Interactions Between Ankyrin-G, Plakophilin-2, and Connexin43 at the Cardiac Intercalated Disc

Priscila Y. Sato, Wanda Coombs, Xianming Lin, Oxana Nekrasova, Kathleen Green, Lori L. Isom, Steven Taffet, Mario Delmar

Rationale: The early description of the intercalated disc defined 3 structures, all of them involved in cell-cell communication: desmosomes, gap junctions, and adherens junctions. Current evidence demonstrates that molecules not involved in providing a physical continuum between cells also populate the intercalated disc. Key among them is the voltage-gated sodium channel complex. An important component of this complex is the cytoskeletal adaptor protein Ankyrin-G (AnkG).

Objective: To test the hypothesis that AnkG partners with desmosome and gap junction molecules and exerts a functional effect on intercellular communication in the heart.

Methods and Results: We used a combination of microscopy, immunochemistry, patch-clamp, and optical mapping to assess the interactions between AnkG, Plakophilin-2, and Connexin43. Immunoprecipitation studies from rat heart lysate demonstrated associations between the 3 molecules. With the use of siRNA technology, we demonstrated that loss of AnkG expression caused significant changes in subcellular distribution and/or abundance of PKP2 and Connexin43 as well as a decrease in intercellular adhesion strength and electric coupling. Regulation of AnkG and of Na$_{1.5}$ by Plakophilin-2 was also demonstrated. Finally, optical mapping experiments in AnkG-silenced cells demonstrated a shift in the minimal frequency at which rate-dependence activation block was observed.

Conclusions: These experiments support the hypothesis that AnkG is a key functional component of the intercalated disc at the intersection of 3 complexes often considered independent: the voltage-gated sodium channel, gap junctions, and the cardiac desmosome. Possible implications to the pathophysiology of inherited arrhythmias (such as arrhythmogenic right ventricular cardiomyopathy) are discussed. (Circ Res. 2011;109:00-00.)

Key Words: Ankyrin-G ■ Plakophilin-2 ■ Connexin43 ■ desmosomes ■ gap junctions
a physical continuum between neighboring cells also populate the intercalated disc. Key among them is the voltage-gated sodium channel (VGSC) complex. Early studies showed that Na\textsubscript{1.5}, the pore-forming subunit of the sodium channel, is abundant in heart, localizes preferentially to sites of end-to-end contact.\textsuperscript{11–13} Consistent with this observation, Na\textsubscript{1.5} was found to coprecipitate with Connexin43 (Cx43)\textsuperscript{14} and with the adherens junction protein N-cadherin.\textsuperscript{24} More recently, we demonstrated that Na\textsubscript{1.5} and PKP2 coexist in the same molecular complex; we further showed that loss of PKP2 expression affects the amplitude and kinetics of the sodium current and the propagation velocity of action potentials in ventricular myocytes.\textsuperscript{15} Overall, we speculate that desmosomes, gap junctions, and sodium channels are not independent but act as a “functional triad” where changes in composition of one can affect the function and integrity of the other, with significant consequences to electric cardiac synchrony.

An important component of the VGSC complex is the cytoskeletal adaptor protein Ankyrin-G (AnkG).\textsuperscript{16–21} Ankyrins can act as organizing centers of subcellular microdomains, clustering protein complexes of complementary function. For example, in heart, Ankyrin-B is primarily present at T-tubules, where it colocalizes with molecules involved in calcium homeostasis (see References 22 and 23).\textsuperscript{22,23} In contrast, AnkG localizes primarily (though not exclusively) to the intercalated disc.\textsuperscript{20,21} The association of AnkG with Na\textsubscript{1.5} is well documented. Yet, whether AnkG can partner with other complexes at the intercalated disc and exert a functional effect on intercellular communication remains undetermined. The studies presented in this report provide evidence for an interaction of AnkG with the desmosomal component PKP2 and with the gap junction molecule Cx43. Our data further demonstrate that AnkG expression is relevant to 2 fundamental functions of the intercalated disc: intercellular adhesion and gap junction-mediated coupling. Finally, we demonstrate that loss of AnkG expression facilitates rate-dependent action potential propagation block in monolayers of neonatal rat ventricular myocytes. Overall, these experiments support the hypothesis that AnkG is a key functional component of the intercalated disc, able to interact with 2 molecular complexes often considered independent: the VGSC and the cardiac desmosome. Possible implications of these findings to the pathophysiology of inherited arrhythmias are discussed.

Methods

Physical interaction between AnkG, PKP2, and Cx43 was assessed by coimmunoprecipitation of proteins from rat adult heart lysates. Immunochemical and functional studies (patch-clamp, optical mapping, and intercellular adhesion strength) were conducted in neonatal rat ventricular myocytes (NRVMs) cultured in standard conditions. The method for intercellular adhesion strength was modified from Reference 24 by adding blebistatin (10 μmol/L) to the media to prevent contraction.\textsuperscript{24} All methods followed those previously used in our laboratories.\textsuperscript{3,15,24} Detailed descriptions are presented in the Online Supplemental Material.

Results

Physical Interaction Between AnkG, PKP2, and Cx43

Previous studies suggested that PKP2 and Cx43 coexist in the same macromolecular complex.\textsuperscript{3} In this report, we confirm this observation and show coimmunoprecipitation of AnkG with PKP2 and, separately, with Cx43. Adult rat heart lysate was presented to beads coated with antibodies to either PKP2, Cx43, or AnkG. IgG-coated beads were used as control. Resulting precipitants were probed for the corresponding proteins. Figure 1 shows the results. For each lane, antibody used as precipitant is noted at the top of the figure (last lane demonstrates the presence of the respective proteins in the heart lysate). Western blots revealed that both PKP2 and Cx43 can coprecipitate with AnkG. Coprecipitation of PKP2 with Cx43 was also observed, consistent with previous studies.\textsuperscript{3} Similar results were obtained in 4 separate experiments. These data indicate a physical interaction (direct or indirect) between the 3 molecules. Interestingly, N-cadherin was not consistently found in the precipitate (Online Figure I), thus indicating that the presence of N-cadherin at concentrations above levels of detection was not a necessary condition for AnkG to pull down PKP2. Next, we tested for interaction of AnkG with PKP2 within the environment of a living cell.

Loss of AnkG Expression and Its Effect on PKP2 Localization

Our results indicate a physical association of AnkG with PKP2. We assessed whether expression of one affects the
abundance or subcellular localization of the other. Monolayers of NRVMs were treated with oligonucleotides that prevented (AnkG-siRNA) or did not prevent (/H9021 siRNA) the expression of AnkG protein. Loss of AnkG expression was confirmed by conventional Western blot (Figure 2A; collective data on AnkG-silencing efficiency is presented in Online Figure II, A). Western blot analysis of the same samples indicated that loss of AnkG expression did not affect the total abundance of PKP2 in the heart cells; an example is shown in panel 2A, and quantification of band densities, relative to loading control, is shown in 2B. Immunofluorescence studies with anti-PKP2 antibodies in cells untreated (Online Figure II, B, and II, C) or treated with the control construct (/H9021 siRNA; Figure 2C, left) revealed the selective staining of bright contours of immunoreactive protein, surrounding areas mostly void of staining, colocalizing with AnkG. The areas of AnkG-PKP2 colocalization probably defined sites of cell-cell apposition, in agreement with previous observations (see also References 20 and 21).20,21 A different profile was observed in cells treated with AnkG-siRNA (Figure 2C, right). In this case, the absence of AnkG immunoreactive protein (top) was associated with a redistribution of the PKP2 signal; though cell borders were still distinguishable, we also observed defined, short-length multiple clusters located at short distances from each other, indicating that a large fraction of the PKP2-immunoreactive protein was localized at sites away from the line of contact between cells. These results, consistently observed in 3 separate preparations, support the hypothesis that AnkG expression is relevant to the subcellular localization of PKP2. These data contrasted with those obtained when AnkG-silenced cells were probed with antibodies for plakoglobin (Figure 3A) or for N-Cadherin (Figure 3B; corresponding AnkG staining in Online Figure III, A, and III, B, respectively); in those cases, the distribution pattern observed in control cells was not different from that noticed after loss of AnkG expression, thus arguing against the notion that AnkG silencing causes a generalized loss of the molecular components normally present at the site of intercellular junctions.

**Reciprocal Regulation of AnkG by Loss of PKP2 Expression**

Data in the preceding paragraph indicate that localization of PKP2 at sites of cell contact is influenced by AnkG expression. Whether this regulation is reciprocal was assessed by our experiments. Monolayers of NRVMs were treated with oligonucleotide constructs targeted to silence PKP2 expression (PKP2-siRNA). Cells treated with a nonsilencing construct (/H9021 siRNA) were used as control. As shown in Figure 4A, PKP2-siRNA treatment led to the loss of PKP2 expression. Interestingly, this treatment also caused a decrease in abundance of immunoreactive AnkG, assessed by Western blot (see Figure 4A; quantification of band densities relative to loading controls in Figure 4B). On this particular, silencing of PKP2 and AnkG were not reciprocal. Fluorescence microscopy analysis (Figure 4C) indicated a significant decrease in overall intensity of AnkG-immunoreactive signal, in agreement with Western blot results. In corollary experiments, we explored whether the same treatment would affect the abundance or distribution of Na\(_{\text{+}}\)1.5. As shown in Figure 5A and 5B, loss of PKP2 expression did not affect Na\(_{\text{+}}\)1.5 abundance; however, we did observe a change in localization of the protein from sites of cell apposition (in control; Figure 5C, left panels) to a more diffuse distribution within the intracellular space in PKP2-siRNA–treated cells (Figure 5C, right panels). The latter may be secondary to loss of AnkG in PKP2-deficient cells (Figure 4C), given the known relevance of AnkG to Na\(_{\text{+}}\)1.5 targeting.19,20 although a direct role of PKP2 on Na\(_{\text{+}}\)1.5 localization cannot be ruled out (see also Reference 25).25
AnkG and Intercellular Adhesion Strength

The preferential localization of AnkG to the area of cell-cell apposition20,21 and its ability to interact with PKP2 in cardiac cells and with E-Cadherin in other systems26 led us to speculate that AnkG may be a necessary component of mechanical junctions between cardiac myocytes. To assess this hypothesis, we adopted a “dispase assay” previously used in other cell systems as an indirect measure of intercellular adhesion strength.24,27 Briefly, confluent NRVM monolayers were exposed to dispase, an enzyme that disrupts binding of cells to the extracellular matrix coating the dish.24 Under control conditions (exposure of cells to \( \Phi \)siRNA), cells were lifted from the anchoring matrix as one sheet. Integrity of the single sheet was preserved even after 1 to 3 minutes of gentle

**Figure 3.** Loss of AnkG expression did not affect abundance/distribution of plakoglobin or N-cadherin in NRVMs. A (left), Western blot for AnkG and plakoglobin from same sample; tubulin was used for each blot as loading control. **Center and right:** Immunodetection of plakoglobin (PG) in cells treated with control (\( \Phi \)siRNA) or AnkG-silencing constructs (AnkG-siRNA). B, Similar conditions. Detection of N-cadherin–immunoreactive proteins. C and D, Quantification of corresponding Western blots. All measurements are normalized to those obtained from untreated cells in same blot. In both cases, \( n=6; P=NS \), \( \Phi \)siRNA versus AnkG-siRNA. UNT indicates untreated. Bars, 20 \( \mu \)m.

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**Figure 4.** AnkG abundance/distribution in PKP2-silenced NRVMs. A, Western blot for AnkG and PKP2 with respective tubulin loading controls; cells untreated (UNT), treated with siRNA for PKP2 (PKP2-siRNA), or treated with nonsilencing construct (\( \Phi \)siRNA). B, Quantification of AnkG band density, corrected individually by loading control and normalized to UNT (\( n=5; P<0.05 \), \( \Phi \)siRNA versus PKP2-siRNA). C, Immunolocalization of PKP2 (top) and AnkG (bottom) in NRVMs treated with \( \Phi \)siRNA or PKP2-siRNA. Bars=20 \( \mu \)m.
agitation (Figure 6A). However, pretreating cells with PKP2-siRNA led to weakening of intercellular adhesion and thus separation of the monolayer sheet into fragments (Figure 6A, column labeled “PKP2-siRNA”; Western blots confirming PKP2 silencing in Online Figure IV, A, and IV, B; fragment quantification is shown in Online Figure IV, C). Interestingly, loss of AnkG expression led to monolayer fragmentation in a manner similar to that observed when PKP2 expression was prevented (Figure 6A; column labeled “AnkG-siRNA”). The number of fragments counted in AnkG-siRNA–treated plates was compared with that recorded from untreated monolayers or from those treated with control (H9021 siRNA) construct. Data are presented in Figure 6B. Silencing was confirmed by conventional Western blots (Online Figure V). These results demonstrate for the first time that AnkG plays an important role in preservation of mechanical continuity between cardiac myocytes. As a next step, we assessed the possible role of AnkG on a separate function of the intercalated disc, that is, gap junction–mediated coupling.

AnkG, Gap Junctions, and the VGSC Complex

Previous studies have demonstrated that loss of mechanical junction integrity can alter Cx43 abundance and/or distribution.1,3,7,28,29 We explored the effect of AnkG knockdown on abundance and localization of Cx43 and on the extent of electric coupling between cells. Figure 7A shows an example of Western blots obtained from NRVM lysates. Average measurements of band densities are shown in Figure 7C. The data show a decrease in total Cx43 abundance, similar to that observed in PKP2–knocked down cells.3 Electrophysiological recordings of junctional current were obtained by dual patch-clamp in cells untreated, treated with ΦsiRNA, and treated with AnkG-siRNA. Loss of AnkG expression was confirmed in parallel cell cultures by Western blot. As shown in Figure 7E, loss of AnkG expression was associated with a significant reduction in the extent of electric coupling between myocytes. These results correlated with a decrease in Cx43 abundance detected by immunofluorescence microscopy (Figure 7A, middle and right panels; corresponding costaining of AnkG is shown in Online Figure III, C). Interestingly, optical mapping experiments showed that loss of AnkG expression did not affect velocity of action potential propagation in the range of pacing frequencies between 1 and 6 Hz. Yet, an increase in the pacing rate led to loss of 1:1 capture. In fact, we failed to command electric activity in all AnkG-knockdown preparations at stimulation frequencies of ≥8 Hz (asterisks), whereas a 1:1 ratio was maintained for 4 of 8 control preparations paced at the same frequency (Online Figures VIII and IX).

Discussion

Studies in heart and other systems ascribe to ankyrins the role of “organizing centers,” clustering molecular complexes of complementary function.16,18,30–32 Previous studies have reported that AnkG localizes preferentially to the intercalated disc. We therefore speculated that AnkG participates in the function of complexes involved in intercellular communication. We focused primarily on the association of AnkG with 2 molecules fundamental to intercalated disc function: PKP2 and Cx43. We further explored the relevance of AnkG...
expression to 2 of the primary functions of intercellular junctions: mechanical adhesion and gap junction–mediated coupling. Finally, given that AnkG associates with more than 1 molecular complex important to cardiac electrophysiology, we assessed the overall impact of AnkG knockdown on the integrity of cardiac myocytes to propagate an action potential. Overall, our data support the notion that AnkG partly defines not only the sodium current properties but also the integrity of cardiac myocytes to propagate an action potential. Our results show a decrease in junctional conductance as well as intercellular adhesion strength consequent to AnkG protein levels. Whether this is consequent to changes in protein half-life and/or functional expression remains to be determined. The data do show that a reciprocal regulation is not large enough to be detected, despite efforts to optimize our experimental conditions. We do recognize the limitations of all Co-IP experiments, where both false-positive and false-negative results are likely; therefore, we pursued experiments to detect the abundance and distribution of relevant proteins in the cellular environment (Figures 2, 3, and 7). Furthermore, the preserved conduction velocity (Online Figure IX), the preserved localization and abundance of N-cadherin and plakoglobin (Figure 3), and the preserved cytoskeletal architecture (Online Figure VII) suggested a degree of specificity in the effects of AnkG silencing rather than a generalized loss of integrity of the junctional and cytoskeletal structure of the cell.

Previous studies have demonstrated that AnkG is necessary for targeting of selected transmembrane proteins to their final destination at the membrane.\textsuperscript{19–21,31} Recently, β4-spectrin and CAMKII have been proposed to form a macromolecular complex at the intercalated disc with AnkG and Nav1.5, as β4-spectrin–dependent targeting of CAMKII has been shown to influence sodium current properties.\textsuperscript{38} Much less is known, however, about the molecular steps that regulate AnkG abundance and its localization. In this report, we show that PKP2 expression is a necessary condition for maintaining AnkG protein levels. Whether this is consequent to changes in protein half-life and/or functional expression remains to be determined. The data do show that a reciprocal regulation is established between the 2 molecular complexes (desmosomes and VGSC), whereby the integrity of one is necessary for the proper function of the other. We speculate that mechanical junctions provide an anchoring point for AnkG to remain at the membrane, thus stabilizing the spectrin-AnkG complex and providing a link to the actin and the desmin cytoskeletal networks. Future experiments will be necessary to address this and other alternative possibilities.

Our results show a decrease in junctional conductance as well as intercellular adhesion strength consequent to AnkG distribution of plakoglobin and of N-cadherin. As such, our results were not consequent to a generalized loss of molecular components present at the intercellular junction. Overall, the intercalated disc emerges as a complex network where 2 molecules may share more than 1 partner and transient associations, particularly with catalytic molecules, are likely. In this temporally and spatially dynamic system, the links between 2 molecules in a living cardiac myocyte cannot be precisely defined. Our data, in combination with others,\textsuperscript{14} do indicate that the molecular associations between intercellular junction proteins and components of the VGSC complex are strong enough to allow for coprecipitation. The data in Figure 1 of this study show that AnkG can coprecipitate with Cx43 and with PKP2 (even if N-cadherin was not found in the precipitate; see Online Figure 1). Previously, we demonstrated the coprecipitation of PKP2 with Cx43\textsuperscript{3} and with Na1.5.\textsuperscript{15} Taken altogether, our data show the existence of intermolecular associations among the 3 complexes, with AnkG emerging as an important component of the intercellular communication between cardiac cells.

It is important to note that the results in Figure 1 and Online Figure 1 do not imply that N-cadherin is completely absent from the complex but rather that the amount present is not large enough to be detected, despite efforts to optimize our experimental conditions. We do recognize the limitations of all Co-IP experiments, where both false-positive and false-negative results are likely; therefore, we pursued experiments to detect the abundance and distribution of relevant proteins in the cellular environment (Figures 2, 3, and 7). Furthermore, the preserved conduction velocity (Online Figure IX), the preserved localization and abundance of N-cadherin and plakoglobin (Figure 3), and the preserved cytoskeletal architecture (Online Figure VII) suggested a degree of specificity in the effects of AnkG silencing rather than a generalized loss of integrity of the junctional and cytoskeletal structure of the cell.

Among the various molecules participating in mechanical coupling,\textsuperscript{8} we chose to focus on PKP2. Our interest in PKP2 follows as a logical consequence of our previous observations indicating that loss of PKP2 expression affects the amplitude and kinetics of sodium current.\textsuperscript{15} Our data led us to hypothesize that a molecular interaction exists between PKP2 and at least 1 component of the VGSC complex. The results presented in this report support this notion. It should be emphasized, however, that the association between AnkG and PKP2 may not be direct but mediated by other proteins. Indeed, ankyrins are promiscuous molecules,\textsuperscript{30} and PKP2 partners with multiple components of the desmosome and adherens junctions.\textsuperscript{33,34} As well as with other catalytic molecules,\textsuperscript{33,35} Yet, it is important to emphasize that loss of AnkG expression had no noticeable impact on the abundance or subcellular
knockdown. Yet, we should emphasize that the role of AnkG in intercellular communication differs from that of molecules directly involved in cell-cell contact (e.g., cadherins or connexins) and is more comparable to that of other molecules, such as PKP2, where an intracellular component provides a scaffolding point for the organization of transmembrane proteins into a functional unit. The detailed molecular steps leading to the final organization of the intercalated disc remain a matter of future investigation (see Reference 37).37 Previous studies identified AnkG as a component of the VGSC complex, PKP2 as part of the desmosome, and Cx43 anchored to ZO-1.21,33,38,39 The image of these 3 complexes as separate from each other, each with its respective adaptor protein, is rapidly changing. PKP2 affects the function of both gap junctions and the VGSC,3,15 AnkG interacts with E-cadherin26 and with PKP2 (Figures 1 and 2), and ZO-1 is originally defined by its interaction with other junctional complexes.40 As such, the current evidence blurs the lines that previously identified junctional complexes are separate and independent. The current picture is instead that of a functional molecular network (see diagram in Online Figure X). This “cross-talk” is also seen at the functional level, where changes in mechanical coupling can also affect electric communication,3,4,15 manipulations affecting intercellular adhesion (PKP2 knockdown; Figure 6A) also change conduction velocity,15 and variations in AnkG abundance affect mechanical and electric coupling between cells. Altogether, the results support the notion of the intercalated disc not as a summation of individual components but rather an organelle, a single functional unit involved in maintaining intercellular communication in the heart. Interestingly, a recent study reported the segregation of Nav1.5 to different “pools” within the cell, guided (at least in part) by the association of the channel molecule with location-specific scaffolding proteins (see Reference 25).25 Whether PKP2 interacts with SAP97 or with other scaffolding molecules of the intercalated disc (in addition to AnkG) and whether changes in desmosomal proteins affect the fraction of Nav1.5 that localizes with the dystrophin/syntrophin complexes41 remain undefined.

Genetic analysis has linked desmosomal mutations to ARVC. Analysis of ARVC-afflicted hearts has demonstrated a loss of gap junction plaques at the sites of cell contact. The latter has led to the hypothesis that loss of Cx43-mediated electric communication may be a major cause of ARVC-

Figure 7. Role of AnkG expression on abundance/localization of Cx43 and Na1.5. A and B, Western blot (left) and immunoreactive signal (center; right) in cells treated with ΦsiRNA (nonsilencing control) or AnkG-siRNA. Tubulin was used as Western loading control. UNT indicates untreated cells. Cells were probed for Cx43 or Nav1.5. C, Quantification of Cx43 band density corrected individually by loading controls. Each measurement normalized to that obtained by UNT cells in same blot (P<0.05. AnkG-siRNA versus ΦsiRNA; P<0.01, AnkG-siRNA versus UNT, n=8). D, Quantification of Nav1.5 band density corrected individually by loading controls. Each measurement normalized to UNT cells in same blot. (P=NS, n=5, 5, and 6 for UNT, ΦsiRNA, and AnkG-siRNA, respectively). E, Dual patch-clamp junctional conductance measurements in cell pairs UNT (n=7) treated with ΦsiRNA (n=12) or AnkG-siRNA (n=16). *P<0.01. Bars=20 μm.
related arrhythmias. Of note, however, studies in genetically modified animals have demonstrated that large decreases in Cx43 abundance do not significantly affect conduction velocity.42–44 Thus, it seems reasonable to speculate that other cellular factors (other than Cx43 localization) may act as arrhythmogenic substrates in ARVC. Our previous studies15 and those in the present study lead us to propose that loss of desmosomal integrity can affect sodium channel function, with deleterious consequences to the electric stability of the heart. We further speculate that changes in the integrity of the VGSC complex may in turn affect mechanical coupling (see Figure 6). In that regard, it is worth noting that several investigators have observed defects in contractility in patients with Brugada syndrome.45 Yet, we do recognize that our results are applicable to the etiology of arrhythmias and/or mechanical impediment in heart disease remains a subject of future investigation. Our data do support the notion that desmosomes, gap junctions, and the VGSC form an interactive complex where changes in composition of one affect the function of the other. This “other” electromechanical coupling, occurring at the intercalated disc, may be relevant not only to the understanding of arrhythmogenesis in rare inherited diseases affecting desmosomal molecules but also in acquired conditions (cardiomyopathies) affecting the integrity of the intercalated disc.47

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**
- The voltage-gated sodium channel complex localizes preferentially the intercalated disc. Ankyrin-G (AnkG) is a part of this complex.
- Mutations in desmosomal molecules are known to be responsible for most familial cases of arrhythmogenic right ventricular cardiomyopathy.

**What New Information Does This Article Contribute?**
- AnkG associates with plakophilin-2, a molecule of the desmosome, and with Connexin43, the major cardiac gap junction protein.
- Loss of AnkG expression weakens intercellular adhesion and reduces gap junction–mediated coupling.
- Loss of plakophilin-2 disrupts AnkG and sodium channel localization and decreases AnkG abundance.
- Plakophilin-2 expression is relevant for stability of the voltage-gated sodium channel complex. AnkG is in turn an important component of the molecular complexes that maintain mechanical and electric continuity in the heart.

The early description of the intercalated disc defined 3 structures involved in cell-cell communication: desmosomes, gap junctions, and adherens junctions. Current evidence shows that molecular complexes that do not provide a physical continuum between cells also populate the intercalated disc. Key among them is the voltage-gated sodium channel, which includes, as a component, the cytoskeletal adaptor protein ankyrin-G. For the most part, the function of the voltage-gated sodium channel has been considered independent from that of other structures involved in cell-cell communication. Yet, this view is rapidly changing. Our data show that Plakophilin 2, a molecule of the desmosome, can affect the integrity and the function of the voltage-gated sodium channel; conversely, loss of expression of ankyrin-G not only affects the sodium current, but it also weakens intercellular adhesion strength and decreases gap junction–mediated communication. These and other results support the notion of the intercalated disc as a single functional unit, where molecules involved in cell excitability are physically and functionally integrated with others involved in maintaining communication between cells. These findings have important implications for understanding the pathophysiology of arrhythmogenic right ventricular cardiomyopathy and related cardiac arrhythmias.
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SUPPLEMENT MATERIAL

MATERIALS AND METHODS

Neonatal Rat Ventricular Monolayers
Neonatal Rat Ventricular Myocytes (NRVM) were obtained as described in.1 One-two day old Sprague-Dawley Rats (Charles River, MA) were euthanized and their hearts removed from the thoracic cavity. The hearts were finely minced, and enzymatically digested.1 The enzymatic fractions were combined to 20mL of supplemented M199 containing 10%FBS. Fractions were spun down and pellets resuspended in supplemented M199 containing 10%FBS, and filtered through a 40µm filter. Cells were subjected to a 2h pre-plating at 37°C 5%CO₂ and the cell suspension was then passed through a 70µm filter. Cells were then counted and plated in 35mm dishes, 18mm coverslips, or 14mm glass-bottom dishes pre-coated with Collagen type IV. BrDU was added to prevent fibroblast proliferation. Media change was performed every 24h for the first 48h and every 48h thereafter.

Co-Immunoprecipitation
Co-IP experiments were performed as previously described.1 Briefly, heart lysates were homogenized in lysis buffer and centrifuged at 3500 rpm for 15min. The supernatant was retained and exposed to Protein A/G beads for 30min at 4°C, followed by centrifugation at 3500rpm for 5min. The pre-cleared supernatant was exposed to beads coated with the antibodies of interest for one hour at 4°C, followed by centrifugation (2500 rpm for 5 min) and re-suspension in lysis buffer. The last step was repeated three times to clear the solvent of unbound material. The final pellet was resuspended in 6x Laemmli buffer and separated by conventional SDS PAGE electrophoresis and transferred for protein detection by immunoblot (see also below). Antibodies used as precipitants were Mouse PKP2 (Meridian Life Science), Rabbit Cx43 (Chemicon), Rabbit Ank-G (Santa Cruz Biotechnologies), PAN-Cadherin (Sigma). Antibodies for protein detection in these experiments were Mouse PKP2 (Meridian), Mouse Cx43-NT1 (Fred Hutchinson Cancer Researach Center), Mouse Ank-G (Neuromab), N-cadherin (BD Transduction).

Western Blot
Western blot analysis of protein content in cardiac cells was performed as previously described.1 Briefly, 35mm NRVM monolayers were harvested in Tris-EGTA containing 5-10X protease inhibitors (Roche). Cells were spun for 3min at 4°C and pellets exposed to the lysis buffer for 30min at 4°C. After spinning for 3min at 4°C, the lysate was combined with 5X Sample Buffer. For PKP2 or Cx43 detection, samples were heated for 5min at 55°C (for Cx43) or 100°C (for PKP2), loaded in a 4-20% 15-well Tris-Glycine gel, and run at 120V. Proteins were transferred to a 0.45µm nitrocellulose membrane (120V; 90min). Membranes were blocked (5%NFM in T-PBS) for 1h at room temperature, and primary antibody incubated overnight at 4°C. Secondary antibodies were used for 45min at room temperature. For Nav1.5 or AnkG detection, samples were heated for 5min at 37°C, loaded in a 4-12% 15-well Tris-glycine gel, and run at 100V. Proteins were transferred to a 0.45µm nitrocellulose membrane at 4°C, 60mA, for 16h. Membranes were then blocked (5%NFM in T-TBS for Nav1.5 or 4%BSA in 0.2%Triton-Phosphate buffer, for AnkG) for 1h at room temperature. Primary antibodies were incubated overnight at 4°C in their respective blocking buffers. Secondary antibodies were used for 45min at room temperature. After the primary detection, membranes were stripped with Restore® (Thermo Scientific, 21059). Membranes were washed with T-PBS and blocked for 1h (5%NFM...
T-PBS) at room temperature. Tubulin antibody was used as loading control. Primary antibodies used were: Rabbit Nav 1.5 (Alomone #ASC-005), Rabbit Ankyrin-G (kind gift from Dr. Vann Bennett- Duke University), Mouse PKP2 (Biodesign), Mouse Plakoglobin (Sigma), Mouse N-cadherin (BD Transduction), Mouse Cx43-NT1 (Fred Hutchinson Cancer Research Center), Mouse Tubulin (Hybridoma Bank at University of Iowa).

Protein Knockdown
Silencing oligos specific for rat PKP2 or AnkG were obtained from Dharmacon (Chicago, USA). Cardiac myocytes were transfected 24h post-plating with ON-TARGETplus SMARTpool siRNA specific for rat PKP2 (CCAAAUCUAGUAACGAAUA, CCACAUCGGUAGCUCGCAU, GAGGAAUUGUCACCGAAU, AGUUAAGAGAAGCGGACU) or rat AnkG (AAGCAGAAGUGGUGCAGUA, GUGCGAAGAUCGACGCCAA, CCUUGUGAGCGGACCGGAUA, UGGCAAUAGUAGCCGAUA). Control for transfection was provided by using ON-TARGETplus Non-targeting siRNA. All transfections were performed according to the manufacturer's protocol using Dharmafect-1. Complete silencing of PKP2 and AnkG was observed at day 5 and day 6 post-transfection, respectively.

Immunofluorescence
Cells plated in 18mm glass coverslips or 14mm glass-bottom dishes were fixed in either 3% paraformaldehyde or cold ethanol for 5min depending on the protein of interest. For staining involving Nav1.5, blocking was done using 0.1% Triton in PBS containing 20% goat serum for 2h at room temperature. Primary antibodies were diluted in 0.1% Triton in PBS containing 5% goat serum and incubated overnight at 4°C. Primary antibodies were then washed with PBS and secondary antibodies were incubated for 1h at room temperature. For staining involving Ankyrin-G, blocking was done using 0.025% Triton in PBS containing 30% sucrose, 3% fish gelatin, and 20% goat serum for 2h at room temperature. Primary antibodies were diluted in 0.025% triton in PBS containing 30% sucrose, 3% fish gelatin, and 5% goat serum and incubated overnight at 4°C. Primaries were then washed with PBS and secondary antibodies were incubated for 1h at room temperature. Primary antibodies used were: Rabbit Nav1.5 (Alomone #ASC-005), Rabbit Ankyrin-G (Santa Cruz Biotechnologies), Mouse PKP2 (Biodesign), Mouse Plakoglobin (Sigma), Mouse N-cadherin (BD Transduction), Mouse Cx43-IF1 (Fred Hutchinson Cancer Research Center), Alexa Fluor 488 Phalloidin (Invitrogen). Secondary antibodies used were: Mouse Alexa Fluor 594, Rabbit Alexa Fluor 488, and DAPI (Molecular Probes). Image acquisition was done using Axioplan 2 bright field microscope (Zeiss) equipped with 63X Plan Apo Lenses or with Zeiss Inverted microscope equipped with 100X objective. All gains for the acquisition of comparable images were maintained constant.

Dispase Assay
The protocol to assess intercellular adhesion strength was modified from2-5. NRVMs were plated to confluency in 35mm dishes. Cells were washed with warm PBS, and then incubated with 1.2-2.4 U/mL Dispase at 37°C/5%CO2 for 4h. Blebbistatin (10µM) was added to the media to decrease cellular contraction. Fragmentation of the monolayer was induced by using an orbital shaker at 70rpm. Imaging of the monolayers was done using a Canon EOS Rebel Xsi Digital Camera containing a Sigma DC 17-70mm 1:2.8-4 MACRO HSM lens. In addition, a HDYA 72mm PL-CIR polarizing lens was used to minimize reflection. The camera was mounted on an inverted tripod and a remote switch was used to take the images to minimize external vibrations. Images were imported to a computer and Photoshop was used to enhance visualization by changing the mode to grayscale and turning the background black.
**Patch Clamp Recordings**
Measurement of junctional conductance between NRVM cell pairs followed conventional protocols.\(^1,6\) Cells were recorded in the dual voltage clamp configuration. Each cell of the pair was independently voltage clamped to the same holding potential (−40mV). Cell 1 was stepped to +20mV, creating a potential difference of +60mV, during repetitive 10 s steps. The current applied by the voltage clamp circuit in cell 2 during the pulse in cell 1 was considered equal and opposite to the junctional current. Junctional conductance was calculated as per Ohm’s Law. Protocols for sodium current recordings as in\(^1\).

**Optical Mapping**
Optical Mapping experiments were performed as previously described.\(^1\) Monolayers were stained with Di-8ANEPPS (40µM) for 15min at 37°C, washed one time with HBSS containing Ca and Mg, placed on a glass holder and kept at a constant temperature of 37°C. HBSS containing Ca and Mg at 37°C was superfused throughout the preparation during the recordings. A dichroic mirror was placed in-between the light source and the preparation, and signal was captured by an 80x80 pixel CCD camera placed under the preparation. An electrode was placed in the center of the dish and pacing stimulus was delivered at 1Hz increments until failure of 1:1 capture. Sampling rate for optical recording was 200-500 frames per second (2-5ms frame intervals) and a complete sample episode lasted 5 seconds (i.e., each movie was 5 seconds in duration). Conduction velocity was calculated from data on the entire 5-second episode. Pacing was done at 5 ms stimulation with 5 mV pulse. Off-line filtration was done using SCROLL software. Details on filtration parameters can be found elsewhere\(^7, 8\).

**Statistical Analysis**
One-way ANOVA analysis followed by Bonferroni or Tukey tests were performed by using SPSS17 (Statistical Package for the Social Sciences, version 17; also known as PASW, Predictive Analytics Software). Statistical consultation was done by the University of Michigan-CSCAR (Center for Statistical Consultation and Research).

**RESULTS**

**AnkG precipitates PKP2 in the absence of detectable N-cadherin**
Co-immunoprecipitation of AnkG with PKP2 is shown in the main manuscript. Here, we sought to determine whether N-cadherin would also be consistently observed in an AnkG precipitate. Beads coated with antibodies against Cx43, AnkG or (in some cases) cadherin were presented to a heart lysate. Each precipitate was then probed with antibodies against PKP2, AnkG or N-cadherin. As shown in Online Figure I, we confirmed the presence of immunodetectable PKP2 in the AnkG precipitate (panels A and B; similar result as in Figure 1 of main manuscript). Yet, N-cadherin was not consistently observed (compare N-cadherin immunoblot in A, where no N-cadherin was found, versus B, where an N-cadherin signal was found in precipitates from AnkG as well as Cx43). These results complement those reported in Figures 1 and 3 of the manuscript. A discussion on the limitations of immunoprecipitation experiments is also presented in the manuscript.

**Analysis of AnkG abundance in NRVMs in control conditions, or treated with AnkG-siRNA**
Studies presented in this manuscript sought to determine the relevance of AnkG expression on the abundance, localization and function of proteins relevant to intercellular communication and/or sodium channel function. Online Figure II-A shows the compiled results obtained from the Western blot analysis of samples that were either untreated (UNT), or treated with oligonucleotides designed to prevent (AnkG-siRNA) or not prevent (ΦsiRNA) AnkG protein.
expression. All values were measured relative to the band density obtained from untreated cells, corrected by a tubulin loading control (see raw data in main manuscript; e.g., Figure 2A and others). Collectively, the density of the bands detected by AnkG antibodies on samples from siRNA-treated cells was <5% of the band density in parallel samples that were untreated, or treated with a non-silencing construct. Moreover, for immunofluorescence experiments assessing the effect of AnkG silencing, cells were co-stained for AnkG and for the protein of interest. As shown in Online Figure II-B-C, AnkG co-localized with PKP2 at sites of cell contact in untreated cells. On the other hand, as shown by data in Online Figure III and also in the main manuscript, negligible AnkG-immuoreactive signal was detected from cells treated with the siRNA construct.

**Intercellular adhesion strength after loss of PKP2 expression**

Loss of AnkG expression led to a decrease in intercellular adhesion strength. Data on that regard are presented in the main manuscript (Figure 6). Examples of fragmentation of NRVM monolayers previously treated with PKP2-siRNA are also shown in the main text. Online Figure IV shows Western blots (panels A and B) confirming the efficiency of the siRNA construct (tubulin used as loading control). Panel C shows the collective data obtained from quantifying the number of fragments observed in the three experimental conditions, at time zero, 1 and three minutes of gentle agitation. Each symbol represents an individual experiment. Notice that the ordinate axis is interrupted to indicate that loss of PKP2 expression caused the disruption of the monolayer into more than 30 fragments, at which point individual fragments were difficult to resolve at the level of resolution of our recording system. Finally, Online Figure V confirms loss of AnkG expression in AnkG-siRNA-treated cells prepared in parallel with those used for the intercellular adhesion experiments presented in the main manuscript.

**Sodium current after loss of AnkG expression in NRVMs.**

Previous studies have documented that AnkG expression is relevant to sodium current properties. Confirmation of these observations in our experimental system (and within the time frame of our studies) is presented here. Panel A of Online Figure VI displays original current traces obtained from NRVMs treated either with siRNA for AnkG, or with a construct that did not affect AnkG expression (ψsiRNA). Panels B, C and D reveal that loss of AnkG expression caused a reduction in sodium current amplitude (B) without changes in steady-state inactivation (C), or recovery from inactivation (D). Panel E presents data on the rate of current decay (measured by fitting an exponential function to the current traces obtained at a voltage command of -40 mV), and Panel F displays Western blot data confirming loss of AnkG expression in AnkG-siRNA-treated cells prepared in parallel with those used for the voltage clamp experiments. Altogether, we observed changes in sodium current properties and Na,v1.5 localization (main manuscript, Figure 7B) consistent with those previously reported. Overall, AnkG silencing caused changes on sodium current amplitude, gap junction communication (Figure 7A of manuscript) and intercellular adhesion strength.

**AnkG knockdown and phalloidin staining**

Our experiments revealed that AnkG had no effect on the abundance or distribution of N-cadherin, and of plakoglobin (Figure 3 of the main manuscript). Further evidence that the observed effects of AnkG knockdown were not reflecting a generalized loss of cell integrity is presented in Online Figure VII. In this case, NRVMs were stained with an antibody against phalloidin. The figure shows that cells treated with AnkG-siRNA showed a preserved cytoskeletal architecture similar to that of cells treated with the control construct (compare panels A and B of Online Figure VII).
AnkG and action potential propagation in monolayers of NRVMs.

Previous studies and those presented here support the notion that AnkG interacts with molecular complexes involved in electrical homeostasis. Yet, the role of AnkG on action potential propagation in cardiac cells remains undefined. Monolayers of NRVMs were paced at various frequencies with an electrode located in the center of the preparation. Conventional optical mapping methods were used to record electrical activity. Results obtained from monolayers exposed to the siRNA were used as control and compared to those obtained from cells treated with the AnkG-siRNA construct. Loss of AnkG expression in the mapped monolayers was confirmed by Western blot following the conclusion of the electrophysiological experiment (Online Figure VIII). Isochrone maps are depicted in Online Figure IX-A. A plot of conduction velocity as a function of pacing frequency is presented in Online Figure IX-B. Small numbers above each data point indicate the number of preparations in which we were able to sustain a 1:1 stimulus:response ratio at that particular pacing frequency. The data show that loss of AnkG expression did not affect the velocity of action potential propagation in the range of pacing frequencies between 1 and 6 Hz. Yet, an increase in the pacing rate led to loss of 1:1 capture. In fact, we failed to command electrical activity in all AnkG-knockdown preparations at stimulation frequencies of 8 Hz or higher (asterisks), whereas a 1:1 ratio was maintained for 4 out of the 8 control preparations paced at the same frequency. These results indicate that AnkG contributes to preservation of action potential propagation at high pacing frequencies.

DISCUSSION

Overall, our results suggest a functional and molecular interaction of AnkG with components of the desmosome, gap junctions and the VGSC complex. These interactions may be direct, or indirect (via additional intermediate molecules). Online Figure X is a diagram not intended as an exact representation of the molecular components but rather, a conceptualization of the fact that desmosomes, sodium channels and gap junctions may represent a functional unit at the intercalated disc.

The observation that AnkG silencing did not affect conduction velocity in NRVM monolayers seems surprising, given the known importance of AnkG on sodium current function and our observations confirming that loss of AnkG expression causes a redistribution of Na,1.5 and a decrease in sodium current amplitude (Online Figure VI; see also), as well as a decrease in junctional conductance. The mechanism(s) underlying these unexpected/interesting experimental observations are presently unknown and warrant further investigation. The mechanistic basis of a ‘propagation reserve’ in intact hearts is also still largely unknown. It is possible that future use of this and other experimental models could serve at some point to elucidate respective mechanisms. Further characterization of the role of AnkG on cardiac propagation awaits availability of an animal model of AnkG deficiency, where electrophysiological parameters can be measured in an intact adult heart. Of note, although the studies of Lowe et al have indicated that AnkG is not relevant for preservation of calcium currents, a possible role of AnkG on the function of other ionic currents in the heart cannot be discarded.
References


Online Figure I: A: Immunoblots of PKP2, AnkG, and N-cadherin (top to bottom) from samples exposed to protein A/G beads coated with rabbit IgG (negative control), Cx43, or AnkG antibodies. Heart lysate (right-most lane) was used as positive control. Blot shown at the bottom is overexposed to further demonstrate the absence of an N-cadherin immunoreactive signal in the precipitates. B: Similar experiment as in A. Immunoblots of PKP2, AnkG, and N-cadherin (from top to bottom) from samples exposed to protein A/G beads coated with rabbit IgG (negative control), Cx43, AnkG, or cadherin antibodies. Heart lysate (right lane) was used as positive control.
Online Figure II. A: Quantification of band densities in immunoblots treated with AnkG antibodies. Bars indicate the density of AnkG-immunoreactive bands in NRVMs. UNT: Untreated. ΦsiRNA, cells treated with an oligonucleotide that does not silence AnkG expression. AnkG-siRNA: cells treated with a construct designed to knockdown AnkG expression. All bands calibrated by density of a loading control (tubulin). Quantification was made relative to density of band in UNT lane, for each blot. Bars represent SEM. B-C: Co-localization of AnkG (B) and PKP2 (C) at sites of cell contact in untreated NRVMs.
Online Figure III. Localization of AnkG-immunoreactive proteins in NRVM monolayers that were treated with a control oligonucleotide (ΦsiRNA) or with AnkG-siRNA. Panels A, B and C depict the same images as those used to illustrate the localization of plakoglobin, N-cadherin and Cx43 (respectively), and presented in the main manuscript (see Figures 3 and 7).
Online Figure IV: Panels A and B: Representative western blots of NRVM monolayers subjected to dispase to assess intercellular adhesion strength. Samples were untreated (UNT) or treated with ΦsiRNA or PKP2-siRNA. The membranes were probed for PKP2 (A) and tubulin (B) as a loading control. C: Numbers of fragments in monolayers previously left untreated (black symbols) or treated with the constructs specified in the inset (red for ΦsiRNA and blue for PKP2-siRNA). Samples that fragmented in more than 30 units are only reported as “>30.” Each data point corresponds to an individual experiment.
Online Figure V: Representative western blot of monolayers subjected to dispase assay under conditions of loss of AnkG expression. Samples of monolayers of NRVMs left UNT or treated with ΦsiRNA or AnkG-siRNA were probed for (A) AnkG and (B) tubulin as a loading control.
Online Figure VI: Sodium currents in NRVMs recorded ~5.5 days after introduction of AnkG silencing construct. A: Sodium current traces obtained from NRVMs treated either with ΦsiRNA or with AnkG-siRNA, as indicated. B: Sodium current density after loss of AnkG. C: Voltage dependence of steady-state inactivation. D: Recovery from Inactivation. E: Time course of current decay. INa,peak (pA/pF) p<0.01 for AnkG-siRNA vs ΦsiRNA. Other comparisons were not significant. N= 7, 6 and 7 for UNT, ΦsiRNA and AnkG-siRNA respectively. F: Western blot for AnkG corresponding to cells used for patch clamp experiments.
Online Figure VII: Representative immunoreactive signal for phalloidin in NRVMs treated with ΦsiRNA and AnkG-siRNA. Images acquired with 63X objective, bars=20µm.
Online Figure VIII: Representative western blot of monolayers that were subjected to optical mapping. Samples of monolayers of NRVMs left UNT or treated with ΦsiRNA or AnkG-siRNA were probed for AnkG (A) and tubulin (B) as a loading control.
Online Figure IX: Loss of AnkG expression and action potential propagation in NRVMs. A: Representative isochrone maps of action potential propagation obtained by optical mapping of monolayers treated with ΦsiRNA or AnkG-siRNA. Each line corresponds to 5ms. B: Plot of conduction velocity as a function of pacing frequencies. Red line, monolayers treated with ΦsiRNA; blue line, AnkG-siRNA. Numbers above each point represent number of cases where 1:1 propagation was maintained during pacing at that specific frequency. Total number of preparations tested was 6 and 8 for AnkG-siRNA and ΦsiRNA groups, respectively.
Online Figure X: Diagrammatic representation of the potential interactions between the VGSC, gap junctions, and desmosomes. Whether the interaction of AnkG with Cx43 and/or PKP2 is direct or indirect still remains to be determined.