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From Fifth Business to Protagonist:

the complex roles of ion channel anchors in cardiac arrhythmia

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Abstract

Proper regulation of cardiac ion channel activity is critical for cellular ion homeostasis and myocyte electrical activity. Recent work has demonstrated that cardiac ion channels are not isolated pores in plasmid membranes, but rather exist within macromolecular signaling complexes. Moreover, within these macro-complexes resides the machinery to finely tune ion channel expression, activity, and signaling. While it is widely-accepted that mutations in ion channel pore-forming genes underlie a number of cardiac arrhythmias, current research is now focusing on the roles of auxiliary subunits in the development of arrhythmia syndromes.

Introduction

Robert Hooke's first microscopic glimpse into multicellular structure revealed a complex biological organization unimagined before the seventeenth century: microscopic "cells" which he believed were the containers of "noble juices" [1]. Since Hooke's original observation of cork, the subcellular organization of vertebrate cells has been defined, revealing a complexity beyond simple membrane-based compartmentalization [2]. While the lipid cell membrane allowed retention of vital cellular components, it produced limited capabilities for communication, as well as decreased access to necessary substrates and the inefficient removal of waste. Whereas early forms of life likely expressed simple transporters necessary for salt and water balance, the evolution of multicellular organisms necessitated the utilization of more complex transmembrane movement. Specifically, the divergence of single-celled life forms into organisms composed of multiple cells types with specific functions required intercellular/intracellular communication, tissue synchronicity, homeostatic measures, and movement. The evolution of specific ion channels, transporters, and pumps became primary mediators of these actions. With the development of the submembranous cytoskeletal network, ion channels were provided with a platform for the micro-compartmentalization of ion channel-mediated activity. This compartmentalization allowed for more rapid and efficient responses to stimuli, ultimately providing for the highly-choreographed release of hormones, excitation-coupling of the heart, and the firing of synapses. While cells in vertebrate organisms express a distinct 'toolbox' of ion channels, it is the specific subcellular localization and regulation of these ion channels that contribute to the unique function of a particular cell type. For example, the precise localization of voltage-

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gated sodium channels (Na_v) to the cardiac intercalated disc regulates the initial upstroke of the cardiac action potential necessary for the heartbeat, while these same channels at nodes of Ranvier and at axon initial segments allow efficient and rapid propagation of electrical impulses that are necessary for neurotransmitter release, muscle movement, and memory.

Recent work has demonstrated that mutations in ion channel genes which disrupt biophysical activity underlie a number of diseases, including a variety of cardiac arrhythmias. The identification of numerous 'channelopathies' has been instrumental in defining the roles of various ion channels in physiology; however, most intriguing has been the observation that mutations that disrupt the assembly and regulation of ion channel complexes also result in disease. While the idea of ion channels consisting of only pore-forming subunits persisted in the early days of ion channel research, it is now recognized that most ion channels function within macromolecular complexes, comprised not only of the pore-forming subunits, but also of associated auxiliary subunits, regulatory enzymes, and targeting proteins. These associated protein partners play vital physiological roles that include feedback ion sensing, enzymatic activity, and the coordination, localization, and targeting of the ion channel assembly.

While much work has clearly defined the role of macromolecular complexes in the regulation of ion channel activity, recent advances in the study of channelopathies have also demonstrated that, in addition to proper channel biophysics and regulation by accessory proteins, functional ion channels and transporters require precise expression and localization to specific membrane domains within the cell. From work on ion channel trafficking defects, it is now appreciated that ion channels and their accessory entourage are not isolated, functionally discrete islands in the sea of membrane lipids, but rather exist within spatially-defined local membrane domains. Dysfunction in the cellular mechanisms involved in ion channel trafficking and retention at specialized membrane domains has emerged not only as a new focus in the field of channelopathy research, but has also become an exciting new discovery in the understanding of the complex mechanisms involved in the regulation of ion channel and transporter function and, ultimately, human physiology and pathology.

This review will highlight the critical role of some anchoring proteins with specific attention on the development of cardiac arrhythmia. While ion channel proteins have had center stage in the study of congenital arrhythmias, supporting roles of their anchoring subunits has come to light in recent years, demonstrating the crucial function of ion channel macromolecular complexes in the regulation of cell excitability.

Yotiao and the modulation of the cardiac potassium channel

The autonomic nervous system regulates cardiac function to adapt to physical activity and emotional stress. Stimulation of the sympathetic nervous system modulates the slowly activating cardiac potassium channel, contributing to cardiac action potential shortening that occurs concomitantly with increases in heart rate. During fight-or-flight scenarios, sympathetic nerves in the heart release norepinephrine, which activates the protein kinase A (PKA) signaling pathway. The slowly activating potassium channel (I_{Ks}) is a critical target of this signaling cascade [3,4]. These unique channels are assemblies of α (KCNQ1) and β (KCNE1) subunits (Figure 1) and activate slowly in response to changes in membrane potential and contribute the essential repolarizing current to the late phase of the cardiac ventricular action potential [5]. β -adrenergic stimulation enhances I_{Ks} current amplitude, contributing to the shortening of the action potential duration; thus ensuring adequate diastolic filling between heart beats in response to sympathetic-induced heart rate elevation. A-kinase-anchoring proteins (AKAPs) are scaffolding proteins involved in the complex organization of protein kinase A and enzymes that regulate the PKA pathway [6,7]. Precise

localization of the molecular complexes of target proteins and regulatory enzymes ensure efficient spatio-temporal regulation of the phosphorylation state of targeted proteins, resulting in distinct cellular events.

AKAP9-encoded Yotiao has been demonstrated to form a macromolecular complex with the slowly activating cardiac potassium channel [8] (Figure 1). A unique missense mutation in Yotiao, S1570L, has been associated with a clinically robust phenotype for LQTS [9]. Specifically, this mutation disrupts the binding between *KCNQ1* and Yotiao, reduces PKA phosphorylation of *KCNQ1*, and eliminates the cAMP-induced response of *KCNQ1* [9]. Moreover, computational models of the ventricular myocyte predict that this missense mutation prolongs the action potential. Together, the reconstituted cellular consequences of S1570L are consistent with delayed repolarization of the ventricular action potential, supporting the role of S1570L-Yotiao as a LQTS-susceptibility mutation.

More recent work has identified a mutation in *KCNQ1*, G589D, associated with a large group of Finnish LQTS patients [10]. Evaluation of this mutation revealed that G589D, present in 508 of 939 established Finnish LQTS patients, disrupts the AKAP signaling complex, preventing β -adrenergic regulation of I_{Ks} [8]. As a result, this mutation creates a trigger of cardiac arrhythmia during sympathetic stimulation. Subsequent *in silico* modeling supported these findings by demonstrating that, while the *KCNQ1* mutation was unlikely to cause QT prolongation at rest, in combination with increased sympathetic activity, could result in QTc prolongation and extrasystoles [11]. More specifically, sympathetic-stimulated increases in I_{Ca} , left uncompensated by increased I_{Ks} , produces the QT prolongation and precipitates calcium-mediated afterdepolarizations and transmural dispersion of repolarization in ventricular cardiomyocytes [11].

In the normal heart, efficient coupling between the sympathetic nervous system and I_{Ks} channel activity provides a protective effect against sympathetic stimulation. This protection is not only due to the enhanced I_{Ks} , but also because current accumulates on a beat-by-beat basis (due to a phosphorylation-induced slowing of I_{Ks} deactivation kinetics as heart rate increases) [12]. Uncoupling of the sympathetic nervous system and I_{Ks} , therefore, contributes to exercise- or stress-induced cardiac events seen in LQT1 and LQT5 patients [13]. Therefore, these mutational data suggest that human mutations that alter the Yotiao/ I_{Ks} channel complex are a likely cause of potentially fatal human arrhythmias.

Ankyrins and the complex organization of cardiac ion channels

Mutations in ankyrin-B are associated with LQT4 and sinus node disease

In 1995, Schott and colleagues reported on a large French kindred with an atypical form of LQTS: sinus node bradycardia, atrial fibrillation, and an abnormal T-wave morphology [14]. Linkage analysis revealed that the newly-named LQT4 (now referred to as “ankyrin-B syndrome”) was associated with chromosome 4q25-27 [14]. Subsequent sequencing of the *ANK2* gene revealed a missense mutation (A4274G) resulting in a substitution of a glutamic acid for a glycine at ankyrin-B residue 1425 (E1425G) [14]. Using the ankyrin-B haploinsufficient mouse (ankyrin-B^{+/-}), Bennett and colleagues were able to demonstrate that the E1425G variant of ankyrin-B represented a loss-of-function mutation [15]. Ankyrin-B^{+/-} mice are viable and share a number of phenotypes with the LQT4 kindred; specifically, sinus bradycardia, conduction defects, and catecholamine-induced polymorphic ventricular arrhythmia associated with syncope and death [15], making them an excellent animal model to evaluate ankyrin-B loss-of-function mutations [15]. Neonatal cardiac myocytes from ankyrin-B^{+/-} mice demonstrate reduced spontaneous contraction rates, abnormal Ca^{2+} transients, and abnormal localization of the Na^+/Ca^{2+} exchanger (NCX) [15-17]. While exogenous expression of wild type ankyrin-B restored myocyte phenotypes, overexpression

of the E1425G ankyrin-B mutant was unable to restore the wild type phenotype [15-17]. Further evaluation of ankyrin-B^{+/-} cardiac lysates revealed that the protein levels of the NCX, the Na⁺/K⁺ ATPase (NKA) and the inositol 1,4,5-trisphosphate (InsP₃) receptor were significantly reduced; with preferential reduction over the T-tubules [15-17]. Specifically, it has been demonstrated that ankyrin-B forms a ternary complex with NCX1, NKA, and the InsP₃ receptor, functionally linking the sarcoplasmic reticulum with the T-tubule [18] (Figure 2A). It is hypothesized that this ternary complex promotes the export of Ca²⁺ from the sarcoplasmic reticulum via InsP₃ receptors and across the plasma membrane through the NCX. The NKA, then exports the Na⁺ generated by the NCX exchange, allowing electroneutral export of Ca²⁺. The loss of electroneutral calcium export could result in an unbalanced entry of sodium through the NCX, resulting in depolarization and the generation of extrasystoles [18].

The sinoatrial node (SAN) is a unique group of specialized cells which regulate cardiac pacing. While our knowledge of the molecular mechanisms underlying cardiac pacemaking is incomplete, it is accepted that pacemaking activity is initiated at the level of a single cell by the highly orchestrated activity of depolarizing and repolarizing currents. Le Scouarnec mapped two large families with a highly penetrant and severe sinoatrial node disease (SND) to *ANK2*, with an identified genetic variant representing a loss-of-function in sinoatrial node cells [19]. Specifically, it was demonstrated that ankyrin-B was highly expressed in the SAN and that ankyrin-B activity was necessary for the posttranslational stability of SAN channels and transporters [19]. Furthermore, ankyrin-B^{+/-} mice mimicked human ankyrin-B SND, displaying sinus bradycardia and heart rate variability [19]. Finally, isolated SAN cells from ankyrin-B^{+/-} mice displayed reduced cell surface expression of the NCX1, NKA, InsP₃ receptor, and, surprisingly, Ca_v1.3 [19] (Figure 2B). Functionally, ankyrin-B^{+/-} SAN cells displayed reduced NCX and Ca²⁺ current, which is most likely due to aberrant membrane expression of Ca_v1.3 and NCX1. Additionally, increased cytosolic Na⁺ resulting from reduced NKA likely contributes to defects in SAN calcium transients and excitability by promoting further inhibition of NCX activity. These data represent the first example of SND involving a non-ion channel protein, and identify ankyrin as a critical link between ion homeostasis, SR calcium cycling, and membrane depolarization in SAN cells.

Ankyrin-G dysfunction is associated with Brugada Syndrome

Two independent groups identified an ankyrin-binding sequence in a cytoplasmic loop that connects Na_v1.2 domains II and III [20,21]. Interestingly, this sequence was found to be conserved across species and is found in most sodium channel gene products. Considering that ankyrin-G had long been implicated in the targeting of voltage-gated Na⁺ channel isoforms in the nervous system [22], it was hypothesized that this ankyrin-G pathway would be conserved in excitable cells [23]. Using a number of biochemical techniques, Mohler and colleagues were able to demonstrate that ankyrin-G was expressed in vertebrate heart and was highly localized to cardiomyocyte membrane domains enriched with Na_v1.5 [23]. Furthermore, it was demonstrated that ankyrin-G and Na_v1.5 associated and that deletion of the ankyrin-binding sequence prevented association of ankyrin-G and Na_v1.5 [23] (Figure 2C). A year after the ankyrin-binding sequence was identified, Priori and colleagues screened a large group of patients with Brugada syndrome for mutations in the ankyrin-binding sequence of the *SCN5A* gene, which encodes Na_v1.5 [23]. A single *SCN5A* missense mutation was identified, resulting in a substitution of a highly conserved glutamic acid with a lysine at amino acid 1053 (E1053K) [23]. Biochemical analyses revealed that E1053K was unable to associate with ankyrin-G and, as a result, was not properly targeted to the membrane surface of cardiomyocytes [23]. While these data demonstrate an ankyrin-G based pathway for Na_v1.5 trafficking in cardiomyocytes, they also support a critical role for the ankyrin family of polypeptides in the regulation of cardiac function.

Syntrophins and the regulation of late sodium current

Syntrophins form a family of intracellular proteins that are critical components of the large dystrophin-associated protein complex (DAPC) that connect to the actin cytoskeleton in skeletal and cardiac muscles [24] (Figure 3). Syntrophins function to recruit different regulatory proteins (kinases, NO-synthase, and ion channels) to dystrophin. A PDZ domain present in all syntrophins allows for interaction with PDZ binding domains in other proteins [25]. In 1998, Gee demonstrated that the PDZ domains of several syntrophins were able to interact specifically with the PDZ binding domains in both Na_v1.4 (skeletal muscle isoform) and Na_v1.5 (expressed in cardiac tissue) [25]. Mutations in the human dystrophin gene, DMD, are the cause of inborn muscular disorders like Duchenne and Becker muscular dystrophies. Additionally, X-linked cardiomyopathies are also common features of DMD mutations. Specifically, such mutations result in cardiac conduction defects, repolarization dysfunction, and heart failure [26].

α 1-syntrophin contains multiple protein interaction motifs that serve as molecular scaffolds for (neuronal) nNOS and the plasma membrane Ca²⁺-ATPase (PMCA). Additionally, α 1-syntrophin interacts with the α -subunit of Na_v1.5. In the heart, NO increases late sodium current (I_{Na}) amplitude [27], which is the primary biophysical mechanism underlying SCN5A-mediated LQT3. Genome-wide association studies have suggested a role of polymorphisms in the nNOS regulator NOS1AP (CAPON) on the QT interval [28,29]. PMCA4b, the cardiac isoform of PMCA, participates in the nNOS complex and inhibits NO production.

Recently, direct sequencing of *SNTA1*, the gene encoding α 1-syntrophin, was performed on a group of LQTS patients who expressed no mutations in the 11 known LQTS-susceptibility genes. Interestingly, a missense mutation, A390V, was identified in a single patient with recurrent syncope and a prolonged QT interval. This mutation occurs in a region of α 1-syntrophin that is highly conserved and postulated to be important for function and structure. Specifically, Ueda and colleagues demonstrated that α 1-syntrophin connects Na_v1.5 to the nNOS-PMCA complex in the heart. Moreover, the A390V mutation disrupts the association with PMCA4b, results in increased direct nitrosylation of Na_v1.5, and increases the late sodium current in both cardiomyocytes and a heterologous expression system. The increase in late I_{Na} was comparable to the increases seen in patients with LQT3 mutations [30]. It should be noted that the dissociation of PMCA4b from the complex that occurs with A390V may affect the transport of PMCA4b in ways that may also prove to be arrhythmogenic. Regardless, these data may account for the observed effects of NOS1AP polymorphisms on the QT interval [28,29] and definitely imply that other channel associated proteins, like PMCA4b, could be candidates for the pathogenesis of LQTS.

Na_v channel β -subunits

The Na_v1 subfamily of voltage-gated sodium channels are responsible for action potential regulation in a number of excitable cells. Na_v1s are multimeric complexes composed of a single, pore-forming α -subunit and one or more β -subunits. In the ventricle, Na_v1s isoforms are located at the intercalated disc, peripheral sarcolemma, at the transverse-tubules [31-34]. While the β -subunits do not form the pore, these proteins play critical roles in the modulation of channel function, in the regulation of channel expression, and in cell adhesion [35]. Since β -subunits play such critical roles in Na_v channel regulation, mutations which disrupt the interaction between α -subunits and β -subunits have been linked with human cardiac arrhythmia. Recently, a mutation in *SCN4B* (L179F) encoding the β 4 subunit of Na_v1.5, has been identified in a patient demonstrating the LQT3 (Brugada syndrome) phenotype [36]. When expressed in HEK293 cells stably expressing the *SCN5A* α -subunit,

the mutant-containing channel complex produced a three-fold increase in late sodium current, resulting in a gain-of-function phenotype consistent with molecular/electrophysiological phenotype reported for other LQTS-associated mutations [36]. Though L179F is a loss-of-function mutation, it is believed to secondarily precipitate a gain-of-function mutation in $\text{Na}_v1.5$ by accentuating the late sodium current [36].

Brugada syndrome is postulated to result from a reduction in the number of functional cardiac $\text{Na}_v1.5$ channels, producing a decrease in the sodium conductance. While mutations in the α -subunits have been demonstrated to cause Brugada syndrome, it is thought that $\beta1$ may play a role in the etiology of this disease as well. A $\text{Na}_v1.5$ α -subunit double mutant ($\text{Na}_v1.5\text{-R1232W/T1620M}$) underlying idiopathic ventricular fibrillation demonstrated that the expression of this mutant alone had no significant effect on sodium current in HEK293 cells [37]. However, when co-expressed with the wild type $\beta1$ subunit, there was a four-fold reduction in current density and a concomitant decrease in α -subunit protein expression [37]. Therefore, the α -subunit loss-of-function mutation required the presence of $\beta1$, possibly indicating that the addition of the $\beta1$ results in the formation of a mutant channel with reduced stability [37]. Another LQT mutation, $\text{Na}_v1.5\text{-D1790G}$, has been shown to disrupt the ability of $\text{Na}_v1.5$ to be modulated by $\beta1$, specifically by preventing association between α and $\beta1$ subunits [38]. Together, these observations suggest that loss of $\beta1$ expression may disrupt normal cardiac function. Whether mutations in $\beta1$ result in arrhythmia in humans remains to be seen. Regardless, these data demonstrate a critical role for β -subunits in the regulation of Na_v1 channel function.

Conclusions

Cardiac action potentials are generated from the coordinated activation of a number of ion channels whose function and localization are tightly regulated. Most ion channels exist and function within macromolecular complexes. Macromolecular complexes allow for rapid and localized regulation of channel activity by phosphorylation and dephosphorylation. Additionally, anchoring proteins target enzymes to particular ion channels or ion channel complexes to distinct microdomains within the cardiomyocyte. Dysfunction in channel localization or activity can result in a disruption of action potential conduction, resulting in arrhythmia. As a result, an expanded view of cardiac “channelopathies” has been revealed. With this new view of congenital arrhythmia, future work should focus on the role of other anchoring proteins for potential roles in the development of human disease. The MAGUK protein SAP97, for example, is highly expressed in the myocardium and associates with voltage-dependent *Shaker* channels $\text{K}_v1.5$ [39,40] and Kir channels [41]. Recently, SAP97 has been demonstrated to form a tripartite complex with K_v4 and CaMKII [42]. In the heart, the main repolarization currents that contribute to the early repolarization phase of the action potential, as well as adaptation to changes in cardiac cycle length, is provided by the transient outward potassium current, I_{to} . Often I_{to} is altered in cardiac diseases, increasing the risk for cardiac arrhythmias. $\text{K}_v4.2$ and $\text{K}_v4.3$ are the main determinants of cardiac I_{to} . Several protein kinases have been demonstrated to regulate cardiac I_{to} current. Inhibition CaMKII accelerates I_{to} inactivation, producing an enhanced fast transient component of the outward current. It is reasonable to propose that mutations in SAP97 or CaMKII which disrupt the formation of this tripartite complex could result in arrhythmia through dysregulation of I_{to} .

In theatre and literature, “fifth business” refers to a character who is neither the villain nor hero, but whose role is necessary for plot resolution. While early theories of channelopathies cast anchoring proteins in this role, recent evidence has shed new light on the functional complexity of these proteins. As our understanding of congenital arrhythmias has evolved,

so too has the concept of anchoring proteins, progressing from minor characters to key players in human physiology and disease.

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Figure 1. The A-kinase-anchoring protein Yotiao modulates the cardiac potassium channel
The slowly-activating potassium channel (I_{Ks}), consisting of *KCNQ1*-encoded α -subunits and *KCNE1*-encoded β -subunits, is anchored by Yotiao to protein kinase A (PKA) and protein phosphatase 1 (PP1). This complex is required for K^+ current regulation by β -adrenergic signaling.

Figure 2. Ankyrin polypeptides in the organization of cardiac ion channels A

In the ventricular cardiomyocyte, ankyrin-B forms a ternary complex with the Na⁺/Ca²⁺ exchanger (NCX1), the Na⁺/K⁺ ATPase (NKA), and the inositol 1,4,5-trisphosphate (InsP₃) receptor, functionally linking the transverse-tubule with the sarcoplasmic reticulum. Ankyrin-B also binds the protein phosphatase, PP2A, providing an additional level of ion channel regulation. **B.** In the sinoatrial node cell, ankyrin-B targets the NCX, NKA, InsP₃ receptor, and potentially Ca_v1.3. **C.** Ankyrin-G associates with the Na_v1.5 and is responsible for targeting Na_v1.5 to the cardiomyocyte plasma membrane.

Figure 3. The dystrophin-associated protein complex

Syntrophin is a critical link between the DAPC and ion channel like Nav1.5 and PMCA.