**Neonatal rat ventricular myocyte (NRVM) cultures**

Cultures of neonatal rat ventricular myocytes were prepared as previously described. Briefly, hearts from 1-2 day old Sprague-Dawley rats (Charles River, MA) were isolated, finely minced, and enzymatically digested. After dissociation, cells were resuspended in supplemented M199 containing 10% FBS and 15 µM vitamin B12. A 2-hour preplating step allowed for the separation of myocytes from other cardiac cells. Cells were then counted and resuspended in supplemented M199 containing 10% Horse serum and 15 µM vitamin B12. Bromodeoxyuridine was added to the growth media to reduce cellular proliferation. Collagen type IV was used to pre-coat 35mm dishes and cells were plated at a density of 1.0 x 10⁶ per monolayer. Media change was performed every 24 hours for the first 48 hours and every 48 hours thereafter. Adenoviral infection was performed concurrent with the first media change.

**Rat Adult Ventricular Myocytes**

Ventricular myocytes were isolated using previously established protocols. Briefly, Sprague-Dawley rats (~200g) were injected with 1500 U/Kg heparin and 162.5U/Kg nembutal. Removed hearts were isolated, finely minced, and enzymatically digested. After dissociation, cells were resuspended in supplemented M199 containing 10% FBS and 15 µM vitamin B12. A 2-hour preplating step allowed for the separation of myocytes from other cardiac cells. Cells were then counted and resuspended in supplemented M199 containing 10% Horse serum and 15 µM vitamin B12. Bromodeoxyuridine was added to the growth media to reduce cellular proliferation. Collagen type IV was used to pre-coat 35mm dishes and cells were plated at a density of 1.0 x 10⁶ per monolayer. Media change was performed every 24 hours for the first 48 hours and every 48 hours thereafter. Adenoviral infection was performed concurrent with the first media change.

**Western blots**

**Detection of proteins in adult hearts**

Samples were fractionated on an 8-16% Tris-Glycine gradient gel (Invitrogen), transferred to nitrocellulose membranes overnight at 62mA, in a transfer buffer (25mM Tris Base, 192mM Glycine, 0.005% SDS, 10% methanol) and blocked with blocking buffer (1% non-fat dry milk, 0.05% Tween-20 in PBS, pH 7.4) for one hour at room temperature or overnight at 4° C. Membranes were then incubated in primary antibodies overnight at 4° C, followed by incubation in secondary antibody for 45 minutes at room temperature. Signal was detected by Chemiluminescence (Pierce). Primary antibodies
used were: mouse anti-PKP2a and b that recognized an epitope on the CT domain of the protein (Biodesign, 1:100), rabbit anti-Nav1.5 (Alomone #ASC-005, 1:200), and rabbit anti-Nav1.5 (Sigma #S0819, 1:500). Secondary antibodies used were: anti-mouse IgG horseradish peroxidase (Sigma #A-4416, 1:5000) and anti-rabbit IgG horseradish peroxidase (Sigma #A-6667, 1:5000). All primary and secondary antibodies were diluted in blocking buffer.

Protein detection in neonatal rat ventricular myocytes (NRVMs).
Western blot for neonatal rat ventricular myocytes in culture followed previously described methods. Briefly, cells were harvested with cold PBS and centrifuged at 13000 rpm for 10 min. Cell pellets were resuspended in 150 µl triton X-100 lysis buffer, briefly sonicated, and incubated on ice for 30 min. A second centrifugation at 13000 rpm for 10 min sedimented cellular debris. After centrifugation, 10 µl of 6XSSB was added to 50 µl of sample and run in a 8-16% tris-glycine gradient gel, transferred to nitrocellulose membrane, and blocked for 1h at room temperature (blocking solution contained 5%milk in T-PBS). Primary antibody was incubated overnight at 4°C and followed by secondary antibody (anti-mouse/rabbit HRP, Sigma) for 45 min at room temperature. Primary antibodies used were: mouse monoclonal anti-PKP2a and 2b (Biodesign), mouse monoclonal anti-beta actin (Sigma), and mouse monoclonal GAPDH (RDI).

PKP2 Silencing
Transfer of oligonucleotides by adenoviral infection
Cardiac myocytes were infected 24 hours post-seeding with adenovirus containing an shRNA for PKP2 (GAAACTCTACCAGATTTGGTTTCTA). An alternative adenovirus construct containing an shRNA shown not to interfere with PKP2, was used as control for infection. The control construct, ΦshRNA, had the following sequence: GAGGTAAGCTGAACTGAATG. Experiments were conducted at 100 MOI for both viruses. All experiments were carried out 5 days after infection. Additional details can be found in Oxford et al.

Non-viral transfection of oligonucleotides
Small interference RNAs (siRNAs) specific for rat PKP2 were purchased from Dharmacon (Chicago, USA). Cardiac myocytes were transfected 24 hours post-seeding with ON-TARGETplus SMARTpool siRNA specific for rat PKP2 (CCAAAUCUGUAACGAAUA, CCACAUCGGUAGCUCGCAU, GAGGAAUUUGUCACGGAAU, AGUUUAAGAAGACGGACUU). Control for transfection was provided by ON-TARGETplus Non-targeting siRNA. All transfections were performed as per manufacturer’s protocol, using Dharmafect 1.

Electrophysiology
Whole Cell cardiac sodium current (I Na) was recorded from adult rat ventricular myocytes after a period of 5 - 6 days in culture. Recorded cells were rod-shaped and appeared striated. All recordings were conducted at room temperature, in a low-sodium extracellular solution containing (in mM): NaCl, 20; MgCl2, 1; CaCl2, 1; CdCl2, 0.1; HEPES, 20; CsCl, 117.5; glucose, 11. The pipette solution contained (in mM): NaCl, 5; CsF, 135; EGTA, 10; MgATP, 5; HEPES, 5. Recordings were performed using an Axopatch-200B Amplifier (Molecular Devices Sunnyvale, CA) and data acquisition and analysis were performed utilizing pClamp10.2 software (Molecular Devices Sunnyvale, CA). Pipette resistances ranged from 2-3 MΩ. Access resistance was compensated to 1-2 MΩ. Input resistance was 500 MΩ to 1 GΩ. To characterize the voltage dependence of the peak I Na, single cells were held at -120mV, and 200 msec voltage
steps were applied from -90 to +30mV in 5 mV increments. Interval between voltage steps was 3 sec. Voltage-dependence of inactivation was assessed by holding cells at various potentials from -130 to -40 mV followed by a 30msec test pulse to -40mV to elicit $I_{Na}$. Recovery from inactivation was studied by holding cells at -120 mV and applying two 20-msec test pulses (S1, S2) to -40mv separated by increasing increments of 2 msec to a maximum S1-S2 interval of 80 msec. The S1-S1 interval was kept constant at 3 sec. All data are presented as mean value ± S.E.M.

Optical Mapping
Optical Mapping measurements were conducted in monolayers at day 6 post-dissociation. Monolayers were stained with a voltage-sensitive dye, di-8ANEPPS (Molecular Probes), at 40 µM for 15 minutes. A custom-made setup was used to observe fluorescence changes which were directly proportional to transmembrane voltage changes. Cultures were maintained at 37°C and quiescent preparations were paced using a bipolar electrode. Pacing frequencies started at 1-2 Hz and were modified, 1-2 Hz at a time, in ascending progression until loss of 1:1 capture. Movies were obtained at 200 or 500 frames/s using an 80x80 pixel CCD camera (SciMeasure Analytical Systems, GA). Signals were filtered and amplified for offline analysis. Conduction velocity analysis was performed using a custom-made software, SCROLL. Phase maps were constructed by performing a Hilbert transformation. Details are described elsewhere.4

Statistical Analysis
Statistical analysis was performed using one-way ANOVA followed by post-ANOVA tests (Tukey or Bonferroni, as noted in the description of results). SPSS 16.0 (Statistical Package for the Social Sciences) was used to conduct all the statistical tests. Statistical consultation was provided by the University of Michigan-CSCAR (Center for Statistical Consultation and research). Comparisons between UNT and ФSHRNA groups at 8, 9, and 10 Hz in Figure 3 of the manuscript were conducted by unpaired Student’s t test, given the lack of data in the shRNA group.
Online Figure I. Sodium current parameters recorded from adult rat ventricular myocytes recorded within 24 hours (D1; black diamonds; n=5) or six days (D6; black circles; n=5) after dissociation. Data labeled “ΦKD” (red; n=6) were collected six days after dissociation, and five days after the cells were transfected with the non-targeted siRNA that was used as a control (see also Figure 2 of the manuscript). Voltage clamp protocols were the same as for Figure 2 of the manuscript, and Figure 4 of this supplement. As opposed to the situation where PKP2 expression was knocked-down, we did not observe either a shift of the voltage dependence of steady-state inactivation (panel A) or a change of the time dependence of recovery from inactivation (panel B) associated with either time in culture, or treatment with control oligonucleotides.

Online Figure II. Western blot of PKP2 obtained from adult rat ventricular myocytes kept in culture for 5-6 days of either untreated (UNT), or treated with an oligonucleotide to knockdown (KD) PKP2 expression. Lane labeled ΦKD corresponds to cells treated with an oligonucleotide that did not affect PKP2 expression. Oligonucleotides were transfected into the cells using DharmaFect-1. GAPDH was used as a loading control. Small numbers on the side indicate the position of estimated molecular weight markers.

Online Figure III. Western blot of PKP2 obtained from adult rat ventricular myocytes kept in culture for 5-6 days of either untreated (UNT), or treated with shRNA to reduce PKP2 expression. Lane labeled ΦshRNA corresponds to cells treated with an oligonucleotide that did not affect PKP2 expression. Oligonucleotides were delivered into the cells by adenoviral infection. GAPDH was used as a loading control. Small numbers on the side indicate the position of estimated molecular weight markers.

Online Figure IV. Sodium current parameters recorded from adult rat ventricular myocytes exposed to PKP2 shRNA. A silencing construct (shRNA) or a construct that failed to silence the PKP2 protein (ΦshRNA) were introduced into single myocytes via adenoviral infection. Examples of sodium currents obtained in control conditions (untreated cells), as well as currents recorded from either shRNA or ΦshRNA treated cells, are shown in Panel A. Average peak current-voltage relations for all three conditions are presented in Panel B. An increase in peak current amplitude was detected in cells treated with the non-silencing virus, perhaps suggesting an effect consequent to the interaction of the virus with its receptor in the myocyte (see, e.g., ref6). It is interesting to note that the adenovirus receptor preferentially localizes to the intercalated disc.6 Whether binding of the virus to its receptor brings about functional changes in $I_{Na}$, remains to be determined. However, it is important to emphasize that the change in $I_{Na}$ caused by exposure to the virus is in the opposite direction to that observed after silencing and as such, it does not obscure the effect of the loss of PKP2 expression. More importantly, the amplitude of the peak voltage-gated sodium current in cells treated with the PKP2 silencing construct was significantly decreased when compared to control (p< 0.001). This result associated with a shift of the voltage dependence of steady-state inactivation toward the more negative values (panel C), as well as a prolongation of the time dependence of recovery from inactivation, from 5.03/20.9 ms (t1/t2) in untreated, and 2.91/13.03 ms (t1/t2) in ΦshRNA-treated cells, to 24.2 ms (t1; best fit by a single exponential) in those cells where PKP2 expression was knocked down (panel D). The changes in the sodium current resulting from loss of PKP2 expression were consistent with those observed when PKP2 was reduced using non-viral technology and different oligonucleotide sequences (Figure 2 in the manuscript).
Online Figure V. Western blot of PKP2 obtained from neonatal rat ventricular myocytes kept in culture for 5-6 days of either untreated (UNT), or treated with shRNA to reduce PKP2 expression. Lane labeled $\Phi$shRNA corresponds to cells treated with an oligonucleotide that did not affect PKP2 expression. Oligonucleotides were delivered into the cells by adenoviral infection. Beta-Actin was used as a loading control. Small numbers on the side indicate the position of estimated molecular weight markers. Each condition tested from four separate monolayers.
REFERENCES


Online Figure II

IB: PKP2

IB: GAPDH
Online Figure III

IB: PKP2

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<td>96</td>
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IB: GAPDH

| 51.5 | 35.3   |

shRNA
A. INa Inactivation

B. I\textsubscript{Na} I-V Relationship

C. Recovery from Inactivation

Online Figure IV

CTRL

ØshRNA

shRNA

20 pA/pF

10 msec

$\phi$shRNA

INa

$\frac{I}{I_{max}}$

Voltage (mV)

Normalized $I_{Na}$

Time (msec)

shRNA

$\phi$shRNA

UNT

$V_m$ (mV)

pA/pF

INa I-V Relationship

Recovery from Inactivation