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Interactions between Ankyrin-G, Plakophilin-2 and Connexin43 at the cardiac intercalated disc

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Abstract

Rationale—The early description of the intercalated disc defined three 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 (VGSC) 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 utilized a combination of microscopy, immunochemistry, patch clamp and optical mapping to assess the interactions between AnkG, plakophilin-2 (PKP2) and Connexin43 (Cx43). Co-immunoprecipitation studies from rat heart lysate demonstrated associations between the three molecules. Using siRNA technology we demonstrated that loss of AnkG expression caused significant changes in subcellular distribution and/or abundance of PKP2 and Cx43, as well as a decrease in intercellular adhesion strength and electrical coupling. Regulation of AnkG and of $Na_v1.5$ by PKP2 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 three complexes often considered independent: the VGSC, gap junctions and the cardiac desmosome. Possible implications to the pathophysiology of inherited arrhythmias (such as arrhythmogenic right ventricular cardiomyopathy; ARVC) are discussed.

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Keywords

Ankyrin-G; Plakophilin-2; Connexin43; Desmosomes; Gap Junctions.

INTRODUCTION

Intercellular communication is essential for proper cardiac function. Mechanical and electrical activities need to synchronize so that the work of individual myocytes transforms into the pumping function of the organ. Mechanical continuity is provided by desmosomes and adherens junctions, whereas gap junctions provide a pathway for passage of ions and small molecules between cells. These complexes preferentially reside at the site of end-end contact between myocytes, within the intercalated disc. Conventionally, the molecules of the intercalated disc have been considered separate entities. Yet, this view is rapidly changing. In 2004, Kaplan et al published breakthrough studies demonstrating that loss of molecular integrity of the desmosome leads to changes in gap junction plaque abundance.[1] Those studies were consistent with previous observations indicating that mechanical coupling is necessary for gap junction formation.[2-4] Cross-talk between molecules involved in cell-cell adhesion and those required for electrical coupling was further supported by our observations, followed by those of others, indicating that the desmosomal protein Plakophilin-2 (PKP2) co-precipitates with the gap junction protein connexin43 (Cx43), and that loss of PKP2 leads to gap junction remodeling.[3,5,6] An interaction between mechanical junctions and gap junctions has been considered an important substrate for arrhythmogenesis,[4] especially in cases of arrhythmogenic right ventricular cardiomyopathy (ARVC) associated with mutations in desmosomal genes.[1,7,8]

The first descriptions of the intercalated disc defined three structures, all involved in cell-cell communication.[9,10] Evidence demonstrates that molecules not involved in providing 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 $Na_V1.5$, the pore-forming subunit of the sodium channel most abundant in heart, localizes preferentially to sites of end-end contact.[11-13] Consistent with this observation, $Na_V1.5$ was found to co-precipitate with Cx43[14] and with the adherens junction protein N-cadherin.[14] More recently, we demonstrated that $Na_V1.5$ and PKP2 co-exist 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.[15] Overall, we speculate that desmosomes, gap junctions and sodium channels are not independent but rather, a “functional triad” where changes in composition of one can affect the function and integrity of the other, with significant consequences to electrical cardiac synchrony.

An important component of the VGSC complex is the cytoskeletal adaptor protein ankyrin-G (AnkG).[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 co-localizes with molecules involved in calcium homeostasis (see[22,23]). In contrast, AnkG localizes primarily (though not exclusively) to the intercalated disc.[20,21] The association of AnkG with $Na_V1.5$ is well documented. Yet, whether AnkG can partner with other complexes at the intercalated disc, and exert a functional effect on intercellular communication, remains undefined. The studies presented here 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 two fundamental functions of the intercalated disc: intercellular adhesion, and gap junction-mediated coupling. Finally, we demonstrate that loss of AnkG

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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 two 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 co-immunoprecipitation of proteins from rat adult heart lysates. Immunochemical and functional studies (patch clamp; optical mapping; intercellular adhesion strength) were conducted in neonatal rat ventricular myocytes (NRVMs), cultured in standard conditions. Method for intercellular adhesion strength was modified from[24] by adding blebistatin (10μM) to the media to prevent contraction. All methods followed those previously used in our laboratories.[3,15,24] Detailed descriptions in "Online Supplemental Material."

RESULTS

Physical interaction between AnkG, PKP2 and Cx43

Previous studies suggested that PKP2 and Cx43 co-exist in the same macromolecular complex.[3] Here, we confirm this observation and show co-immunoprecipitation 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 co-precipitate with AnkG. Co-precipitation of PKP2 with Cx43 was also observed, consistent with previous studies.[3] Similar results were obtained in four separate experiments. These data indicate a physical interaction (direct or indirect) between the three 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 pulldown 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 indicated a physical association of AnkG with PKP2. Next, 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 (ΦsiRNA) the expression of AnkG protein. Loss of AnkG expression was confirmed by conventional western blot (Figure 2A; collective data on AnkG-silencing efficiency, 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,C) or treated with the control construct (ΦsiRNA; Figure 2C, left) revealed the selective staining of bright contours of immunoreactive protein, surrounding areas mostly void of staining, co-localizing with AnkG. The areas of AnkG-PKP2 co-localization likely defined sites of cell-cell apposition, in agreement with previous observations (see also[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

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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 three 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 Figures 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 non-silencing construct (ΦsiRNA) 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 $Na_v1.5$. As shown in Figure 5A and 5B, loss of PKP2 expression did not affect $Na_v1.5$ 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 $Na_v1.5$ targeting,[19,20] although a direct role of PKP2 on $Na_v1.5$ localization cannot be ruled out (see also ref[25]).

AnkG and intercellular adhesion strength

The preferential localization of AnkG to the area of cell-cell apposition,[20,21] and its ability to interact with PKP2 in cardiac cells and with E-Cadherin in other systems,[26] 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 utilized 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 ΦsiRNA), cells lifted from the anchoring matrix as one sheet. Integrity of the single sheet was preserved even after 1-3 minutes of gentle agitation (Figure 6A). However, pre-treating 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,B; fragment quantification 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 to that recorded from untreated (UNT) monolayers, or from those treated with control (ΦsiRNA) construct. Data are presented in Figure 6B. Silencing was confirmed by conventional western blots (Online Figure V). These

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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] Here, we explored the effect of AnkG knockdown on abundance and localization of Cx43, and on the extent of electrical 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 associated with a significant reduction in the extent of electrical coupling between myocytes. These results correlated with a decrease in Cx43 abundance detected by immunofluorescence microscopy (Figure 7A, middle and right panels; corresponding co-staining of AnkG in Online Figure III-C). Overall, these data are similar to those previously obtained from cell systems, and from cardiac tissue, after loss of integrity of mechanical junctions,[1,3-5,7,29] and support the notion of AnkG as a functional element of cell-cell communication in the heart. Additional experiments confirmed that loss of AnkG expression caused a redistribution of $Na_v1.5$ (Figure 7B) and a reduction in sodium current amplitude (Online Figure VI), in agreement with previous studies.[20] Of note, staining of NRVMs with anti-phalloidin antibodies demonstrated general preservation of cytoskeletal architecture in AnkG-silenced cells (Online Figure VII). 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 electrical activity in all AnkG-knockdown preparations at stimulation frequencies of 8Hz or higher (asterisks), whereas a 1:1 ratio was maintained for 4 out 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. Here, we focused primarily on the association of AnkG with two molecules fundamental to intercalated disc function: PKP2 and Cx43. We further explored the relevance of AnkG expression to two of the primary functions of intercellular junctions: mechanical adhesion, and gap junction-mediated coupling. Finally, given that AnkG associates with more than one molecular complex important to cardiac electrophysiology, we assessed the overall impact of AnkG knockdown on the ability 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 mechanical and electrical coupling and as such, is a molecule relevant to impulse propagation in the heart.

Among the various molecules participating in mechanical coupling,[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.[15] Our data led us to hypothesize that a molecular interaction exists between PKP2

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and at least one component of the VGSC complex. The results presented here 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,[30] and PKP2 partners with multiple components of the desmosome and adherens junctions[33,34] as well as with other catalytic molecules.[33,35] Yet, it is important to emphasize that loss of AnkG expression had no noticeable impact on the abundance or subcellular 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 two molecules may share more than one partner and transient associations, particularly with catalytic molecules, are likely. In this temporally- and spatially-dynamic system, the links between two molecules in a living cardiac myocyte cannot be precisely defined. Our data, in combination with others,[14] do indicate that the molecular associations between intercellular junction proteins and components of the VGSC complex are strong enough to allow for co-precipitation. The data in Figure 1 of this study show that AnkG can co-precipitate with Cx43 and with PKP2 (even if N-cadherin was not found in the precipitate; see Online Figure I). Previously, we demonstrated the co-precipitation of PKP2 with Cx43[3] and with $Na_v1.5$.[15] Taken altogether, our data show the existence of inter-molecular associations between the three 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 I 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 positives 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.[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.[36] Much less is known, however, about the molecular steps that regulate AnkG abundance, and its localization. Here, 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 two 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 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

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organization of transmembrane proteins into a functional unit. The detailed molecular steps leading to the final organization of the intercalated disc, remains a matter of future investigation (see[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 three 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-cadherin[26] and with PKP2 (Figures 1-2), and ZO-1 is in fact, originally defined by its interaction with other junctional complexes.[40] As such, the current evidence blurs the lines that previously identified junctional complexes as 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 electrical 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 electrical 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 paper reported the segregation of $Na_v1.5$ to different "pools" within the cell, guided (at least in part) by the association of the channel molecule with location-specific scaffolding proteins (see[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 $Na_v1.5$ that localizes with the dystrophin/syntrophin complexes,[41] remains 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 electrical communication may be a major cause of ARVC-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 studies,[15] and those here, lead us to propose that loss of desmosomal integrity can affect sodium channel function, with deleterious consequences to the electrical 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 Brugada syndrome patients.[45] Yet, we do recognize that our studies are conducted in isolated cell systems, far from conditions that can be directly comparable to those of a diseased heart. Whether 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,[46] but also in acquired conditions (cardiomyopathies) affecting the integrity of the intercalated disc.[47]

Supplementary Material

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Non-Standard Abbreviations and Acronyms

ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy
AnkG	Ankyrin-G
AnkG-siRNA	AnkG knockdown
Cx43	Connexin43
Hz	Hertz
Na_v1.5	Alpha-subunit of the sodium channel, 1.5
NRVM/s	Neonatal Rat Ventricular Myocytes
PKP2	Plakophilin 2
PKP2-siRNA	PKP2 knockdown
PG	Plakoglobin
ΦsiRNA	Non-target knockdown
UNT	Untreated cells
VGSC	Voltage-Gated Sodium Channel Complex
ZO-1	Zona Occludens-1

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NOVELTY AND SIGNIFICANCE

What is known?

- The voltage–gated sodium channel (VGSC) complex localizes preferentially at the intercalated disc. Ankyrin-G (AnkG) is a part of this complex.
- Also resident of the intercalated disc are desmosomes, and gap junctions.
- Mutations in desmosomal molecules are known to be responsible for most familial cases of arrhythmogenic right ventricular cardiomyopathy (ARVC).

What new information does this article contribute?

- AnkG associates with plakophilin-2 (PKP2), a molecule of the desmosome, and with Cx43, the major cardiac gap junction protein.
- Loss of AnkG expression weakens intercellular adhesion, and reduces gap junction-mediated coupling.
- Loss of PKP2 disrupts AnkG and sodium channel localization and decreases AnkG abundance.
- PKP2 expression is relevant for stability of the VGSC complex. AnkG is, in turn, an important component of the molecular complexes that maintain mechanical and electrical continuity in the heart.

The early description of the intercalated disc defined three 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 (VGSC), which includes as a component the cytoskeletal adaptor protein ankyrin-G (AnkG). For the most part, the function of the VGSC has been considered independent from that of other structures involved in cell-cell communication. Yet, this view is rapidly changing. Our data show that PKP2, a molecule of the desmosome, can affect the integrity and the function of the VGSC; conversely, loss of expression of AnkG 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. The possible implications of these results on the pathophysiology of cardiac arrhythmias, with particular focus on ARVC, is discussed.

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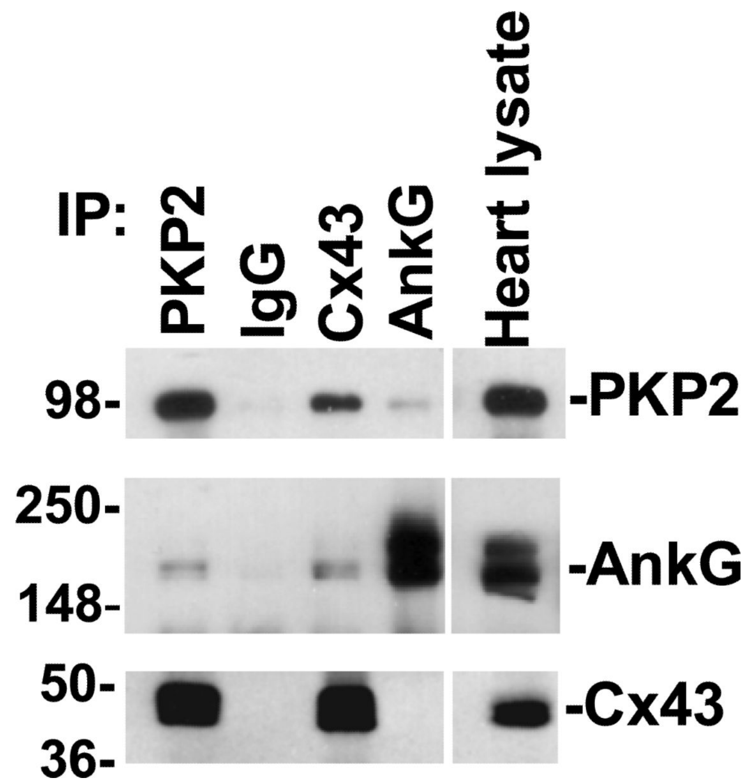

Figure 1.

Immunoblots of PKP2, AnkG, and Cx43 (top to bottom) from samples exposed to protein A/G beads coated with PKP2, rabbit IgG (negative control), Cx43, or AnkG antibodies. Heart lysate (far right lane) used as positive control.

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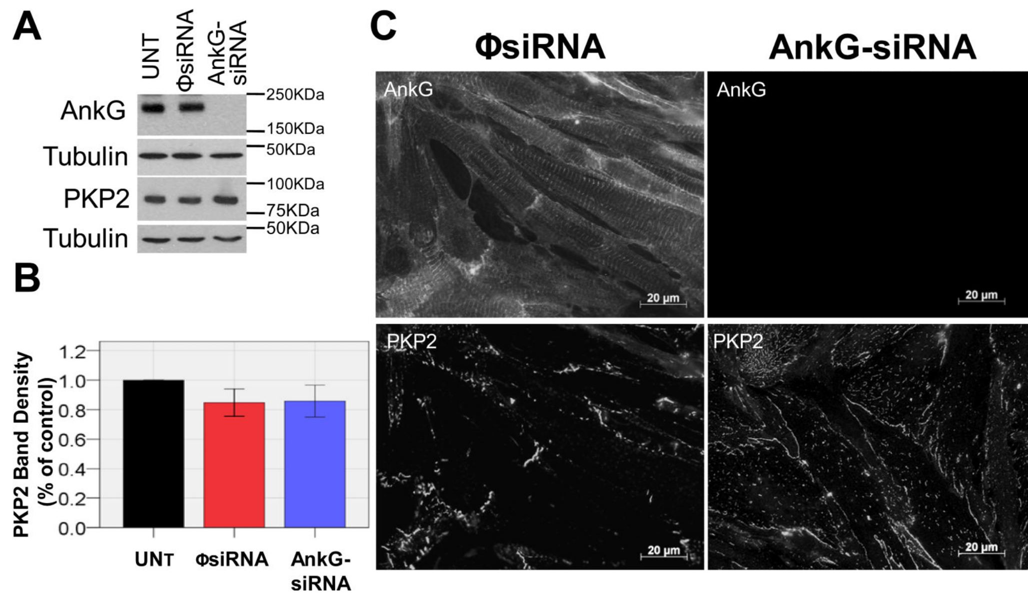

Figure 2.

Loss of AnkG expression leads to PKP2 relocalization. Panel **A**: Western blot of NRVMs untreated (UNT) or treated with an oligonucleotide that prevents, or fails to prevent AnkG expression (AnkG-siRNA and ΦsiRNA). Cumulative data in **B**. All measurements normalized to those from untreated cells in same blot (n=8; p=NS ΦsiRNAvsAnkG-siRNA). **C**: Immunolocalization of AnkG (top) and PKP2 (bottom) immunoreactive proteins in cells treated with control oligonucleotide (ΦsiRNA) or AnkG-siRNA. Loss of AnkG expression (right column) led to PKP2 redistribution. Bars, 20μm.

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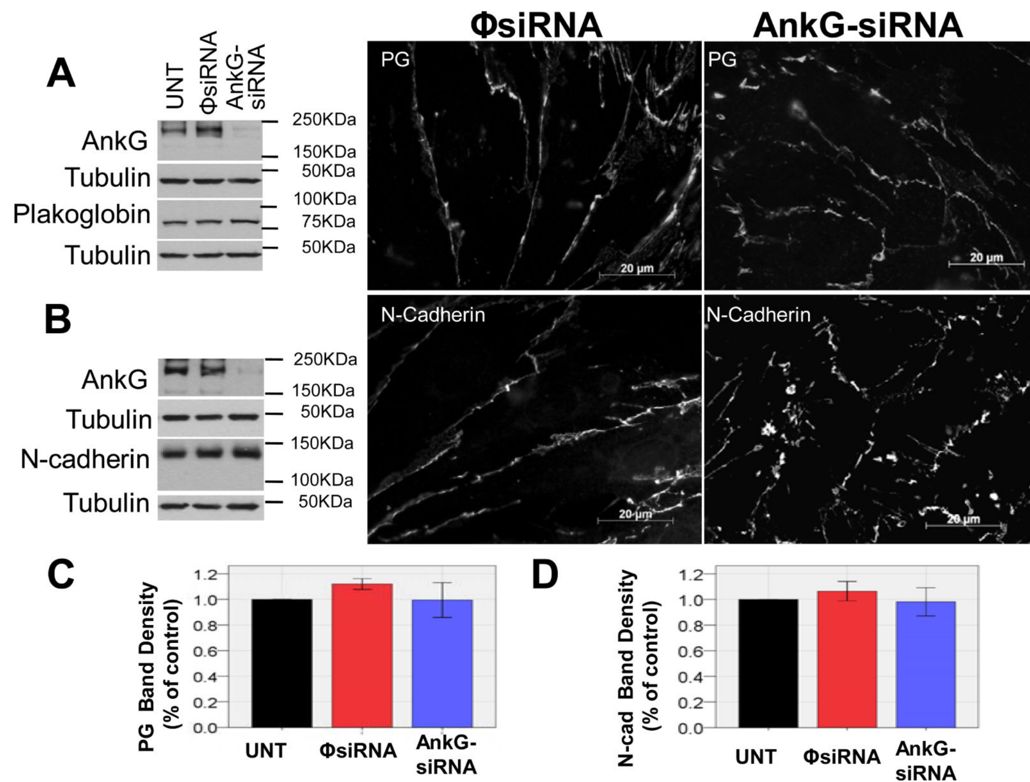

Figure 3.

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 used for each blot as loading control. Center and right: immunodetection of plakoglobin (PG) in cells treated with control (ΦsiRNA) or AnkG-silencing constructs (AnkG-siRNA). **B**: Similar conditions. Detection of N-cadherin-immunoreactive proteins. **C**-**D**: Quantification of corresponding Western blots. All measurements normalized to those obtained from untreated cells in same blot. In both cases, n=6; p=NS ΦsiRNAvsAnkG-siRNA. Bars, 20µm.

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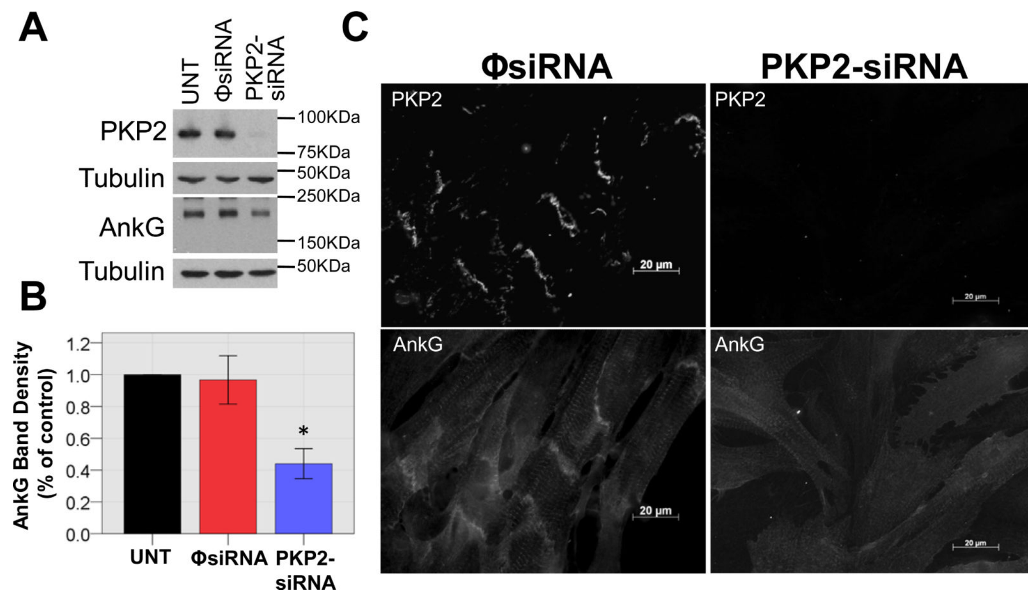

Figure 4.

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 with non-silencing construct (ΦsiRNA). **B:** Quantification of AnkG band density, corrected individually by loading control and normalized to UNT (n=5; p<0.05 ΦsiRNA vs PKP2-siRNA). **C:** Immunolocalization of PKP2 (top) and AnkG (bottom) in NRVMs treated with ΦsiRNA or PKP2-siRNA. Bars=20µm.

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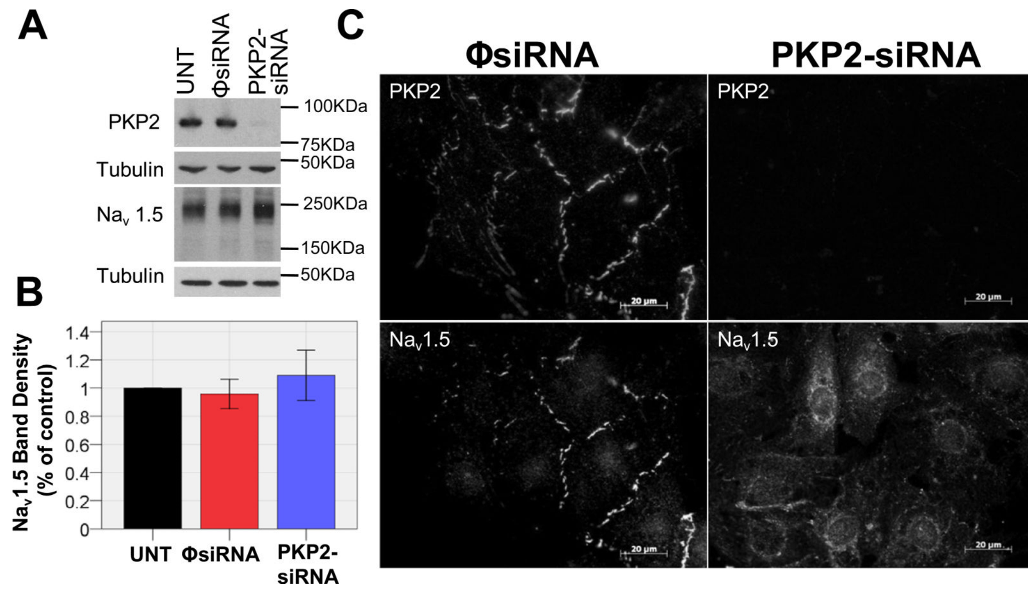

Figure 5.

Loss of PKP2 expression leads to Na$_v$1.5 remodeling. **A:** Western blot for PKP2 and Na$_v$1.5 with respective tubulin loading controls in NRVMs untreated (UNT), treated with ΦsiRNA, or PKP2-siRNA. **B:** Quantification of Na$_v$1.5 band density (n=6; pNS; ΦsiRNA vs PKP2-siRNA). **C:** Immunolocalization of PKP2 (top) and Na$_v$1.5 (bottom) in NRVMs treated with ΦsiRNA or PKP2-siRNA. Bars=20μm.

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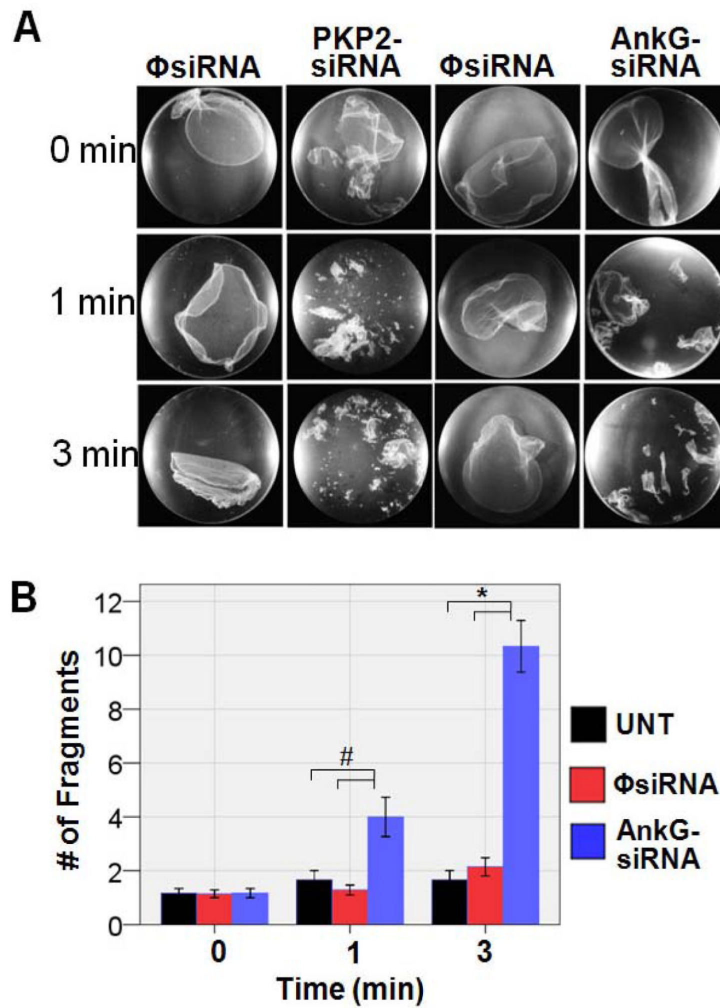

Figure 6.

Loss of AnkG expression weakens intercellular adhesive strength. Cells treated with 1.2-2.4 U/mL dispase 4h to release them from attachment to matrix. **A**: Pictures of monolayers after dispase treatment (time zero) and after 1 and 3 minutes of gentle shaking (field diameter, 35mm). **B**: Bars indicate number of fragments found at different times in experiment. An intact, lifted monolayer was counted as 1 fragment; UNT n=6, ΦsiRNA n=7, AnkG-siRNA n=6 (#, $p<0.05$; *, $p<0.01$).

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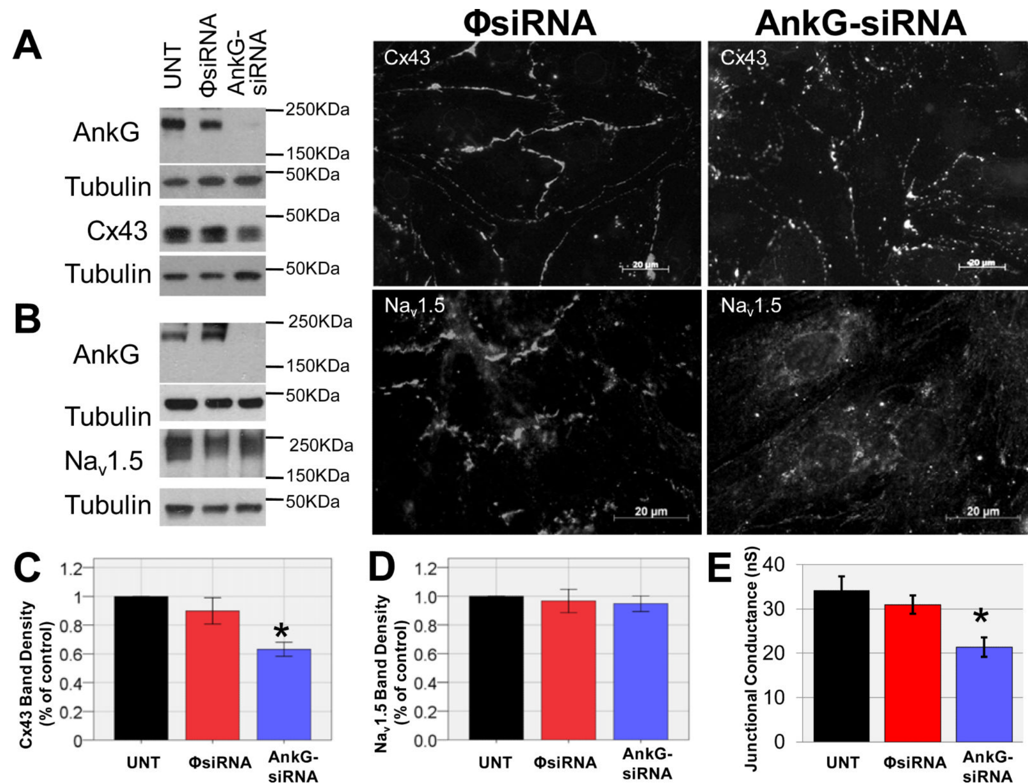

Figure 7.

Role of AnkG expression on abundance/localization of Cx43 and $Na_v1.5$. **A and B**: Western blot (left) and immunoreactive signal (center; right) in cells treated with ΦsiRNA (non-silencing control) or AnkG-siRNA. Tubulin used as Western loading control. UNT: Untreated cells. Cells 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 vs ΦsiRNA; $p<0.01$ AnkG-siRNA vs 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=5,5,6 for UNT, ΦsiRNA, 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=20µm.