Ankyrin-G Coordinates Intercalated Disc Signaling Platform to Regulate Cardiac Excitability In Vivo

Michael A. Makara, Jerry Curran, Sean C. Little, Hassan Musa, Iuliia Polina, Sakima A. Smith, Patrick J. Wright, Sathya D. Unudurthi, Jed Snyder, Vann Bennett, Thomas J. Hund, Peter J. Mohler

Rationale: Na_1.5 (SCN5A) is the primary cardiac voltage-gated Na channel. Na_1.5 is critical for cardiac excitability and conduction, and human SCN5A mutations cause sinus node dysfunction, atrial fibrillation, conduction abnormalities, and ventricular arrhythmias. Further, defects in Na_1.5 regulation are linked with malignant arrhythmias associated with human heart failure. Consequently, therapies to target select Na_1.5 properties have remained at the forefront of cardiovascular medicine. However, despite years of investigation, the fundamental pathways governing Na_1.5 membrane targeting, assembly, and regulation are still largely undefined.

Objective: Define the in vivo mechanisms underlying Na_1.5 membrane regulation.

Methods and Results: Here, we define the molecular basis of an Na channel regulatory platform in heart. Using new cardiac-selective ankyrin-G^{-/} mice (conditional knock-out mouse), we report that ankyrin-G targets Na_1.5 and its regulatory protein calcium/calmodulin-dependent kinase II to the intercalated disc. Mechanistically, β_IV- spectrin is requisite for ankyrin-dependent targeting of calcium/calmodulin-dependent kinase II-δ; however, δ-spectrin is not essential for ankyrin-G expression. Ankyrin-G conditional knock-out mouse myocytes display decreased Na_1.5 expression/membrane localization and reduced I_{Na} associated with pronounced bradycardia, conduction abnormalities, and ventricular arrhythmia in response to Na channel antagonists. Moreover, we report that ankyrin-G links Na channels with broader intercalated disc signaling/structural nodes, as ankyrin-G loss results in reorganization of plakophilin-2 and lethal arrhythmias in response to β-adrenergic stimulation.

Conclusions: Our findings provide the first in vivo data for the molecular pathway required for intercalated disc Na_1.5 targeting/regulation in heart. Further, these new data identify the basis of an in vivo cellular platform critical for membrane recruitment and regulation of Na_1.5. (Circ Res. 2014;115:929-938.)

Key Words: ankyrin ■ arrhythmia (mechanisms) ■ cell biology ■ mouse mutant ■ Nav1.5 ■ protein trafficking ■ sodium channels ■ targeting

SCN5A-encoded Na_1.5 is the principal voltage-gated Na channel in heart. Na_1.5 regulates the rapid upstroke of the cardiac action potential, and human SCN5A mutations are linked with multiple forms of human cardiovascular disease, including sinus node dysfunction, atrial fibrillation, conduction defects, and ventricular arrhythmias. Na_1.5 dysfunction is further linked with arrhythmias associated with acquired heart failure. Based on the role of Na_1.5 in health and disease, therapies to target select Na_1.5 properties have remained at the forefront of cardiovascular medicine. Unfortunately, the molecular pathways underlying Na_1.5 regulation remain largely undefined partially because of lack of essential in vivo data.

Editorial, see p 901

In This Issue, see p 897

Na_1.5 is principally regulated by membrane voltage. However, more recent data demonstrate that Na_1.5 is secondarily modulated by the calcium/calmodulin–dependent kinase IIδ (CaMKIIδ) for acute action potential modulation and propagation. Importantly, elevated CaMKII activity in heart disease is associated with increased pro-arrhythmic Na_1.5-dependent late sodium current (I_{Na,L}). The mechanisms underlying CaMKIIδ-dependent regulation of Na_1.5 were unknown until recent work revealed phosphorylation of the channel (Na_1.5 serine 571) at baseline and hyper-phosphorylation of the channel in disease. However, the
cellular pathways underlying this local regulation have remained undefined.

Ankyrin polypeptides play critical roles in ion channel and transporter targeting in excitable and nonexcitable cells. Ankyrin-R (ANK1) links membrane transporters to the cytoskeleton in erythrocytes, and human ANK1 mutations cause hereditary spherocytosis. Ankyrin-B (ANK2) is critical for ion channel and transporter targeting in heart, brain, and pancreas, and ankyrin-B dysfunction has been linked with sinus node disease, atrial fibrillation, ventricular arrhythmia, and diabetes mellitus. Finally, ankyrin-G (ANK3) plays central roles for ion channel targeting in neurons. Although in vitro work supports an association between ankyrin-G and Na1.5 in heart, little is known about the role of this complex in vivo. Further, a link between ankyrin-G/Na1.5 and CaMKII δ is undefined. Finally, the functional pathophysiological consequences for disrupting any of these putative complexes are unknown.

We report the molecular basis of a novel signaling platform in heart that couples CaMKII δ with Na1.5. Our in vivo data demonstrate that ankyrin-G serves as an intercalated disc receptor for both Na1.5 and βν spectrin, a molecule originally identified in brain and linked with neurological disease. Mice harboring a conditional null allele for ankyrin-G in heart (cKO) are surprisingly viable, but display decreased Na1.5 expression, membrane localization, and I Na,L associated with bradycardia, conduction abnormalities, QRS prolongation, and ventricular arrhythmias in response to NaL channel antagonists. Further, ankyrin-G cKO mice show loss of βν spectrin recruitment to the intercalated disc membrane. βν spectrin C-terminal domain associates with CaMKII δ, and ankyrin-G cKO mice, as well as βν spectrin mutant mice lacking the C-terminal domain (qv4J), show defects in CaMKII δ targeting and CaMKII δ-dependent regulation of I Na,L. Finally, we report that the ankyrin-G–dependent protein platform links NaL channels with broader intercalated disc signaling/structural nodes, as in vivo ankyrin-G loss results in remodeling of plakophilin-2, a resident desmosome protein critical for intercalated disc integration with the intermediate filament-based cytoskeleton. Together, our findings identify a novel molecular platform critical for the membrane recruitment and regulation of Na1.5 in heart. These findings further provide new insight into the pathways underlying cardiac excitability in health and disease.

Methods

Biochemistry

Immovilized glutathione-S-transferase-fusion proteins were incubated with 100 μg left ventricular heart lysate overnight in pull-down buffer at 4°C. The samples were washed 3× in pull-down buffer, eluted, and proteins were separated by SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis). The gels were transferred to nitrocellulose and immunoblotted. Nitrocellulose blots were included to stain nuclei.

Results

Generation of Mice With Cardiac-Specific Deletion of Ankyrin-G

Based on its role in assembly of excitable domains in the nervous system, and in vitro links with Na1.5 in myocytes, we hypothesized that ankyrin-G may serve as a molecular platform for cardiac Na1.5 signaling. To test this role in vivo, we

Figure 1. Generation of cardiac-specific ankyrin-G null mouse. A, Scheme for generation of cardiac conditional ankyrin-G null mouse (cKO). LoxP sites were inserted to flank exons 22 to 23 in ankyrin-G gene (Ank3). Mice homozygous for loxP insertion (f/f) were crossed with mice expressing Cre recombinase under control of the αMHC promoter. Cre-mediated excision of exons 22 to 23 resulted in the production of a premature stop codon after exon 21. B, Flox (f) allele (434 bp) and wild-type (WT; +) allele (366 bp) identified by PCR. C, Cre expression (+ Cre) evidenced by band at 300 bp not observed in WT animals (− Cre) and in no template control (Ctrl). D–E, Ankyrin-G expression in WT and cKO mouse tissue lysates. Note that unlike heart, other tissues examined express multiple molecular weight forms of ankyrin-G. F, Data from experiments in D–E were quantified and expressed relative to WT tissue (corrected for actin expression; n=4/genotype, P<0.05). G–H, Expression of ankyrin-G (green) and N-cadherin (red) in WT and cKO myocytes (Bars=10 μm; arrow notes intercalated disc). DAPI (blue) was included to stain nuclei.
generated a conditional null mutant allele where exons 22 to 23 the mouse ankyrin-G gene (Ank3) is flanked by LoxP sites (Ank3f/f) and therefore are deleted in the presence of Cre recombinase (Figure 1A–1C). We selectively eliminated ankyrin-G in postnatal cardiomyocytes by using αMHC-Cre knock-in mice; homozygous conditional knockout mice are referred to as αMHC-Cre; Ank3f/f or cKO.αMHC-Cre; Ank3f/f wild-type (WT) age- and sex-matched littermates were used as control mice. Surprisingly, cKO mice were viable, displayed no gross differences in size, weight, feeding, and grooming, and showed no apparent deficits in motor function, unlike mice harboring selective deletion of cerebellar ankyrin-G.20 Immunoblots from whole heart lysates showed elimination of ankyrin-G in cKO heart (Figure 1D). Selective loss of ankyrin-G in the heart was confirmed by immunoblot from cortex, cerebellum, skeletal muscle, and kidney of control and cKO mice where we observed no difference in ankyrin-G expression (Figure 1E and 1F). At the level of the single ventricular myocyte, ankyrin-G is enriched at the intercalated disc along with N-cadherin (Figure 1G). Ankyrin-G expression at the intercalated disc (and minor population at transverse-tubule) was eliminated from cKO ventricular myocytes (Figure 1H).

**Ankyrin-G cKO Mice Display Abnormal Na, Channel Targeting and Function**

Ankyrin-G is linked with voltage-gated Na channel function in cerebellum.20 We, therefore, examined Na, 1.5 expression, localization, and function in ankyrin-G cKO hearts. We observed a significant decrease in Na, 1.5 expression in cKO hearts by immunoblot (Figure 2A and 2B; P<0.05). Although primarily localized to the intercalated disc, Na, 1.5 is also found in secondary populations at the peripheral sarcolemma.23,24 In line with immunoblot data, Na, 1.5 expression was significantly reduced in cKO compared with WT myocytes (Figure 2C and 2D). Moreover, consistent with the localization of ankyrin-G, we observed selective loss of Na, 1.5 from the intercalated disc in cKO myocytes (Figure 2C–2F). Na, 1.5 immunostaining at the peripheral sarcolemma in cKO myocytes was unaffected (Figure 2C–2F). However, we observed populations of intracellular Na, 1.5-positive puncta in ankyrin-G cKO myocytes (Figure 2F). This loss was specific for Na, 1.5 as expression levels of Ca, 1.2, Na/Ca exchanger 1, and ankyrin-B were unchanged between WT and cKO hearts (Online Figure I). Consistent with these data, we observed =50% reduction in peak INa in cKO myocytes compared with myocytes from WT littermates (Figure 2G–2J). Although INa was reduced in cKO myocytes, we observed no difference in Na, 1.5 steady-state voltage-dependent inactivation or recovery from inactivation (Online Figure II). Finally, consistent with prior findings in Scn5a-/- mice,25 loss of INa in cKO myocytes resulted in a significant decrease in action potential amplitude and maximum upstroke velocity (Vmax; Online Figure III). Together, these findings demonstrate an in vivo requirement of ankyrin-G for cardiac Na, 1.5 membrane expression and function.

**Ankyrin-G Recruits βIV Spectrin to the Myocyte Intercalated Disc**

Ankyrin partners with spectrin polypeptides to bridge membrane and cytoskeletal structures.26 In the nervous system, although ankyrin-G and βIV spectrin are required for assembly of the axon initial segment, ankyrin-G seems to serve as the primary organizing protein. To investigate the functional relationship between ankyrin-G and βIV spectrin in heart, we
tested the localization of \( \beta_{IV} \) spectrin in ankyrin-G cKO mice. In WT myocytes and heart, \( \beta_{IV} \) spectrin was concentrated at the intercalated disc membrane (Figure 3; Online Figure IV).\(^8\) However, \( \beta_{IV} \) spectrin was nearly absent from the intercalated disc membrane of cKO myocytes (Figure 3; Online Figure IV). Instead, we observed populations of \( \beta_{IV} \) spectrin-positive puncta near the perinuclear region in cKO myocytes (Figure 3). Thus, ankyrin-G is required for the recruitment of \( \beta_{IV} \) spectrin to the myocyte intercalated disc.

\( \beta_{IV} \) Spectrin Is Not Required for Ankyrin-G or Nav1.5 Targeting in Myocytes

We tested the converse requirement of \( \beta_{IV} \) spectrin for ankyrin-G and Nav1.5 targeting in heart. For these experiments, we used a \( \beta_{IV} \) spectrin mutant mouse model (qv4J mice) harboring a premature stop codon in the 10th spectrin repeat, resulting in a truncated polypeptide lacking ankyrin-G-binding activity (located in 15th repeat spectrin; see Figure 4A).\(^{18,19}\) As expected, a glutathione-S-transferase-\( \beta_{IV} \) spectrin fusion protein harboring the qv4J mutation lacked binding activity for ankyrin-G and Nav1.5 (Figure 4B). In contrast to findings in neurons,\(^{27} \) qv4J myocytes displayed no significant difference in ankyrin-G or Nav_1.5 expression compared with control hearts by immunoblot (Figure 4C and 4D). Further, we observed no difference in intercalated disc proteins N-cadherin or \( \beta \)-catenin in qv4J hearts (Figure 4C and 4D). In line with immunoblot data, we observed no difference in ankyrin-G or Nav1.5 localization at the intercalated disc between control and qv4J myocytes (Figure 4E–4H). However, consistent with the role of the \( \beta_{IV} \) spectrin C-terminus in CaMKII\(\delta\) targeting,\(^4\) qv4J myocytes displayed a significant decrease in CaMKII\(\delta\) expression (Figure 4C and 4D; \( P<0.05 \)). In summary, our findings define a requirement of ankyrin-G for \( \beta_{IV} \) spectrin targeting to the intercalated disc, whereas ankyrin-G is targeted to the disc independent of \( \beta_{IV} \) spectrin-binding.

Ankyrin-G Recruits CaMKII\(\delta\) to the Myocyte Intercalated Disc

\( \beta_{IV} \) spectrin, via a short C-terminal motif, associates with CaMKII\(\delta\).\(^3\) \( \beta_{IV} \) spectrin mutant mice lacking this C-terminal motif display aberrant CaMKII\(\delta\) intercalated disc targeting.\(^4\) Based on the loss of \( \beta_{IV} \) spectrin in ankyrin-G cKO hearts (Figure 3), we hypothesized that ankyrin-G cKO hearts would display decreased CaMKII\(\delta\) expression and abnormal intercalated disc targeting. We observed reduced CaMKII\(\delta\) expression by immunoblot in cKO versus control hearts (Figure 5A and 5B). In cKO heart, we observed selective loss of CaMKII\(\delta\) at the intercalated disc (location of \( \beta_{IV} \) spectrin) versus other myocyte membrane populations (ie, transverse-tubules, Figure 5C and 5D; Online Figure V). Thus, ankyrin-G, via \( \beta_{IV} \) spectrin, controls the intracellular targeting of CaMKII\(\delta\) to the intercalated disc.

Ankyrin-G Recruits CaMKII\(\delta\) to Regulate Na_1.5 Phosphorylation

In response to sympathetic stimulation, CaMKII\(\delta\) phosphorylates Na_1.5 via serine 571 to regulate Na_1.5-dependent late current (\( I_{Na,L} \)).\(^{1,26} \) Ankyrin-G recruits both Na_1.5 and CaMKII\(\delta\) to the intercalated disc (Figures 2 and 5). In line with these data, we observed reduced Na_1.5 serine 571 phosphorylation by both immunoblot and immunostaining in cKO myocytes compared with control cells (Online Figure VIA–VID). When corrected for peak \( I_{Na,L} \), we did not observe differences in \( I_{Na,L} \) between WT and cKO myocytes at baseline (Online Figure VII). However, consistent with the loss of CaMKII\(\delta\)-dependent regulation of \( I_{Na,L} \) in the absence of ankyrin-G, we observed a significant difference in isoproterenol-induced-\( I_{Na,L} \) between WT and cKO myocytes (Online Figure VII). Specifically, although WT myocytes displayed nearly a 2-fold increase in \( I_{Na,L} \) in response to isoproterenol, we observed no statistical change in \( I_{Na,L} \) in cKO myocytes:isoproterenol, regardless of the voltage (Online Figure VII; \( P<0.05 \)). Our combined data support an in vivo ankyrin-G–based platform with dual roles for Na_1.5 channel recruitment and CaMKII\(\delta\)-dependent regulation.

Ankyrin-G cKO Mice Display Bradycardia, Conduction Defects, and Arrhythmia

Impaired Na_1.5 function is associated with phenotypes of sinus node dysfunction, conduction defects, and ventricular arrhythmia.\(^1\) We, therefore, tested the role of the ankyrin-G–dependent disc platform for cardiac electric regulation. Conscious ankyrin-G cKO mice monitored by ECG telemetry exhibited significant reduction in resting heart rate compared with control mice (Figure 6A–6C). Impaired atrioventricular (AV) conduction in cKO mice was also readily apparent in cKO mice as noted by an increase in PR interval compared with control mice (Figure 6D–6F); intra-atrial conduction also reduced as evidenced by increased P wave duration, \( P<0.05 \). Moreover, cKO mice displayed a significant increase in the QRS interval, consistent with delayed intraventricular conduction (Figure 6D, 6E, and 6G). In summary, in vivo findings strongly support a role of the ankyrin-G–based protein complex in regulation of Na_1.5 channel function and cardiac excitability.

Flecainide Induces Bradycardia, QRS Prolongation, and Arrhythmia in cKO Mice

Class 1C anti-arrhythmics (Na_1.5 channel antagonists) may be used in clinical practice to identify individuals with Na_1.5 channel abnormalities.\(^{29} \) Moreover, these compounds (eg, flecainide) have been used to uncover Na_1.5 channel-based arrhythmia mechanisms in animal models.\(^{30,31} \) To test the functional relevance of Na_1.5 channel dysfunction in the ankyrin-G cKO mouse, we analyzed ECGs of control and cKO mice after flecainide administration.\(^{32,33} \) At baseline, cKO mice displayed...
bradycardia compared with control mice (Figure 7A, 7B, and 7E). Flecainide reduced heart rate in both control and cKO mice (Figure 7A–7E). However, the effect of flecainide on heart rate was significant and potentially pathological in cKO mice as this intervention resulted in an overall ≈30% decrease in heart rate compared with a normal mouse at baseline (Figure 7E; P<0.05). Further, in cKO but not control mice, flecainide caused AV block and ventricular arrhythmia (Figure 7F–7H). Specifically, 10% of control mice displayed AV block in response to flecainide, whereas we recorded AV

Figure 4. Ankyrin-G targets Na$_{1.5}$ to the intercalated disc independent of βIV spectrin. A Diagram of βIV spectrin domains and location of qv$^{4J}$ truncation upstream of ankyrin-G-binding site. B, Glutathione-S-transferase (GST)-βIV spectrin associates with Na$_{1.5}$ (via ankyrin-G). βIV spectrin representing the qv$^{4J}$ mouse truncation lacks binding activity for ankyrin-G and thus Na$_{1.5}$. C–D, WT and qv$^{4J}$ hearts display no difference in expression of ankyrin-G or Na$_{1.5}$ by immunoblot. CaMKIIδ levels are reduced in cKO hearts consistent with qv$^{4J}$ allele lacking the CaMKIIδ-binding site (see A). Levels in C are normalized for actin expression (n=4/genotype; P<0.05). E–H, WT and qv$^{4J}$ myocytes display no difference in ankyrin-G or Na$_{1.5}$ localization. Bar=10 μm.
block in ≈73% of cKO mice (Figure 7F–7G; P<0.05). Of the mice showing AV block, the incidence of block was >140-fold in cKO mice (control, 0.019±0.019 episodes/min; cKO, 2.75±0.98 episodes/min; P<0.05). Moreover >80% of cKO mice showed arrhythmia, including bigeminy and nonsustained ventricular arrhythmia, compared with 10% of control mice (Figure 7H; P<0.05). Consistent with conscious ECG recordings, anesthetized cKO mice displayed prolonged QRS interval compared with control mice (Online Figure VIII). This QRS interval difference between genotypes was further accentuated by flecainide administration (Online Figure VIII; P<0.05). Ankyrin-G cKO mice also showed increased PR interval duration after flecainide (Online Figure IX; P<0.05). Together, these data strongly support the role of ankyrin-G-protein complex in Na+, channel regulation in vivo, as well as link Na+ channel-based mechanisms with observed cardiac electric phenotypes in the ankyrin-G cKO mouse.

Ankyrin-G cKO Mice Display Defects in Plakophilin-2 Expression and Cellular Organization

Beyond membrane ion channels, in vitro work supports roles of ankyrin-G for membrane protein regulation at cell junctions.17,34 We therefore tested the in vivo requirement of ankyrin-G for expression and localization of key intercalated disc proteins as well as Na+ β-subunits that have been previously linked with ankyrin polypeptides.35 Notably, we observed no difference in expression or localization of disc proteins N-cadherin and β-catenin, or Na+ channel β2 and β4 subunits between control and cKO hearts (Figure 8A–8K; Online Figure X). In contrast, plakophilin-2 showed a 50% increase in expression in cKO hearts (Figure 8A and 8B). Further, we observed a striking redistribution of plakophilin-2 from the intercalated disc of cKO myocytes to the cytosolic regions of cKO myocytes (Figure 8K and 8L; Online Figure
XI). Although prior work proposed a link for ankyrin-G in targeting of connexin43,17 we observed no difference in connexin43 or ZO-1 expression by immunoblot (Figure 8C and 8D). Together, our new in vivo data clearly support a role of ankyrin-G in intercalated disc protein organization.

Ankyrin-G cKO Mice Display Catecholamine-Induced Arrhythmia and Death

Ankyrin-G cKO mice display several pro-arrhythmic ECG phenotypes associated with reduced $I_{Na}$ (Figures 6 and 7). However, based on the additional observed molecular defects in ankyrin-G cKO animals (Figure 8), we tested cKO animals for arrhythmia susceptibility in response to elevated adrenergic stimulation. Ankyrin-G cKO mice showed arrhythmia phenotypes after a standard intraperitoneal epinephrine injection protocol to mimic catecholaminergic stress (Online Figure XII).12 Specifically, the majority (=62.5%) of cKO mice displayed multiple instances of ventricular arrhythmia, and ≈38% of cKO mice died of malignant arrhythmias after this protocol (Online Figure XII; 3/8 cKO versus 0/6 control; $P<0.05$). The cKO mice also displayed decreased AV conduction compared with WT mice (increased PR interval; $P<0.05$; n=5/genotype) in these experiments. Examples of sustained premature ventricular contractions and polymorphic ventricular arrhythmia in cKO mice are shown in Online Figure XIIB and XIIC. We recorded no incidence of ventricular arrhythmia (0%) or death (0%; Online Figure XIIA and XIIIB) in WT animals using this protocol. In summary, we conclude that loss of the cardiac ankyrin-G–based protein platform results in pro-arrhythmic ECG phenotypes at rest and arrhythmia and death in response to catecholaminergic stress. Of note, as catecholamine-based arrhythmias in cKO mice were observed in the absence of elevated CaMKIIδ or $I_{Na,L}$, we tested for potential alterations in myocyte calcium handling in cKO myocytes. Consistent with whole animal data (eg, premature ventricular contractions), cKO myocytes displayed increased incidence of spontaneous Ca waves compared with WT myocytes (Online Figure XIII; $P<0.05$).

Ankyrin-G cKO Mice Display Cardiac Structural Phenotypes

Ankyrins are linked with assembly and maintenance of structural domains in erythrocytes, epithelia, and neurons.36–38 Further, our data, and earlier data from Delmar and colleagues,17 link ankyrin-G with plakophilin-2, a protein associated with cardiac structural remodeling in humans and mice.31,39,40 We, therefore, examined ankyrin-G hearts for changes in cardiac structure or function at baseline and in aging. At 8 weeks, we observed no difference in heart weight/body weight (or tibia length) or systolic or diastolic wall thickness between WT and cKO mice (Online Figure XIV). Although we observed a nonsignificant trend for increased systolic and diastolic LV diameter in cKO mice (p=NS), cKO mice displayed a small, but significant decrease in ejection fraction compared with WT littermates (Online Figure XIV). Notably, cKO mice displayed obvious cardiac phenotypes with age (≈9 months) as indicated by a further depression in ejection fraction, increase in systolic and diastolic chamber diameters, and reduction of both the anterior and posterior wall thickness compared with WT littermates (Online Figure XV).

Discussion

Voltage-gated $Na_\text{v}$ channels are critical for the rapid upstroke of the cardiac action potential and cardiac conduction. Defects in $Na_\text{v}$ channel function are linked with a host of congenital and acquired forms of human disease, including sinus node dysfunction, atrial fibrillation, conduction disorders, ventricular arrhythmia, and heart failure.1–3 Despite these compelling human disease linkages and decades of elegant $Na_\text{v}$ channel biophysical studies, the field still lacks significant fundamental knowledge of the regulatory mechanisms governing the function of this critical molecule. Here we use in vivo animal...
models to define the cellular pathway underlying the targeting of Na\(_{v}\)1.5 and its regulatory molecule, CaMKII\(\delta\), to the intercalated disc membrane. Ankyrin-G, a cytoskeletal adapter protein, associates with Na\(_{v}\)1.5 and recruits the channel to the myocyte membrane. Loss of ankyrin-G results in defects in Na\(_{v}\) channel expression, localization, and function. We further show that ankyrin-G, via the recruitment of \(\beta_{IV}\) spectrin, also targets CaMKII\(\delta\) to the intercalated disc. As CaMKII\(\delta\) phosphorylates Na\(_{v}\)1.5 to modulate cardiac myocyte excitability in health and disease,\(^{8,28}\) our findings provide data that support the mechanisms underlying the biogenesis of this membrane signaling domain. Finally, our findings confirm in vivo protein pathways for ankyrin-G and Na\(_{v}\)1.5 in heart. Specifically, as described by Delmar and colleagues\(^{17}\) in cultured myocytes, our in vivo data link ankyrin-G with the desmosomal protein plakophilin-2. Further, our data associate ankyrin-G with the disc protein \(\beta_{IV}\) spectrin.

Ankyrin-G cKO mice were surprisingly viable, but display bradycardia, AV conduction defects, QRS prolongation, and arrhythmia associated with flecainide or epinephrine. Consistent with our proposed mechanism of I\(_{Na}\) dysfunction in these animals, Na\(_{v}\)1.5 is linked with impulse propagation through the sinoatrial node,\(^2\) the AV node,\(^{41}\) and the ventricular myocardium.\(^{42}\) Prior in vivo work links Na\(_{v}\)1.5 dysfunction with bradycardia and reduced sinoatrial node pacemaker potential.\(^{42}\) Moreover, consistent with our findings, flecainide has been previously shown to evoke ventricular arrhythmias in multiple mouse models of Na\(_{v}\) channel deficiency.\(^{30,31}\)

Although our findings clearly link ankyrin-G with Na\(_{v}\)1.5 targeting, they reveal new ankyrin-G associated pathways that are relevant to cardiac electric, signaling, and structural roles in both health and disease. Genetic mutation in the ankyrin-binding motif of Na\(_{v}\)1.5 has been previously linked with loss of myocyte I\(_{Na}\), abnormal Na\(_{v}\) channel targeting, and human Brugada syndrome.\(^{21}\) However, to date, ankyrin-G (ANK3) variants have not yet been linked with human arrhythmia. Based on past findings in other tissues, as well as work in this study, a pure loss-of-function ANK3 allele may be incompatible with life. Mice simply lacking Ank3 in the cerebellum are frail and display severe neurological defects. Moreover, human ANK3 variants have been linked with bipolar disease, schizophrenia, and autism.\(^{33-47}\) Ankyrin-G is required for normal retinal protein trafficking and essential for lateral membrane biogenesis in columnar epithelia.\(^{37,46}\) Based on our findings, we predict that more subtle variants may cause sinus node disease, arrhythmia, and even structural heart disease because of defects in intercalated disc infrastructure.

As noted earlier, our data demonstrate that ankyrin-G recruits \(\beta_{IV}\) spectrin to the intercalated disc. In other cell types, \(\beta\)-spectrins, through their association with \(\alpha\)-spectrins and actin, form critical submembrane cytoskeletal infrastructure. In fact, defects in spectrins have been linked with a host of pathologies in humans and animals.\(^26\) Moreover, \(\beta_{IV}\) spectrin associates with and targets a subpopulation of CaMKII\(\delta\) to the intercalated disc to phosphorylate Na\(_{v}\)1.5.\(^{4,8}\) Deletions in CaMKII\(\delta\) phosphorylation of Na\(_{v}\)1.5 have now been linked with multiple forms of heart failure in humans and animal models.\(^8,47\)

Our in vivo data support a role of ankyrin-G in organization of the intercalated disc. Plakophilin-2 is a key disc protein, linking desmosomal cadherins with desmoplakin and the intermediate filament system. Human plakophilin-2 loss-of-function variants are linked with arrhythmogenic right ventricular cardiomyopathy and Brugada syndrome.\(^{48,49}\) Although cardiac phenotypes in the cKO model are distinct from human arrhythmogenic right ventricular cardiomyopathy (increased levels of PKP2 in cKO model versus reduced PKP2 levels in arrhythmogenic right ventricular cardiomyopathy),\(^39\) the unexpected new data on structural defects in ankyrin-G cKO hearts and association with plakophilin-2 alterations are noteworthy. However, the specific relationship between ankyrin-G and plakophilin-2, as well as the potential link between these pathways and the observed structural defects in cKO mice, will require additional investigation. Interestingly, unlike plakophilin-2, we did not observe alterations in expression of intercalated disc proteins N-cadherin, connexin43, ZO-1, or \(\beta\)-catenin in cKO mice by immunoblot. Notably, Delmar and colleagues\(^{50}\) previously showed reduced connexin43 expression in ankyrin-G siRNA transfected neonatal myocytes. Although future experiments will be important in defining the relationship between these molecules (ie, compensatory changes versus direct protein partners), our findings clearly

---

Figure 8. Plakophilin-2 organization is disrupted in cKO myocytes. A–B, Immunoblots of myocyte intercalated disc proteins in wild-type (WT) versus cKO heart. Note that plakophilin-2 levels are significantly increased in cKO hearts (n=4/genotype; P<0.05). C–K, Confocal imaging of WT (left) and cKO (right) myocytes labeled with antibodies for resident intercalated disc proteins. Note that although primarily localized to the disc of WT myocytes, plakophilin-2 is clustered in the perinuclear region of cKO myocytes (arrowheads; Bar=10 μm).
implicate ankyrin-G as a multifunctional regulatory molecule in the heart. Further, our work demonstrates that phenotypes observed in the ankyrin-G cKO mouse likely extend far beyond simple $I_{Na}$ deficiency.

Our in vivo findings demonstrate a critical role for ankyrin-G for intercalated disc Na$\n_{1.5}$ targeting. However, Na$\n_{1.5}$ targeting to nonintercalated disc membranes has been proposed as ankyrin-independent, relying instead on unique cellular machinery, including syntrophin/dystrophin, SAP97, caveolin-3, MOG1, and FGFI2.\(^{11}\) Based on the essential role of Na$\n_{1}$ channels for myocyte excitability, we propose that the verterobate has evolved multiple mechanisms for Na$\n_{1.5}$ membrane targeting. Future studies that explore the specific roles of ankyrin-G versus other targeting proteins in ion channel trafficking versus membrane scaffolding will be important to explore the relative contribution of each protein in dictating cardiac excitability and function using in vivo models. Additionally, it will be important to define the identity of populations of Na$\n_{1.5}$-positive puncta in cKO myocytes (blue arrows in Figure 2F, ie, endosomes, T-tubule populations, etc). Further, based on the in vivo link between ankyrin-G and plakophilin-2, it will be important to further investigate the mechanistic roles of ankyrin-G in regulation of intermedi-filaments at the intercalated disc. Finally, it will be important to investigate the role of ankyrin-G for targeting other Na$\n_{1}$ channels in heart (eg, brain Na$\n_{1}$, channel gene products).

Sources of Funding

This work was supported by the National Institutes of Health (HL084583, HL083422, HL114383 to PJ. Mohler; HL114893 to T.J. Hund); Saving Tiny Hearts Society (P.J. Mohler), American Heart Association (P.J. Mohler), and James S. McDonnell Foundation (T.J. Hund).

Disclosures

None.

References


33. Leoni AL, Gavillet B, Rougier JS, et al. Variable na(v)1.5 protein expression from the wild-type allele correlates with the penetrance of cardiac conduction disease in the scn5a mouse model. PLoS ONE. 2010;5:e9298
Ankyrin-G Coordinates Intercalated Disc Signaling Platform to Regulate Cardiac Excitability In Vivo

Michael A. Makara, Jerry Curran, Sean C. Little, Hassan Musa, Iuliia Polina, Sakima A. Smith, Patrick J. Wright, Sathya D. Unudurthi, Jed Snyder, Vann Bennett, Thomas J. Hund and Peter J. Mohler

Circ Res. 2014;115:929-938; originally published online September 19, 2014;
doi: 10.1161/CIRCRESAHA.115.305154

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/11/929

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/09/19/CIRCRESAHA.115.305154.DC1
Supplemental Material

Supplemental Methods

Generation of cardiac ankyrin-G-deficient mice. Mutant ankyrin-G<sup>f/f</sup> mice were generated by the introduction of LoxP sites flanking exons 22-23 of Ank3 as described. These animals were then crossed with mice expressing Cre under the cardiac promoter α-myosin heavy chain (αMHC-Cre) resulting in specific loss of ankyrin-G in adult cardiac myocytes (cKO; NEO selection cassette removed from line). For all experiments, 8-10 week WT and cKO male mice (C57/Bl6 background) were used. Qv<sup>αMHC</sup> mice (C57/Bl6) harboring truncated β<sub>IV</sub> spectrin allele were also utilized for experiments. All animal studies were performed in accordance with the American Physiological Society Guiding Principles for Research Involving Animals and Human Beings, and approved by The Ohio State University Institutional Animal Care and Use Committee. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Immunoblots and antibodies. Whole heart, brain (cortex or cerebellum), muscle and kidney lysates, following quantitation by BCA assay (Pierce), were loaded into 4-15% precast gels and transferred to nitrocellulose membranes. Membranes were blocked for >1hr at room temperature in 5% milk and incubated in primary antibody overnight at 4°C. Primary antibodies included ankyrin-G (1:1,000), Na<sub>V</sub>1.5 S571 (1:500), Na<sub>V</sub>1.5 (1:500), CaMKIIδ (1:500, Badrilla), β<sub>IV</sub> spectrin (1:1000, gift from M. Komada, Tokyo Institute of Technology, Yokohama, Japan), N-Cadherin (1:2000, Invitrogen), connexin43 (1:1000 Invitrogen), plakophilin-2 (1:500 Abcam), β-catenin (1:2000, BD Biosciences), ZO-1(1:1000, Invitrogen), ankyrin-B<sup>3</sup> (1:2000), Na/Ca exchanger (1:500, Swant), Ca<sub>V</sub>1.2 (1:1000, Invitrogen), actin (1:2000, Santa Cruz), Nav channel β2 and β4 (1:400; NeuroMabs), GAPDH (1:5000, Fitzgerald). Secondary antibodies
used were donkey anti-mouse-HRP and donkey-anti-rabbit-HRP (Jackson Laboratories). Densitometric analysis was performed using Image Lab software and all data was normalized to GAPDH or actin.

Immunofluorescence. Cardiomyocytes (left ventricle) were isolated from WT and cKO hearts as described. Isolated cells were fixed and permeabilized in 100% ethanol at -20°C. Cells were blocked in 3% bovine serum albumin and 1% fetal goat serum in PBS. Cells were stained with primary antibody overnight in blocking solution. Cells were stained with secondary antibodies in blocking solution for >1 hr at room temperature. Secondary antibodies included Alexa-conjugated donkey anti-mouse 488, 568 and donkey anti-rabbit 488, 568. Cells were imaged on a LSM 780 confocal microscope (Carl Zeiss). Myocytes were imaged using identical confocal settings between genotypes. At least 20 myocytes were examined for each staining protocol.

Electrocardiography. Electrocardiogram (ECG) recordings were obtained from both conscious and anesthetized mice. For conscious ECG recordings, >20g WT and cKO mice were implanted with ETA-F10 radiotelemeters as described. Following surgery, mice recovered for seven days prior to ECG recordings. ECGs were recorded at rest and following epinephrine administration (2 mg/kg, I.P.). Average resting heart rates were obtained from continuous 2 hr ECG recordings. Arrhythmia was defined as sustained if >2 seconds as described. ECG traces were analyzed using P3 Plus software (Ponemah). For anesthetized surface ECG recordings, mice were anesthetized with 2% isoflurane and oxygen at rate of 1.0 L/min. Mice were placed in the prone position on a heated pad to maintain body temperature. Anesthesia maintained at 1% isoflurane at 1.0 L/min. Subcutaneous electrodes were placed in the lead II configuration and ECGs were recorded on a Powerlab 4/30 (AD Instruments). Baseline ECG was recorded for 3 minutes after anesthesia. Flecainide (20 mg/kg, i.p.) was administered at 3 min after anesthesia and ECGs were recorded per protocol of Knollmann and colleagues. ECG traces were analyzed using LabChart 7 Pro (AD Instruments).
**Electrophysiology:** $I_{\text{Na}}$ currents were recorded utilizing a patch-clamp configuration using an Axopatch 200B amplifier and Digidata 1440A digitizer on left ventricular myocytes. Data acquisition and analysis was performed using pCLAMP software (ver.10.3; Molecular Devices, Sunnyvale, CA). Sodium currents ($I_{\text{Na}}$) were recorded at room temperature (20-22 °C) with pipette resistances <2.8 MΩ when filled with pipette filling solution containing (in mM): NaCl (5), CsF (135), EGTA (10), MgATP (5), Hapes (5), pH 7.2. The extracellular bathing solution contained (in mM): NaCl (5), MgCl2 (1), CaCl2 (1.8), CdCl2 (0.1), glucose (11), CsCl (132.5) and Hapes (20); pH was maintained at 7.4 with CsOH at room temperature. Appropriate whole-cell capacitance and series resistance compensation ($\geq$60%) was applied along with leak subtraction. To assess the $I_{\text{Na}}$ density, cells were held at -160 mV and stepped to various test potentials from -100 to 30mV in 5 mV increments, with 200 ms duration pulses and 2800 ms interpulse intervals. Voltage-dependence of inactivation was assessed by holding the cells at -160 mV followed by a 300 ms test pulse from -140 to -40 mV in 5 mV increments; interpulse interval was 2700 ms. Recovery from inactivation was studied by holding cells at -160 mV and applying two 20 ms test pulses (S1, S2) to -45 mv, separated by increasing increments of 1 ms to a maximum S1-S2 interval of 50 ms. The S1-S1 interval was kept constant at 2000 ms. $I_{\text{Na}}$ late was determined by quantifying the persistent current during an active voltage pulse at time range of 100-200msec after the activation of $I_{\text{Na}}$ and was expressed as a percentage of $I_{\text{Na}}$ peak amplitudes. Prior to quantification, baseline was set to zero. Action potentials were measured as described.4, 5

**Calcium Measurements.** Myocyte calcium measurements were performed as described.9

**Sinus node myocytes.** Sinoatrial node myocytes were prepared and stained as described.10
Statistics. *P* values were determined with the unpaired Student *t* test (2 tailed) for single comparisons. Multiple comparisons were analyzed by use of 1-way ANOVA. The Bonferroni test was used for post hoc testing (SigmaPlot 12.0). If the data distribution failed normality tests with the Shapiro-Wilk test, rank-based ANOVA and the Dunn multiple-comparisons test were performed. Incidence of arrhythmia was analyzed by Chi Square Test. A *p*-value <0.05 was considered statistically significant.

References

Online Fig. I. Ankyrin-B, Cav1.2, and NCX expression levels are not altered in cKO heart. (A-C) Immunoblots and normalized expression levels of myocyte proteins in WT versus cKO heart (n=4/genotype; p=N.S.).
Online Fig. II. A) Voltage-dependent inactivation (h' curve) and B) time-dependent recovery of INa in WT (n=10) and cKO (n=10) myocytes. We observed no significant difference in either property between genotypes (N.S.).
Online Fig. III. Ankyrin-G cKO myocytes display defects in action potential amplitude and Vmax compared with WT myocytes, consistent with reduced INa (n>8/genotype; p<0.05.).
Online Fig. IV. Heart sections from ankyrin-G cKO mouse (B) display reduced βIV spectrin intercalated disc staining (arrows) compared with WT heart (A) (bar equals two microns).
Online Fig. V. Heart sections from ankyrin-G cKO mouse display reduced CaMKIIδ intercalated disc staining compared with WT heart (bar equals ten microns). Image on right is magnified view of CaMKIIδ localization.
Online Fig. VI. Ankyrin-G cKO myocytes display reduced phosphorylation of Na\textsubscript{v}1.5 pS571. (A-B) Immunostaining of Na\textsubscript{v}1.5 pS571 in WT and cKO myocytes. Bar=10 microns. (C-D) Na\textsubscript{v}1.5 pS571 levels are significantly reduced in cKO hearts compared with hearts of WT mice. Levels are normalized for GAPDH (n=4/genotype; p<0.05).
Online Fig. VII. Iso-induced enhancement of \( I_{\text{Na,L}} \) in WT but not cKO myocytes. A) Transient and late INa currents evoked in response to 200 ms duration voltage clamp steps to -25 mV in the absence (black trace) and presence (red trace) of Iso in WT (A) and AnkG KO (B) myocytes. C) Bar graph of \( I_{\text{Na,L}} \) at multiple experimental voltages ±Iso. WT: -ISO (black), +Iso (blue). AnkG cKO: –Iso (red), +Iso (gray). D) \( I_{\text{Na,L}} \) was significantly increased in the presence of ISO in WT cells at several of the tested voltages while currents from AnkG cKO myocytes were insensitive to ISO treatment. \( n \geq 6 \) for all conditions.
Online Fig. VIII. Ankyrin-G cKO mice display significant increase in QRS duration following flecainide challenge (p<0.05).
Online Fig. IX. (A) Following flecainide, AnkG cKO mice display increased PR intervals. $N>5$/ genotype; $p<0.05$. 
Online Fig. X. Nav channel beta subunit expression is not altered in cKO heart. (A-C) Immunoblots and normalized expression levels of myocyte proteins in WT versus cKO heart. (n=4/genotype; p=N.S.).
Online Fig. XI. Heart sections from ankyrin-G cKO mouse display normal plakophilin 2 (PKP2) intercalated disc staining (arrows) compared with WT heart (bar equals ten microns) but increased intracellular staining.
Online Fig. XII. Ankyrin-G cKO mice display arrhythmias and death. (A) ECG of WT mice post-injection of epinephrine (2 mg/kg, I.P.). (B-C) Ankyrin-G cKO mice following identical epinephrine administration display arrhythmia. (B) cKO mouse exhibiting ectopic ventricular beats (red arrowheads). (C) Ankyrin-G cKO mouse presenting polymorphic ventricular arrhythmia just prior to death (bottom). (D-E) Frequency of ventricular arrhythmias and death in WT and cKO mice following epinephrine injection (p<0.05). Bars equal 200 msec.
Online Fig. XIII. Ankyrin-G cKO myocytes display increased calcium waves compared with myocytes from WT littermates. N>10/genotype; *p<0.05.
Online Fig. XIV. Ankyrin-G cKO mice display minor cardiac structural phenotypes at 8-10 weeks. Representative short-axis M-mode images of wild-type (A) and cKO (B) mice at 8 weeks of age. The ratios of HW/BW (C) and HW/TL (D) are preserved as well as no change in anterior wall thickness (E) or LV diameters (F) in cKO mice. However, cKO mice display a small but significant decrease in fractional shortening (G) and ejection fraction (H) compared to WT mice. For each measurement n>5/genotype; *p<0.05.
Online Fig. XV. Ankyrin-G cKO mice display structural abnormalities and reduced ventricular function with age. Representative short-axis M-mode image of 9 month WT (A) and cKO (B) mice. cKO mice display an increase in both the systolic (C) and diastolic (D) left ventricular chamber diameter and a decrease in both the posterior wall (LVPW, E) and anterior wall (LVAW, F) thickness. Consistent with chamber dilation and thinned walls, cKO mice display a significant reduction in performance as indicated by reduced fractional shortening (G) and ejection fraction (H). For each measurement n>5; *p<0.05.