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Ankyrin-based Cellular Pathways for Cardiac Ion Channel and Transporter Targeting and Regulation

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Abstract

The coordinate activities of ion channels and transporters regulate myocyte membrane excitability and normal cardiac function. Dysfunction in cardiac ion channel and transporter function may result in cardiac arrhythmias and sudden cardiac death. While the past fifteen years have linked defects in ion channel biophysical properties with human disease, more recent findings illustrate that ion channel and transporter localization within cardiomyocytes is equally critical for normal membrane excitability and tissue function. Ankyrins are a family of multifunctional adapter proteins required for the expression, membrane localization, and regulation of select cardiac ion channels and transporters. Notably, loss of ankyrin expression in mice, and ankyrin loss-of-function in humans is now associated with defects in myocyte excitability and cardiac physiology. Here, we provide an overview of the roles of ankyrin polypeptides in cardiac physiology, as well as review other recently identified pathways required for the membrane expression and regulation of key cardiac ion channels and transporters.

Keywords

ankyrin; Nav1.5; intercalated disc; transverse-tubule; arrhythmia; sodium channel; targeting

1. Introduction

The excitability of neurons, cardiomyocytes, and endocrine cells is regulated by the expression and function of voltage-gated ion channels in the plasma membrane. In the heart, voltage-gated sodium, potassium, and calcium channels regulate cardiomyocyte excitability including the generation and propagation of action potentials. For example, voltage-gated sodium channels determine the amplitude and slope of action potentials along with controlling conduction velocity throughout the cardiac tissue. Dysfunction of the cardiac sodium channel results in increased risk for ventricular arrhythmias such as Brugada syndrome and long QT syndrome type 3 (reviewed in [1]). These life-threatening cardiac arrhythmias may arise from mutations in the sodium channel that alter gating properties, decrease protein expression, or cause the mis-localization of sodium channel subunits.

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Notably, while many properties of cardiac voltage-gated sodium channels have been well established over the past twenty years (i.e. biophysical properties, critical structural domains, signaling motifs), the specific mechanisms underlying the membrane targeting, stabilization, and local regulation of these essential membrane proteins have received less attention. Over the past decade, findings from mice and humans have implicated ankyrin polypeptides in ion channel and transporter targeting in cardiomyocytes. In fact, ankyrin dysfunction has been linked with defective targeting of voltage-gated sodium channels, as well as Na/Ca exchanger, Na/K ATPase, and Kir6.2 in heart. Moreover, ankyrin dysfunction for cardiac membrane protein targeting has been linked with potentially fatal human arrhythmias. This review will focus on the role of ankyrin polypeptides in cardiac ion channel and transporter targeting, with an emphasis on the role of ankyrin-G for voltage-gated sodium channel localization. Additionally, we will detail known mechanisms for regulating ankyrin function and specificity in excitable cells. Finally, we will describe current knowledge on the mechanisms underlying ankyrin function in membrane protein delivery, as well as post-transcriptional mechanisms that modulate ankyrin-interactions in excitable cells.

2. Ankyrins

Ankyrins are a family of adaptor proteins that link integral membrane proteins including ion channels, transporters, and cell adhesion molecules to the actin/spectrin cytoskeleton. The ankyrin family consists of numerous ankyrin polypeptides resulting from tissue-specific alternative splicing (reviewed in [2]) of three ankyrin genes (*ANK1*: ankyrin-R, *ANK2*: ankyrin-B, *ANK3*: ankyrin-G). The prototypical ankyrin consists of three functional domains: the membrane-binding domain, spectrin-binding domain, and C-terminal regulatory domain (Fig.1). The membrane-binding domain is made up of 24 ANK repeats that assemble as a suprahelical spiral[3]. The ANK repeat is a common protein motif (33 amino acid motif, comprised of two alpha-helices) that mediates protein-protein interactions. ANK repeats of the membrane-binding domain mediate ankyrin interactions with integral membrane proteins such as the voltage-gated sodium channels, sodium/calcium exchanger (NCX), inositol_(1,4,5)-trisphosphate receptor (IP₃ receptor), ATP-sensitive potassium channel subunit Kir6.2, and the L1 family of cell adhesion molecules [4–11]. The spectrin-binding domain interacts with β -spectrin thereby tethering ankyrin-associated integral membrane proteins to the cytoskeleton[12]. The spectrin-binding domain also interacts with the regulatory subunit of protein phosphatase 2A (PP2A) suggesting that another function of ankyrin is to organize local signaling networks [13]. Ankyrin interactions with β -spectrin and integral membrane proteins are partially regulated by the C-terminal regulatory domain. This domain most likely regulates ankyrin specificity for particular interacting proteins and directs ankyrin subcellular targeting [14–16]. The functional significance of this domain is highlighted by the prevalence of human disease-associated variants within this domain of ankyrin-B [17].

The heart expresses protein products of all three ankyrin genes including the 190 kDa isoform of ankyrin-G, the 160 and 220 kDa isoforms of ankyrin-B, and the 210 kDa isoform of ankyrin-R. While the molecular basis for ankyrin-R function in heart has yet to be fully elucidated, there is some understanding as to how ankyrin-B and ankyrin-G function in heart. Specifically, ankyrin-B is important for the proper targeting and stability of NCX, IP₃ receptor, and sodium/potassium ATPase (NKA) at membrane junctions of the transverse-tubules (T-tubules) with sarcoplasmic reticulum (SR) [5,18]. Ankyrin-B also regulates the protein expression and membrane targeting of K_{ATP} channel subunit Kir6.2 to T-tubules in addition to modulating K_{ATP} channel ATP sensitivity [8,9,19]. In contrast, as addressed in greater detail below, ankyrin-G is important for the protein expression and proper targeting of the voltage-gated sodium channel Na_v1.5 to intercalated disc membranes [4,7].

3. Ankyrin-Dependent Targeting of Cardiac Voltage-Gated Sodium Channels

The voltage-gated sodium channel (Nav) consists of a pore-forming α -subunit and one or more auxiliary β -subunits [20]. In addition to alternative splice variants, there are ten different α -subunits encoded by different genes that individually produce a ~260 kDa membrane protein. The α -subunits display differential tissue, cellular, and subcellular expression patterns. A prototypical α -subunit has four domains (DI–DIV) that contain six α -helical transmembrane segments (S1–S6) (Fig.2). The S4 segment is the voltage sensor and the membrane-embedded loop between segments S5 and S6 confers ion selectivity. The transmembrane and extracellular domains of the α -subunits share a significant degree of homology. In contrast, the intracellular domains are more divergent and account for the α -subunit's unique biophysical properties and expression patterns. By itself, the α -subunit harbors the fundamental properties of a sodium channel (pore formation, ion selectivity, and rapid inactivation), while the β -subunits modulate the channel's biophysical properties in addition to regulating channel expression and localization in the plasma membrane [21]. Four genes encode the β -subunits that are single-pass transmembrane proteins with an extracellular immunoglobulin domain that mediates homophilic interactions between adjacent β -subunits.

In heart, the most prevalent sodium channel α -subunit is TTX-resistant Nav1.5. This subunit is predominantly expressed at the intercalated disc membrane [4,22,23] where gap junctions, adherens junctions, and desmosomes link neighboring cardiomyocytes both electrically and mechanically. Expression of Nav1.5 at the intercalated disc facilitates action potential propagation throughout the working myocardium. Nav1.5 channels have also been detected on T-tubules and the peripheral sarcolemma. Additional α subunits expressed on T-tubules include the TTX-sensitive Nav1.1, Nav1.2, Nav1.3, Nav1.4, and Nav1.6 [23,24]. These subunits are notably less abundant than TTX-resistant Nav1.5, but still estimated to account for 10–20% of the total sodium current in adult cardiomyocytes.

There are four Nav channel β -subunits: β 1, β 2, β 3, and β 4. All four subunits including an alternative β 1A isoform are expressed in the heart [25]. An individual β -subunit has a single-pass transmembrane domain, an extracellular domain with an Ig domain, and an intracellular domain. The rates of activation, inactivation, and recovery from inactivation for α -subunits can be altered by β -subunits depending on the α -subunit and the cell-type background. In addition, β -subunits can increase the membrane expression of α -subunits. For example, the β 1-subunit increases membrane expression of a brain sodium channel by 2 to 4 fold in a heterologous cell line [26]. Finally, the significance of β -subunits for proper sodium channel activity and normal cardiac physiology is highlighted by multiple studies that link cardiac β -subunits mutations to cardiac arrhythmia disorders [27,28]. While Nav β -subunits are clearly important for cardiac I_{Na} , it will be important in the future to define how and where α - and β -subunits co-assemble in the biosynthetic pathway. Moreover, it will be interesting to determine how β -subunits may recruit signaling molecules to the larger sodium channel complex. Finally, similar to α -subunits, Nav β -subunits are differentially localized in cardiomyocytes [24]. For example, the β 3-subunit has been found at the SR/T-tubule junction and the β 4-subunit at the intercalated disc, while the β 1- and β 2-subunits have been detected at both the intercalated discs and SR/T-tubule junctions. There has yet to be studies in the heart that address whether β -subunits selectively associate with specific α -subunits. In fact, this interaction may be partially dictated by their co-localization at particular membrane domains.

The interaction between ankyrin-G and voltage-gated sodium channels was initially characterized in neurons. A nine amino acid motif in the DII/DIII intracellular loop of the

sodium channel mediates binding to ankyrin-G [29,30]. In neurons, ankyrin-G binding is necessary for the proper targeting and retention of sodium channels at the axon initial segment and nodes of Ranvier. In a mouse model lacking ankyrin-G in the cerebellum, sodium channels are no longer enriched at the axon initial segments of cerebellar Purkinje neurons [31]. As a result, there is a decrease in action potential generation and the cerebellar neurons degenerate over time [31]. A similar ankyrin-binding motif has been identified in the potassium channel subunits KCNQ2/3 and the expression of these subunits at axon initial segments is also dependent on ankyrin-G [32–34].

Similar to neuronal sodium channels, the cardiac sodium channel $\text{Na}_v1.5$ harbors an ankyrin-binding motif in the DII/DIII intracellular loop (Fig.2). This motif is important for the proper targeting and retention of $\text{Na}_v1.5$ to intercalated discs [4,7]. Reduction in ankyrin-G levels decreases $\text{Na}_v1.5$ membrane expression and total sodium current density in individual cardiomyocytes by 50–65% [7]. Likewise, $\text{Na}_v1.5$ membrane expression and total sodium current density are attenuated by a mis-sense variant (E1053K) in the ankyrin-binding domain (Fig.2) that abolishes ankyrin-G/ $\text{Na}_v1.5$ interactions [4]. Interestingly, this variant was identified in a patient with Brugada syndrome, a cardiac disorder associated with decreased I_{Na} , precordial ST segment elevation, right bundle branch block, and fatal arrhythmias [4].

Finally, ankyrin-G may play a regulatory role for $\text{Na}_v1.5$ in heart by recruiting signaling proteins to the intercalated disc [35]. For example, recent work demonstrates that ankyrin-G associates with β_{IV} spectrin in heart at the intercalated disc. Moreover, β_{IV} spectrin recruits CaMKII to regulate $\text{Na}_v1.5$ activity via a conserved residue in the DI–DII linker (S571, one of likely multiple phospho-regulatory sites on $\text{Nav}1.5$). In fact, β_{IV} spectrin mutant mice lacking the CaMKII-binding motif display aberrant CaMKII intercalated disc targeting, defects in $\text{Nav}1.5$ S571 phosphorylation, I_{Na} , and myocyte/cardiac excitability[35].

4. Ankyrin-based intracellular trafficking pathways

While many of the mechanisms that regulate intracellular trafficking of ankyrin remain unknown, some insight was gained from a recent study that demonstrated an interaction between ankyrin-B and EH (Eps15-homology) domain (EHD) containing proteins. EHD proteins regulate endosomal anterograde and retrograde trafficking, in addition to membrane protein recycling and lipid homeostasis [36]. In this recent study, it was demonstrated that ankyrin-B interacts with four EHD proteins (EHD1–4) that are expressed in the heart [37]. EHD protein expression was upregulated in ankyrin-B null cardiomyocytes suggesting that the loss of ankyrin-B function (i.e. membrane expression of ion channels and transporters) triggers a compensatory response in EHD upregulation. In this study, a link was drawn between EHD3 protein expression and ankyrin-B dependent NCX membrane expression. Specifically, overexpression of EHD3 increased NCX current density in an ankyrin-B dependent manner, while decreasing EHD3 protein expression through siRNA treatment reduced NCX current density [37]. These findings demonstrate that EHD3 partially regulates NCX membrane expression and it can be inferred that EHD3 is involved in the intracellular trafficking of ankyrin-B, although more detailed experiments are needed to evaluate this hypothesis.

With the identification of Golgi-specific ankyrin isoforms, it has been suggested that ankyrins play an active role in the process of intracellular transport for some proteins [38]. A recent study provided compelling evidence that the Golgi-specific isoform of ankyrin-R is required for the trafficking of the sodium/potassium ATPase (NKA) from the endoplasmic reticulum (ER) to the trans-Golgi network [39]. Specifically, the ankyrin-binding domain in the α -subunit of NKA is both necessary and sufficient for ankyrin-dependent intracellular

transport. α -subunits lacking this domain are no longer properly trafficked and overexpression of this domain acts as a dominant negative by competing with endogenous NKA for ankyrin-R binding, thereby causing the accumulation of endogenous NKA in the ER [39]. The minimal ankyrin-binding domain was sufficient to restore intracellular transport of a protein construct between the ER and trans-Golgi network. Interestingly, the binding domain was not sufficient to advance the trafficking of this protein construct beyond the trans-Golgi network suggesting that the function of the Golgi-specific ankyrin-R isoform may be restricted to a particular step along the biosynthetic pathway of NKA. Given the abundance of ankyrin isoforms and their differential localization throughout the biosynthetic pathway from the ER to plasma membrane to recycling endosomes, there is a tremendous amount of opportunity for discovery in this area of research.

5. Ankyrin-independent Pathways for Cardiac Sodium Channel Targeting

While ankyrin-based pathways have recently been linked with Nav1.5 membrane targeting in heart, many additional studies have identified ankyrin-independent cellular pathways for cardiac voltage-gated sodium channel organization and membrane expression including Nav β -subunits (see discussion above), syntrophin, MOG1, 14-3-3, and plakophilin. The proceeding section provides a brief introduction to these proteins, as well as a description of their potential roles for Nav1.5 membrane targeting and/or regulation in cardiomyocytes.

MOG1 is a small protein that has been shown to function as a guanine nucleotide release factor that regulates nuclear protein trafficking via its interaction with the small GTPase Ran [40]. MOG1 mRNA is most highly expressed in the heart and in cardiomyocytes MOG1 displays both nuclear and cytosolic expression [41,42]. Interestingly, MOG1 co-localizes with Nav1.5 at intercalated discs of adult cardiomyocytes and has been co-precipitated with Nav1.5 [42]. MOG1 interacts with Nav1.5 intracellular loop DII/DIII and this interaction increases membrane expression of the channel [42]. In fact, MOG1 overexpression in neonatal cardiomyocytes increases Nav1.5 current density by 2-fold without having any apparent effect on the channel's biophysical properties [42]. Whether the molecular basis for MOG1 function is through increased channel transport or reduced channel turnover is still unknown. Nevertheless, MOG1 is another factor that contributes to the optimal membrane expression of Nav1.5.

Syntrophin proteins are ~59 kDa adaptor molecules that link ion channels and signaling molecules to the dystrophin-glycoprotein complex (DGC) through their interactions with the C-terminus of dystrophin. In addition to interacting with signaling molecules such as neuronal nitric oxide synthase, α and β syntrophin proteins directly interact with Nav1.5 through a PDZ domain in the channel's C-terminus [43]. In the *mdx* mouse (mouse model of Duchenne muscular dystrophy and dystrophin deficiency), there is a pronounced reduction in the expression of proteins that directly interact with dystrophin such as syntrophin [44]. Interestingly, Nav1.5 protein expression is likewise reduced in *mdx* hearts, thereby causing a 29% reduction in total sodium current density in individual *mdx* cardiomyocytes [45]. This decrease in sodium current density results in conduction defects, which manifest as prolonged QRS complexes on electrocardiograms of *mdx* mice [45]. Not surprisingly, electrocardiograms of Duchenne muscular dystrophy patients also reveal conduction defects [46,47]. While many of the cardiac disorders manifested in these patients such as dilated cardiomyopathy clearly reflect structural deficiencies associated with the loss of dystrophin, these patients also display life-threatening arrhythmias that may reflect the loss of dystrophin-associated ion channels that are important for normal conduction velocity such as Nav1.5.

The family of 14-3-3 proteins consists of small adaptor proteins that are ubiquitously expressed, interact with over 200 binding proteins, and have been implicated in almost every cellular process including intracellular trafficking of membrane proteins. While 14-3-3 proteins were initially characterized by their selective binding preference for phosphorylated residues, they also bind to non-phosphorylated protein targets. 14-3-3 proteins form homo- and hetero-dimers with each subunit binding to a discrete phospho-serine or phospho-threonine residue [48]. In the heart, all 14-3-3 isoforms ($\beta, \gamma, \epsilon, \eta, \tau, \zeta$) are expressed except 14-3-3 σ . Recently, 14-3-3 η was identified as a binding partner of Nav1.5 by yeast 2-hybrid analysis and this interaction was confirmed with co-immunoprecipitation assays [22]. This interaction was mapped to residues 417–467 in the DI/DII intracellular loop of Nav1.5 [22]. In addition, 14-3-3 η was found to co-localize with Nav1.5 at the intercalated disc. Based on patch-clamp experiments in transfected COS-7 cells, 14-3-3 η had no effect on total sodium current density suggesting that Nav1.5 membrane expression is not regulated by 14-3-3 η [22]. While co-expression of 14-3-3 η shifted channel inactivation towards a negative potential and delayed recovery from inactivation, the actual molecular basis for 14-3-3 η activity in channel function has yet to be characterized [22]. Considering 14-3-3 proteins have been shown to bind to ER retention motifs, it is possible that 14-3-3 η facilitates the anterograde transport of Nav1.5 by masking an ER retention motif in the DI/DII intracellular loop.

Finally, plakophilin-2 is an intracellular adaptor protein that is a part of the desmosomal protein complex that provides mechanical stability and structural organization to intercalated discs. Plakophilin-2 links the desmosomal cadherins, which connects neighboring cardiomyocytes, to the intermediate filament desmin through another linker protein called desmoplakin. Mutations to plakophilin-2 have been linked to arrhythmogenic right ventricular cardiomyopathy (ARVC), a cardiac disorder characterized by the replacement of right ventricular myocardial tissue with fibrous fatty tissue leading to electrical instability and ventricular arrhythmias [49]. A recent study demonstrated that plakophilin-2 interacts with Nav1.5. Specifically, Nav1.5 was isolated from heart lysate using an antibody to or a GST-protein of plakophilin-2 [50]. In addition to the co-localization of these proteins at intercalated discs, plakophilin-2 silencing with shRNA reduced sodium current density and altered the channel's biophysical properties [50]. Plakophilin-2 silencing also decreased action potential propagation in a monolayer of ventricular cardiomyocytes as determined by optical mapping experiments [50]. Whether there is a direct or indirect interaction between plakophilin-2 and Nav1.5 has yet to be determined. These findings would clarify whether plakophilin-2 directly contributes to Nav1.5 membrane expression/stability or whether the reduction in sodium current density may be a byproduct of desmosomal structural defects.

6. Conclusions

The past decade has witnessed many exciting and new findings that link dysfunction in ion channel and transporter targeting with severe human disease including cardiac arrhythmia, neurological disorders, cystic fibrosis, and diabetes. Notably, ankyrin polypeptides have been linked with ion channel and transporter targeting in a number of excitable cells including neurons, cardiomyocytes, and pancreatic beta cells. While these findings illustrate the importance of ankyrins for normal metazoan physiology, a number of questions remain regarding ankyrin regulation *in vivo*. For example, despite the fact that ankyrins were discovered nearly thirty years ago, we have yet to resolve the specific roles for ankyrins in membrane protein targeting. Are ankyrins simply static membrane adapters that retain membrane structure in collaboration with spectrin- and actin-based cytoskeletal elements (i.e. similar to ankyrin-R)? Alternatively, do ankyrins play active roles in the post-ER membrane trafficking of ion channels/transporters? Finally, a major unresolved issue in ankyrin biology is *in vivo* specificity of ankyrins for protein targets. For example, while

ankyrin-B binds to and targets Na/K ATPase in heart, ankyrin-G targets the same protein in lung and kidney. Thus, the fidelity of ankyrin/protein interactions is most likely maintained by a combination of the unique binding motifs in ankyrin and the target protein in addition to other regulatory mechanisms. In closing, understanding the mechanisms by which specific ankyrin gene products segregate with target proteins *in vivo* must be a significant new research direction in the field of ankyrin biology.

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Figure 1.

Ankyrin domain organization and associated proteins. Canonical ankyrins display an amino-terminal membrane-binding domain comprised of 24 consecutive *ANK* repeats (blue), a spectrin-binding domain (red), a death domain (green), and C-terminal domain (black). Validated binding partners for cardiac ankyrin-B and ankyrin-G are noted below the domain of interaction. Note that both Na/K ATPase and EHD1-4 may require interaction sites on both membrane- and spectrin-binding domains.

Figure 2.

Schematic representation of the sodium channel α -subunit Nav_V1.5 and targeting/membrane regulatory proteins. Illustrated are validated Nav1.5 binding partners, shown with binding sites on Nav1.5. Note that for plakophilin, caveolin-3, and Nedd-4-like protein (binds C-terminal domain), binding sites are not illustrated due to space/lack of binding data.