STRUCTURAL INSIGHTS INTO THE REGULATORY MECHANISM OF THE RYANODINE RECEPTOR AND ITS DISEASE-ASSOCIATED MUTANTS

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Calcium is a ubiquitous second messenger in cells that plays a vital role in the control of cellular and physiological processes as diverse as cell division, memory and learning, fertilization and muscle contraction. Opening of the sarcoplasmic reticulum (SR) Ca$^{2+}$-release channel, the ryanodine receptor (RyR), in response to mechanical or chemical stimuli via the dihydropyridine receptor (DHPR) is a crucial step in the process of muscle excitation-contraction coupling. I have determined the first high-resolution structure of a folded domain of RyR1 (RyR1A). The structure adopts a β-trefoil fold that is similar to the homologous suppressor domain of the inositol 1,4,5-trisphosphate receptor (IP$_3$R). I identified a loop region in RyR1A concentrated with malignant hyperthermia (MH)- and central core disease (CCD)-associated mutations that have been implicated in perturbing inter-domain interactions with downstream regions of RyR. More recently I have used nuclear magnetic resonance (NMR) spectroscopy to study the structure and dynamics of the cardiac isoform (RyR2) A domain and its mutants. I detected a dynamic α-helix that undergoes an α-helix to β-strand switch in the catecholaminergic polymorphic ventricular tachycardia (CPVT)-associated mutant, RyR2A Δ exon 3. This dynamic helix is localized at an interface with electron dense columns in the cryo-EM map of the tetrameric receptor that connect with the pore region, suggesting that this dynamic helix may also interact with downstream regions of RyR to gate the channel. My high-resolution structural studies in collaboration with others have shed light on the structural underpinnings of RyR function and dysfunction in human disease.
Acknowledgments

I would like to thank my supervisor, Dr. Mitsu Ikura, for all his help and encouragement throughout my graduate studies. I would like to also thank my supervisory committee members, Dr. David MacLennan and Dr. Gil Prive. David, it was an honor to get to know and work with one of the pioneers in our field. I enjoyed our discussions about past RyR research as well as exciting future discoveries yet to come. I am grateful for your encouragement and helpful suggestions throughout my PhD studies. Gil, you were instrumental in providing direction and purpose throughout my graduate studies, especially when experiments didn’t always work. Your technical expertise and timely suggestions helped me through many obstacles.

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I would like to dedicate this thesis to my parents Uriel and Alma Amador and to my fiancé Melinda Hurd. You always believed in me.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>$^2\text{H}$</td>
<td>Deuterium</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>Carbon 13 isotope</td>
</tr>
<tr>
<td>$^{15}\text{N}$</td>
<td>Nitrogen 15 isotope</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström or $10^{-10}$ m</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ARVD2</td>
<td>Arrhythmogenic right ventricular dysplasia type 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>$\beta\text{ME}$</td>
<td>$\beta$-mercaptoethanol</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CCD</td>
<td>Central core disease</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CICR</td>
<td>$\text{Ca}^{2+}$-induced-$\text{Ca}^{2+}$-release</td>
</tr>
<tr>
<td>CPVT</td>
<td>Catecholaminergic polymorphic ventricular tachycardia</td>
</tr>
<tr>
<td>Cryo</td>
<td>Cryogenic</td>
</tr>
<tr>
<td>CSI</td>
<td>Chemical shift index</td>
</tr>
<tr>
<td>CSP</td>
<td>Chemical shift perturbation</td>
</tr>
<tr>
<td>DHP</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E-C</td>
<td>Excitation-contraction</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>\textit{Escherichia coli}</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FKB</td>
<td>FK506-binding protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HS</td>
<td>Hot spot</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IP3R</td>
<td>inositol 1,4,5-trisphosphate receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MALS</td>
<td>Multi angle light scattering</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MH</td>
<td>Malignant hyperthermia</td>
</tr>
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<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
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</tr>
<tr>
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<td>Nonidet P40</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser enhancement spectroscopy</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>R1</td>
<td>Longitudinal relaxation</td>
</tr>
<tr>
<td>R2</td>
<td>Transverse relaxation</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>S^2</td>
<td>Generalized order parameter</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco(endo)plasmic reticulum Ca^{2+}-ATPase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SPRY</td>
<td>tyrosine kinase spore lysis A (SplA) and the mammalian RyR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SOICR</td>
<td>Store overload-induced Ca(^{2+}) release</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TROSY</td>
<td>Transverse relaxation-optimized spectroscopy</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
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Chapter 1

Introduction

This chapter has been reformatted from the original publication: Amador, F.J., Stathopulos, P.B., Enomoto, M., Ikura, M. (2013) Ryanodine Receptor Calcium Release Channels: Lessons from structure-function studies. FEBS J. Epub 2013/02/19. DOI: 10.1111/febs.12194
1.1 Calcium as a cellular messenger

The early ocean was full of ions (Na\(^+\) and Cl\(^-\) (~1-2\%) and Mg\(^{2+}\) (~0.1\%)) that were present at higher concentrations than Ca\(^{2+}\) (~0.04\%) \cite{1}. Their intracellular control was crucial to the evolution of life. Moreover, the control of Ca\(^{2+}\) was critical because of its ability to readily interact with biological molecules. Its unique physical and chemical properties such as flexible coordination chemistry, high affinity for carboxylate oxygen and rapid binding kinetics made it toxic to cells at high concentrations \cite{2}. Therefore, the earliest forms of life had to maintain a low intracellular concentration of Ca\(^{2+}\) \([\text{Ca}^{2+}]_i\) of ~100 nM, which is 10,000-20,000 times lower than the extracellular milieu \cite{3}. This defense mechanism meant to keep cells alive serendipitously created the Ca\(^{2+}\) signaling mechanism that is vital to cellular function.

Of the 1.4 kg of calcium (Ca\(^{2+}\)) found in the human body, less than 1\% is free to circulate in the blood and extracellular spaces to reach cells and regulate their most important processes \cite{4}. This minute amount of free Ca\(^{2+}\) has extraordinary consequences when it comes to human physiology. Sidney Ringer was first to demonstrate its physiological role while studying the contraction of isolated rat hearts in the late 19\textsuperscript{th} century \cite{5}. His pioneering work was largely forgotten until the 1940’s when Heilbrunn \cite{6} and Bailey’s \cite{7} work reignited the field. Since then, this small molecule has been shown to regulate such diverse physiological functions as cell proliferation and differentiation, secretion, fertilization and muscle contraction (reviewed in \cite{3, 4, 8}).
A simplified Ca\(^{2+}\) signaling mechanism can be thought of two competing systems, ‘on’ and ‘off’ [9] (Figure 1.1).

![Diagram of simplified Ca\(^{2+}\) signaling system.](image)

**Figure 1.1 Simplified Ca\(^{2+}\) signaling system.**

Stimuli are sensed by receptors on the cell surface, which generate Ca\(^{2+}\) mobilizing signals, such as inositol 1,4,5-trisphosphate (IP3). These act on various ‘ON’ mechanisms (i.e. Ca\(^{2+}\)-release channels) to increase intracellular Ca\(^{2+}\) concentration. The increased level of Ca\(^{2+}\) activates Ca\(^{2+}\)-sensitive processes (e.g. muscle contraction), after which, ‘OFF’ mechanism restore Ca\(^{2+}\) to its resting level. Figure taken from (Berridge et al., 2000)

The ‘on’ system is responsible for introducing Ca\(^{2+}\) into the cytosol, while the ‘off’ system is responsible for removing Ca\(^{2+}\) from the cytosol. During the ‘on’ system, Ca\(^{2+}\) enters down its concentration gradient via specialized channels found on the plasma membrane or on the membrane of internal Ca\(^{2+}\) stores such as the endoplasmic or sarcoplasmic reticulum (ER/SR). Voltage-gated, ligand-gated and store-operated
channels are examples of the former while the inositol 1,4,5-trisphosphate receptor (IP$_3$R) and ryanodine receptor (RyR) are examples of the latter. A Ca$^{2+}$ signal is generated as a result of this increased [Ca$^{2+}$]. Buffering proteins such as parvalbumin, calbindin and calretinin shape the duration and amplitude of this signal [9]. They also limit the spatial diffusion of Ca$^{2+}$ signals, such as in neurons, where it is confined to synapses. As well as binding to buffers, Ca$^{2+}$ binds to sensors that activate diverse cellular functions. Well-known sensors are troponin C (TnC), Calmodulin (CaM), S100A and neuronal Ca$^{2+}$ sensor (NCS) which all have the Ca$^{2+}$-binding EF-hand motif [10]. The ‘off’ system terminates the Ca$^{2+}$ signal by restoring [Ca$^{2+}$], to its resting level. It does so by pumping or extruding Ca$^{2+}$ out to the external environment or into internal stores. On the plasma membrane, the Na$^+$/Ca$^{2+}$ exchanger (NCX) transports out 1 Ca$^{2+}$ for 3 Na$^+$ [11], while the plasma membrane Ca$^{2+}$-ATPase (PMCA) pump actively transports Ca$^{2+}$ out of the cytosol [12]. The sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) pump is responsible for reloading the ER/SR with Ca$^{2+}$ [13]. Tight control and integration of these two systems allow cells to use the transient increase in [Ca$^{2+}$], as a means to carry out most of its cellular functions. Although, Ca$^{2+}$ signaling is responsible for many cellular processes, I will now expand on the mechanisms involved in muscle contraction.

### 1.2 Human muscle anatomy and physiology

Human skeletal muscle contains some of the most specialized cellular architecture and cell types in the human body. A skeletal muscle is made up of bundles of individual muscle cells called fascicles, these in turn are made up of muscle fibers and lastly these are made up of myofibrils, which contain the two contractile filaments, actin and myosin [14].
1.3 Identification of Excitation-contraction (E-C) coupling molecular components

The process by which membrane depolarization leads to muscle contraction is called excitation-contraction (E-C) coupling and by the 1960s, the most pressing question regarding E-C coupling was how an action potential (AP) could initiate contraction of the fibre’s center in 1-2 msec when they are 50-100 µm in diameter [15]. Diffusion of an activating factor (e.g. Ca$^{2+}$) alone would take hundreds of seconds to reach the center. An answer emerged from the combined work of several groups, which showed that invaginations of the plasma membrane called transverse (T)-tubules penetrated deep inside the muscle fibre and surrounded each myofibril [16-18]. Further experiments showed that T-tubules were electrically identical to the plasma membrane [19-21]. In addition to being close to the contractile elements, T-tubules were flanked on either side by the terminal cisternae of the SR, forming what is commonly referred to as a triad (Figure 1.2).
Figure 1.2 Triad architecture.

A schematic of T-tubule formation by invagination of the sarcolemma is shown on the left panel. The close apposition of the terminal cisternae of the SR to the T-tubule allows depolarization to activate the calcium release channels of the SR (red figures). The arrangement of a T-tubule flanked by two terminal cisternae is termed a triad. The dashed line indicates a section through a triad, giving rise to the micrograph on the right. Arrows point to electron dense ‘feet’ spanning the gap between the two membranes, which are in fact the calcium release channels of the SR. The panel on the right is taken from (Franzini-Armstrong, 1973).

A finding that the T-tubule membrane was a rich source of an L-type Ca\(^{2+}\) channel known as the dihydropyridine receptor (DHPR) was initially overlooked and it wasn’t until subsequent experiments demonstrated that DHPR might be the voltage sensor in E-C coupling [22-25]. This was confirmed when cell lines derived from a DHPR \(\alpha_{15}\)-null dysgenic mouse lacking E-C coupling and charge movement were recapitulated by injecting cDNA of the \(\alpha_1\)-subunit [26, 27]. Electron micrographs of triads revealed a 10 nm gap between the T-tubules and the terminal cisternae spanned by an unknown electron ‘density’ [16]. Studies in the late 1980s identified this ‘density’ as a high molecular weight protein with a high affinity for the plant alkaloid ryanodine and as the SR Ca\(^{2+}\)-release channel [28-30]. Electron microscopy studies showed that the ryanodine receptor (RyR), as it became known, aligned with four DHPR molecules (a
tetrad) in a repeating pattern that saw every other RyR associated with a tetrad [31]. In contrast to skeletal muscle, RyRs and DHPRs in cardiomyocytes do not adhere to this strict alignment. This distinction proves crucial for the regulation of SR Ca^{2+}-release in heart versus skeletal muscle, as will be explained below.

With these important elements identified, a sequence of events could now outline the mechanism of E-C coupling (Figure 1.3). The AP propagates along the plasma membrane and down the T-tubules where it is sensed by DHPR. Coupling between DHPR and RyR causes the latter to open and release Ca^{2+} from the SR down its concentration gradient into the myoplasm. Myoplasmic Ca^{2+} binds to TnC, initiating movement of tropomyosin, resulting in actin and myosin interaction, and force development. These ‘on’ mechanisms quickly give way to ‘off’ mechanisms that terminate SR Ca^{2+}-release and pump myoplasmic Ca^{2+} back into the SR (SERCA) and out of the cell (NCX) ensuring myocytes are ready for another AP.
An action potential (AP) propagates down the T-tubule membrane where it is detected by the dihydropyridine receptor (DHPR), causing it to undergo a conformation change that couples to opening of the SR Ca\(^{2+}\)-release channel, the ryanodine receptor (RyR1). Ca\(^{2+}\) enters the myoplasm down its steep concentration gradient and binds to troponin C, initiating movement of tropomyosin, actin and myosin interaction and force development. Ca\(^{2+}\) is pumped (SERCA1) or extruded (NCX) out of the cell, ensuring the cell is ready for another AP.

1.4 Ryanodine receptors

1.4.1 Discovery, purification and expression of RyRs

Several groups successfully monitored the solubilization, purification and isolation of RyRs from striated muscle using \(^3\)H-ryanodine \([32-34]\), a plant alkaloid and a potent insecticide \([35]\). These groups showed that RyRs form homotetramers with molecular weights of ~2.2MDa and each monomer consists of ~5000 amino acids.
In mammals, three different isoforms of RyRs exist. The first to be studied and cloned was RyR1 \[36, 37\]. It is primarily expressed in skeletal muscle. RyR2 is highly expressed in cardiac muscle \[38, 39\] but is also found in smooth muscle and the nervous system \[40, 41\]. RyR3 is expressed at low levels in a variety of cell types including the nervous system and skeletal muscles of the diaphragm \[41\]. All three isoforms share \(~66\%\) sequence identity, the largest variation occurring in three ‘divergent regions’ encompassing residues 4254-4631 (D1), 1342-1403 (D2) and 1872-1923 (D3) in RyR1. Non-mammalian vertebrates such as chickens and bullfrogs express two RyR isoforms, RyRα and RyRβ \[42, 43\], while lower organisms only express a single isoform \[44, 45\]. Knockout studies of RyR demonstrate its critical role in development and physiology. Mice missing \(RyR1\) die perinatally due to respiratory failure \[46\], \(RyR2\)-knockout mice die early during embryonic development \[47\], while mice missing \(RyR3\) are viable \[48-50\] but exhibit impaired social behavior \[51-53\].

1.4.2 Disease Mutations

Several inheritable human diseases are associated with RyR mutations. Currently, none are associated with RyR3 mutations, but recent studies of neurodegenerative disorders such as Alzheimer’s Disease (AD) suggest that overexpression of RyR3 as well as altered expression of alternatively spliced variants of RyR2 and RyR3 may play a role in the remodeling of neuronal \(Ca^{2+}\) signaling which lead to AD \[54, 55\].

Mutations in RyR1 have been linked with malignant hyperthermia (MH) \[56-59\], central core disease (CCD) \[60-62\] and multiminicore disease \[63\]. MH is a pharmacogenetic disorder characterized by a rapid rise in body temperature, muscle
rigidity and rhabdomyolysis in severe cases. An MH episode is usually triggered by a volatile anesthetic or a muscle relaxant, but in some cases stress can also be a trigger [64]. Administering dantrolene, a hydantoin derivative, effectively treats MH episodes. Since the first clinical use of dantrolene in the late seventies [65], the mortality rate for MH episodes has decreased from ~80% to 5% today [66]. Although dantrolene inhibits RyR1 and studies have shown a direct interaction with RyR1 [67, 68], its molecular mode of action remains unresolved. CCD is a congenital myopathy that usually presents during infancy and can lead to death. It is characterized by regions devoid of mitochondria in muscle fibers that histologically appear as circular ‘cores’ after oxidative staining [69, 70]. Patients usually present with delayed motor development and hypotonia. Currently there are no known treatments for CCD.

Mutations associated with RyR2 can give rise to diseases associated with cardiac arrhythmias. Catecholaminergic polymorphic ventricular tachycardia (CPVT) gives rise to bidirectional ventricular tachycardia that can result in sudden death [71]. These episodes are usually triggered by physical or emotional stress and patients do not present any structural evidence of myocardial disease [72]. Another cardiac disease associated with RyR2 mutations is arrhythmogenic right ventricular dysplasia (ARVD2). It is characterized by replacement of cardiac tissue with ‘fibrofatty’ deposits [73].

Well over 500 RyR mutations have been identified with a link to human disease. Most of these tend to cluster in three distinct regions on the protein: the N-terminal region (1-600), central region (~2100-2500) and the C-terminal region (~3900-5000). It should be noted, however, that mutations have been found outside these regions (Figure 1.4). Mutations associated with a specific disease tend to cluster in particular regions.
Figure 1.4 Localization of disease-associated mutations on RyR1 and RyR2.
Vertical lines representing a disease mutation are shown along the linear sequence of both RyRs. RyR1 mutations are more dispersed, whereas RyR2 mutations are found in three or four mutation hot spots. Domains where experimentally determined structures or homology models exist are outlined. Figure taken from (Yuchi et al., 2013) [74] within RyRs. For instance, most MH mutations are found in the N-terminal and central region, while those of CCD are clustered in the C-terminal region [75]. Most mutation studies point to a gain-of-function hypothesis, whereby RyRs are rendered hypersensitive to activation either from the cytosolic [76] or luminal side of the receptor [77]. However, there are some mutations that reduce RyR activity; these are typically associated with CCD and are found in the pore-forming region of RyR1 [78-80]. Many of them lead to uncoupling of activation via DHPR and/or reduced sensitivity to activating agents such as Ca\(^{2+}\), caffeine and 4-CmC [81, 82]. Models at the functional and molecular level have tried to explain RyR function and dysfunction brought about by disease-associated mutations. Chen and co-workers have put forward a functional model that examines the effect of SR Ca\(^{2+}\) on the regulation of RyRs [83]. It is known that SR Ca\(^{2+}\) overload can result in spontaneous Ca\(^{2+}\) waves in the absence of membrane depolarization, also known as store overload-induced Ca\(^{2+}\) release (SOICR) [77]. RyRs are activated by a certain level of free luminal Ca\(^{2+}\), typically ~ 95% of ER store capacity and is known as the activation threshold. Mutations in RyR1 and RyR2 have been shown to lower this threshold, making them hypersensitive to activating stimuli and resulting in Ca\(^{2+}\) leak
into the myoplasm [84, 85]. Additionally, ARVD2 and dilated cardiomyopathy (DCM) mutations have recently been shown to lower the termination threshold which results in greater Ca\(^{2+}\) fractional release (activation threshold – termination threshold) during SOICR [86]. The resultant increased myoplasmic Ca\(^{2+}\) and decreased SR Ca\(^{2+}\) would give rise to the observed disease phenotypes associated with RyR1 and RyR2 mutations. Ikemoto and Yamamoto have proposed a molecular model for the increased sensitivity or ‘leakiness’ of RyRs, known as the ‘zipper’ model [87, 88]. In this model they suggest that the N-terminal and central regions interact with each other. This interaction is thought to stabilize the channel in its closed state. Mutations clustered at interfaces between the N-terminal and central regions of RyR would weaken these normal interactions involved in the allostery of the channel, thereby destabilizing the closed state and rendering the channel more sensitive to stimuli. Several caveats exist for this model. The first is that it is based on the study of smaller peptides [89] as opposed to the larger folded N-terminal and central domains. Also, it relies on a binary interaction between the N-terminal and central regions for controlling the opening and closing of RyRs. However, as has been recently demonstrated by high-resolution structural studies, the N-terminal domain is involved in at least six interfaces (see below).

### 1.4.3 Cryo-electron microscopy (Cryo-EM) studies reveal tetrameric structure of RyR

Advances in single-particle cryo electron microscopy (cryo-EM) have accelerated our understanding of the structure/function relationship in RyRs. Initial cryo-EM studies of RyR1 isolated from rabbit skeletal muscle examined the channel in the closed state at a resolution of ~2.4 nm [97, 98]. Subsequent studies have pushed the resolution to ~ 10 Å
These later studies reveal a tetrameric structure that is made up of a large cytoplasmic domain (~80% volume), with the remainder of the structure encompassing the transmembrane (TM) region (Figure 1.5A and B). The structure exhibits a four-fold symmetry axis along the pore of the channel. The cytoplasmic region measures $270 \times 270 \times 100$ Å, while the smaller TM region measures $120 \times 120 \times 60$ Å. The former is full of cavities that separate distinct regions from one-another and ostensibly provide access to a host of RyR regulators for docking onto the structure [75]. Several cytoplasmic subregions have been identified and are thought to play a role in the allosteric modulation of RyR. These include the ‘clamp’ and ‘handle’ regions as well as the ‘central rim’ [100, 101] (Figure 1.5A and B). A study characterizing the open and closed state of RyR1 at 10.2 Å revealed not only a change in channel pore size but also considerable changes in the conformation of the cytoplasmic region [102]. Specifically, the clamp region moved down towards the SR membrane by ~8 Å, while the central rim moved up towards the T-tubules by 4 Å. In addition there is evidence for a twisting motion in the TM region [102]. These studies reveal that RyR undergoes allosteric coupling between the distinct regions in its transition from open to closed state, with easy access for its regulators to modulate this coupling.

The insertion of green fluorescent fusion proteins (GFP) or antibody epitopes into full length RyRs has facilitated approximations of the location of several RyR regions and accessory proteins in the tetrameric receptor (reviewed by Kimlicka and Van Petegem in [103]). A caveat of these studies, however, is that the chromophoric center of fluorescent proteins inserted into RyR which report on relative localization can be as far away as 45 Å from the insertion site inferred from primary sequence [104].
Figure 1.5 Molecular architecture of tetrameric RyR1.
Cryo-EM map at 9.6 Å resolution of tetrameric RyR1 in the closed state is viewed from the cytoplasm (A) and side (B). Subregions are numbered and colored for one RyR1 subunit. Morphological regions (clamp, handle and column) are highlighted as well. Figure taken from (Serysheva et al., 2008).

1.4.4 Regulators

Since ~80% of the RyR is found facing the cytosol and it contains many cavities, a wide array of proteins, small molecules and post-translational modifications are able to regulate the function of RyR either in a stimulatory or inhibitory fashion. Additionally, regulation via the luminal side of the receptor is also of vital importance. RyRs together with proteins on either side of the SR membrane such as DHPR, PKA, FKBP, CaM, CaMKII, triadin, junction, and calsequestrin form a macromolecular complex that receives and integrates cellular signals to regulate SR Ca$^{2+}$-release [105].

1.4.4.1 Ca$^{2+}$, Mg$^{2+}$ and ATP

Studies using skinned muscle fibres were first to reveal the complex interplay between Ca$^{2+}$, Mg$^{2+}$ and ATP in the regulation of RyRs. Although Ca$^{2+}$ is regarded as a potent activator of RyRs, it is unable to activate RyR1 in the presence of Mg$^{2+}$. However, if myoplasmic Mg$^{2+}$ is lowered well below its physiological level of 1 mM to 0.5 mM, RyR1 spontaneously opens and depletes the SR of Ca$^{2+}$ [106]. This occurs because RyR1 is strongly activated by myoplasmic ATP, even in the near absence of [Ca$^{2+}$]$_i$ (<10 nM) [107].

Studying isolated mammalian RyRs in SR vesicles in the absence of Mg$^{2+}$ further clarified these findings by showing that RyR2 is activated by Ca$^{2+}$ binding to an activating (A) site with a dissociation constant ($k_a$) of ~ 1 µM and inhibited by Ca$^{2+}$ binding to a low affinity ($k_i >$ 1 mM) inhibitory (I) site [108, 109]. This produces a bell-
shaped Ca\(^{2+}\) dependence curve for RyR2 activation (Figure 1.6A). This differs in two ways for RyR1: (i) as stated before ATP activates the channel even in the absence of Ca\(^{2+}\); and (ii) the affinity of the I-site for Ca\(^{2+}\) is much higher (k\(_i\) ~ 0.1 mM) meaning that the curve shows increased RyR1 activity at low [Ca\(^{2+}\)]\(_i\) and marked inhibition at 0.1-1 mM Ca\(^{2+}\) (Figure 1.6B) [108, 109].

The introduction of Mg\(^{2+}\) adds another layer to RyR regulation. In RyR1 and RyR2 Mg\(^{2+}\) competes with Ca\(^{2+}\) at both the A- and I-sites. However, the affinity for Mg\(^{2+}\) at the A-site is ~40-1000 fold lower than Ca\(^{2+}\), furthermore Mg\(^{2+}\) does not activate the channel [110]. Therefore, the presence of physiological levels of Mg\(^{2+}\) (1 mM) shifts the activation dependence to higher [Ca\(^{2+}\)]\(_i\) for both RyR1 and RyR2 (Figure 1.6A and B). In contrast, the affinity for Mg\(^{2+}\) at the I-site is identical to Ca\(^{2+}\) and the inhibitory ability of Mg\(^{2+}\) is equal to Ca\(^{2+}\). Therefore the I-site is really a ‘Mg\(^{2+}\)-I-site’ since Mg\(^{2+}\) will occupy it since levels of Mg\(^{2+}\) in the myoplasm are 1 mM while Ca\(^{2+}\) never reaches these levels. Interestingly, this Mg\(^{2+}\) inhibition is not significant for RyR2 because affinity at this site is so low (k\(_i\) > 1 mM) compared to RyR1 (k\(_i\) ~ 0.1 mM). However, it is significant for RyR1, since Mg\(^{2+}\) will be bound to the I-site no matter how high [Ca\(^{2+}\)]\(_i\) reaches.

The net effect therefore is that a small rise in [Ca\(^{2+}\)]\(_i\) will activate some RyR2 and the resulting Ca\(^{2+}\)-release will recruit others. In contrast, RyR1 cannot open solely due to an increase in [Ca\(^{2+}\)]\(_i\); in order to fully activate RyR1, another mechanism involving the dihydropyridine receptor (DHPR) is required to overcome the inhibitory action of Mg\(^{2+}\).
**Figure 1.6 Schematic of the Ca\(^{2+}\) dependence of RyR1 and RyR2 activation.**

*Left panel* RyR2 in the absence of cytoplasmic Mg\(^{2+}\) (black curve) shows half maximal activation at ~ 1 µM Ca\(^{2+}\) and half inhibition at > 1 mM Ca\(^{2+}\); giving rise to a bell-shaped Ca\(^{2+}\) dependence. The presence of 1 mM Mg\(^{2+}\) (red curve) shifts the Ca\(^{2+}\) dependence of activation to higher [Ca\(^{2+}\)] and lowers the maximal activation to that seen at 1 mM Ca\(^{2+}\) in the absence of Mg\(^{2+}\) (dashed line). *Right panel* In the absence of Mg\(^{2+}\) (black curve), ATP activates RyR1 even at low [Ca\(^{2+}\)] and is inhibited half maximally at 0.1 mM Ca\(^{2+}\). In the presence of 1 mM Mg\(^{2+}\) (red curve), peak activation of RyR1 is so low that it does not exceed the level seen at 1 mM Ca\(^{2+}\) in the absence of Mg\(^{2+}\) (dashed line). Figure was adapted from (Lamb, 2000) \[111\]

### 1.4.4.2 Dihydropyridine receptors

DHPR is the voltage sensor in myocytes. It is comprised of 5 subunits but only two are essential for E-C coupling in skeletal cells: the II-III loop of the \(a_{1S}\) subunit and the C-terminal tail of the \(\beta_{1a}\) subunit \[112\]. The II-III loop is well characterized as the critical interaction site for E-C coupling \[27, 113-116\]. Depolarization is sensed by charged residues, which cause a conformational change in the loop that is physically transmitted to RyR1 \[117\]. One role of the \(\beta_{1a}\) subunit is to traffic the \(a_{1S}\) subunit to the T-tubule membrane; further, new studies show that it might also contribute directly to E-C coupling via its C-terminal tail \[112, 118\]. Although DHPR is a key component of E-C coupling, two distinct mechanisms exist for triggering RyR opening and subsequent Ca\(^{2+}\)
release from the SR into the cytosol. In cardiomyocytes, depolarization of the plasma membrane results in the opening of the DHPRs, which allows extracellular Ca\(^{2+}\) into the cytosol. This influx is sensed by RyR2, which binds Ca\(^{2+}\) and enables opening of the channel and a further influx of Ca\(^{2+}\) from the SR into the cytosol. This process is known as Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release (CICR) [119, 120]. In skeletal muscles RyRs are intimately tied to DHPRs allowing a direct link between the two receptors [25, 27]. In this scenario, two components of the DHPR interact with RyR1 in response to depolarization of the surface membrane. The sensitivity of DHPR to voltage changes at the plasma membrane results in a conformational change that is sensed by RyR1. It is believed that the interaction between DHPR and RyR1 stabilizes an open state of RyR1 that has lower affinity for Mg\(^{2+}\) and thus removes its potent inhibitory affect. Therefore, depolarization of the plasma membrane alone is sufficient to open RyR1.

Downstream from the N-terminal domain (NTD) is a region encoding three ‘SPRY’ domains, so named because they were identified in both Dictyostelium discoideum tyrosine kinase spore lysis A (SplA) and the mammalian RyR [121]. SPRY domains normally function as protein-protein interaction motifs and are composed of a β-sandwich [122]. Although the precise function of SPRY domains in RyRs is still unknown, several reports support the idea that SPRY2 (residues S1085-V1208 in RyR1) plays a role in E-C coupling and, in fact, may be the binding partner for DHPR [123-125].

### 1.4.4.3 EF-hand containing proteins

Calmodulin (CaM) is a small (17kDa) EF-hand containing Ca\(^{2+}\) binding protein that is able to fine-tune the activity of RyRs. It binds to the receptor in both its Ca\(^{2+}\)-
bound (Ca\textsuperscript{2+}-CaM) and Ca\textsuperscript{2+}-free states (apo-CaM) \cite{126, 127}. Two overlapping yet distinct bindings sites are proposed for apo- and Ca\textsuperscript{2+}-CaM on the tetrameric receptor \cite{128}, which is in close proximity to residues 3614-3643 in RyR1 and 3581-3612 in RyR2 \cite{129, 130}. Each RyR isoform is affected differently by CaM. Apo-CaM weakly activates RyR1, while Ca\textsuperscript{2+}-CaM inhibits RyR1 \cite{126, 127, 130, 131}. For RyR2, both apo-CaM and Ca\textsuperscript{2+}-CaM inhibit its activity \cite{132, 133}. The opposite effects of apo- and Ca\textsuperscript{2+}-CaM on RyR1 activity may be due to the structural consequences of Ca\textsuperscript{2+} binding to CaM. The structural fold of apo-CaM allows it to bind to the “activating” binding site while Ca\textsuperscript{2+} binding to CaM results in its structural rearrangement allowing it to bind to the “inhibitory” site. S100A1 is another EF-hand containing protein that binds and regulates RyRs. It enhances the activity of both RyR1 and RyR2 \cite{134, 135}. A crystal structure of Ca\textsuperscript{2+}-CaM bound to a RyR1 peptide (3614-3643) \cite{136} and an NMR structure of S100A1 bound to two RyR1 peptides (3616-3627) \cite{137} reveal that both proteins compete for the identical binding site by interacting with the same hydrophobic residues. A simplified model for apo-CaM, Ca\textsuperscript{2+}-CaM and S100A1 regulation proposes that under resting conditions, S100A1 primarily regulates RyR1, allowing it to potentiate maximal Ca\textsuperscript{2+} release from the SR when it is stimulated. As Ca\textsuperscript{2+} becomes elevated in the cytosol, Ca\textsuperscript{2+}-CaM begins to compete out S100A1, thereby promoting channel inactivation \cite{138}.

1.4.4.4 \textit{FKBP}

FKBPs are small cytosolic proteins capable of binding immunosuppressive drugs such as FK506 and rapamycin. FKBP12 and FKBP12.6 (named according to their mass) bind to all three isoforms \cite{139} of RyR but generally the former is associated with RyR1
FKBPs bind to RyRs with a stoichiometry of 4 FKBPs to 1 RyR tetramer [140, 142] and stabilize the closed state of the channel [141, 143]. Removal of FKBPs, either by immunosuppressive drugs or a FKBPs genetic deficiency results in higher RyR open probability (P₀) and mean open times [143, 144].

1.4.4.5 Phosphorylation by PKA and CaMKII

It has been known and generally accepted that phosphorylation modulates the activity of RyRs [145]. The specifics of which sites are phosphorylated [146] and which kinase, PKA [90, 91] or CaMKII [147-149], are responsible for hyperphosphorylation is up for debate. The results however are generally accepted; elevated phosphorylation in the heart leads to increased SR Ca^{2+} leak, which contributes to an increased disposition for arrhythmias [147, 148, 150]. A study by Marks and colleagues suggested that the increased β-adrenergic levels that occur during stress or exercise lead to PKA-dependent hyperphosphorylation of RyR residues (S2030 and S2809 in RyR2 and S2843 in RyR1) [90]. Furthermore, this causes FKBPs to dissociate from RyRs, producing leaky channels. These leaky channels reduce SR Ca^{2+} content, which result in smaller Ca^{2+} transients and weaker heartbeats, ultimately manifesting as cardiac arrhythmias. However, dissociation of FKBPs due to PKA-dependent hyperphosphorylation in failing hearts has not been verified by other groups [93, 151].

A clearer picture has emerged for the role of CaMKII dependent phosphorylation of RyR2. Studies ranging from single channel experiments [152] to transgenic animals [153] show consistent alterations of RyR function as a consequence of CaMKII activity. Single channel experiments show increased RyR2 P₀ upon phosphorylation by CaMKII,
while transgenic mice overexpressing CaMKII showed marked hypertrophy, elevated Ca\textsuperscript{2+} frequencies, pronounced SR Ca\textsuperscript{2+} leak and susceptibility for arrhythmias [153, 154]. Furthermore, mice engineered with the RyR2 S2814A mutation, which is unable to be phosphorylated by CaMKII, were protected from inducible arrhythmias [155]. Taken together these studies suggest that CaMKII-dependent phosphorylation markedly modifies RyR2 function and cardiac Ca\textsuperscript{2+} signaling.

Recent studies of mice with Duchenne muscular dystrophy (DMD), *mdx* mice, have also implicated CaMKII-dependent phosphorylation of RyR2 in promoting ventricular tachycardia (VT) in these animals [156]. This is significant since VT is the second most common cause of death in patients with DMD. A recent study has shown that CaMKII blockade with the inhibitor KN-93 or genetic inhibition of RyR2 S2814 prevent VT in *mdx* mice [156]. As a consequence, CaMKII and RyR2 have become targets for treatment of various cardiac-associated disorders.

### 1.5 Comparison with the inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R)

In the same year that RyR1 was cloned, another Ca\textsuperscript{2+} release channel, the inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R) from rat and mouse had its primary structure determined [129, 157]. These studies revealed that RyR and IP\textsubscript{3}R share ~17% sequence identity overall; however, the identity increases to ~35% within the predicted transmembrane region of the two proteins. It was also revealed that IP\textsubscript{3}Rs are about half the size of RyRs. Despite the difference in molecular weight, the two receptors show similarities in their function and regulation. Both permeate Ca\textsuperscript{2+} and are regulated by it in a bell-shaped manner [158, 159]; both are transmembrane proteins that share a high
sequence homology in their ion-conducting pore and both have conserved domains outside the TM region. These include the internal RyR and IP$_3$R homology (RIH) [160] and mannosyl-transferase, IP$_3$R, RyR (MIR) domains [161].

Three isoforms of IP$_3$Rs (IP$_3$R1, IP$_3$R2 and IP$_3$R3) are also present in mammals [162-164]. They are ubiquitously expressed and have distinct cellular distribution patterns. In the nervous system, IP$_3$R1 is predominantly expressed, whereas most other cell types express multiple isoforms [165-168]. IP$_3$R-knockout studies reveal that IP$_3$R1 deficient mice die in utero or by the weaning period [169], whereas IP$_3$R2 or IP$_3$R3 deficient mice exhibit physiological abnormalities such as decreased olfactory mucus secretion and hypoglycemia [170, 171]. Although many studies have linked mutations on the RyR genes to several human diseases, only one mutation in IP$_3$R has been associated with human disease [172].

Both RyR and IP$_3$R functional genes have been identified in a variety of multicellular eukaryotes ranging from Caenorhabditis elegans to human [105, 173]. Recently, putative RyRs and IP$_3$Rs have also been observed in unicellular organisms such as Salpingoeca rosetta, Monosiga brevicollis and Capsaspora owczarzaki [174, 175] as well as in pathogenic unicellular parasites including Trypanosoma brucei, Trypanosoma cruzi, Leishmania infantum and Leishmania major [160]. These putative IP$_3$R/RyR channels are found in several subcellular compartments such as the ER, alveolar sacs and contractile vacuole complex, where they play a role in exocytosis, trichocyst biogenesis and basic Ca$^{2+}$ homeostasis [176]. A recent study that examined the co-evolution of these two channels suggests that RyRs arose from ancestral IP$_3$R-like channels by incorporating promiscuous ‘RyR’ and ‘SPRY’ domains via horizontal gene transfer [177].
These genomic analyses suggest that RyRs and IP$_3$Rs have co-evolved from a common ancestor in unicellular species.

Despite the large difference in size, the two receptors share a similar architecture consisting of a N-terminal domain (NTD), followed by a central regulatory domain that has various binding partners in both proteins, yet is nearly twice as large in RyRs, a transmembrane domain (TMD) that contains the Ca$^{2+}$-conducting pore and a C-terminal tail (CTT) (Figure 1.7A). The phylogenetic relationship of RyRs and IP$_3$Rs in unicellular and multicellular eukaryotes suggests that the NTD of IP$_3$R evolved from a lower organism RyR (Figure 1.7B). This indicates that the inositol 1,4,5-trisphosphate (IP$_3$) binding ability of IP$_3$Rs was not always inherent, and may have been attained during evolution. In fact, Arg and Lys residues involved in IP$_3$ coordination are less conserved in the lower eukaryotes, suggesting a mechanism whereby IP$_3$Rs evolutionarily acquired dual control by Ca$^{2+}$ and IP$_3$. 
Figure 1.7 Domain organization and genomic analyses of NTD from RyRs and IP$_3$Rs.

A: Domain architecture of RyR and IP$_3$R. Numbering used is from rabbit RyR and rat IP$_3$R1. NTD, N-terminal domain; SD, suppressor domain; IBC, IP$_3$-binding core; TMD, transmembrane domain; CTT, C-terminal tail. B: A neighbor-joining phylogenetic tree showing the relationships of the NTD from uni- and multi-cellular eukaryote homologues of IP$_3$Rs and RyRs. The numbers in parentheses represent the amino acid residues of the RyR and IP$_3$R homologues. Bootstrap values greater than 40 are shown at the nodes. The scale bar represents amino acid substitutions per site. Multiple sequence alignments were made with MUSCLE [178] using the default parameters. Neighbor-joining analysis was carried out using MEGA 5 [179].
1.6 Conclusion and future prospective

Since the discovery that RyRs were the ‘density’ spanning the gap between the SR and T-tubular membrane, an abundance of information has accumulated about their function and regulation in SR Ca\(^{2+}\)-release. RyRs respond to either chemical (e.g. Ca\(^{2+}\)) or physical signals (e.g. DHPR) to release Ca\(^{2+}\) into the myoplasm to carry out its function in muscle contraction. Throughout this process, the activity of RyRs is constantly regulated and fine-tuned by a myriad of proteins, small molecules and post-translational modifications that allow RyRs to respond to cellular signals and therefore generate SR Ca\(^{2+}\) transients matched to the needs of skeletal or cardiac muscle.

Cryo-EM studies of functional tetrameric RyRs, in the open and closed state, have provided the first structural clues as to how these giant receptors function; by revealing allosteric movements during open and closed states or the location of several binding partners on the tetrameric structure. High-resolution structural studies using X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy have recently augmented the “low” resolution data of cryo-EM of both wild-type (wt) and mutant RyRs. Despite these recent advances, many questions remain. From a structural biology perspective we will need to determine the atomic-resolution structures of various parts of the receptor, particularly those enriched with mutations. Moreover, elucidation of the structural basis for receptor interaction with various binding partners such as DHPR, FKBP and CaM is required. Finally, it will be critical to decipher the exact mechanism of ion translocation within the TM-domain to fully understand the complex nature of these giant receptors and their role in human physiology and disease.
1.7 Thesis rationale and organization

RyRs were first identified in 1987 as the SR Ca^{2+}-release channels and within a short period of time, low-resolution cryo-EM studies revealed its overall structure; many of its regulators and effectors were identified and studied; and RyR dysfunction was implicated in several lethal human diseases. By the time I began my graduate studies, the cryo-EM structures had been refined to ~9.6Å, allowing sausage-like domains and many cavities to be visualized in the cytoplasmic portion of the receptor; the effect of many RyR regulators had been established; the importance of posttranslational modifications such as phosphorylation was a hot topic and the functional consequences of many disease-associated mutations had been established. However, the underlying mechanism of the RyR structure-function relationship with and without regulators as well as their dysfunction associated with several human diseases was not well defined.

High-resolution structural data was needed to help fill this void. Clearly, the size of RyR did not make it feasible to carry out these studies on the entire protein. However, our lab had recently been successful in determining the X-ray crystal structures of two homologous domains in the NTD of IP_3R. More importantly, the NTD domain in RyR is rich in disease-associated mutations. A collaboration with Dr. David MacLennan’s laboratory was initiated before I arrived in the fall of 2007 with the goal of determining high-resolution structures of several RyR domains. His lab had optimized the expression and purification of several RyR1 constructs, including some from the NTD that were homologous with the IP_3R structures recently solved by our lab.
When I joined the lab, several constructs for RyR1 were optimized for expression and purification; also initial structural studies using X-ray crystallography or NMR were underway. In my first year of graduate work, I optimized the expression and purification of several RyR2 constructs from the distal N terminus. Preliminary X-ray crystallography trials and NMR studies were not promising enough to obtain structural information. Therefore it was decided that I would focus my attention on the RyR1 constructs. Soon after, I determined the first high-resolution structure of a folded domain for any isoform of RyR, the distal N terminus of RyR1 (RyR1A). I identified a loop concentrated with several charged residues and disease-associated mutations, which I called the ‘hot spot’ (HS)-loop, suggesting that this loop could be a site of interaction with downstream regions of RyR1 or other proteins. I also studied the effect of several disease-associated mutations on the structural stability of RyR1A using NMR methods. Soon after my structure was solved, the lab of Dr. Filip Van Petegem solved the corresponding structure in RyR2. They also solved the structure of the entire NTD, which highlighted the structural importance of the HS-loop in RyR function. This work is presented in Chapter 2.

In my last year of study, I went back to the RyR2A construct and focused on optimizing the sample for NMR analysis. I was successful in obtaining novel structural and dynamic information for RyR2A and four of its disease-associated mutants. This was a collaboration with Dr. Filip Van Petegem’s laboratory that had previously solved structures of RyR2A and its mutants. My study revealed a dynamic α-helix not seen in any of the crystal structures that was capable of switching to a β-strand in the RyR2A Δ exon 3 mutant. Furthermore, this flexible loop was shown to be very close to electron
dense ‘columns’ in the cryo-EM map that extend down towards the pore, suggesting a link between the pore and N terminus. This work is presented in Chapter 3.

In-between these two studies I generated many constructs for both RyR1 and RyR2 that encompassed the first 1200 residues. Some of these were of a region flanked by SPRY domains 1 and 2, which I call the AEN domain. Initial biophysical studies revealed that the constructs were folded and stable at room temperature, making them amenable for structural studies. Initial NMR studies have proved promising. Recently a homology model of this region has been generated and published. This region is thought to be structurally homologous to the recently published structure of the RyR1 and RyR2 phosphorylation domain. Chapter 4 summarizes these studies and findings. In Chapter 5 general discussion and future direction of RyR projects are outlined.
Abstract

A critical step in excitation-contraction (E-C) coupling is the release of Ca$^{2+}$ from the sarcoplasmic reticulum (SR) by the ryanodine receptor (RyR). Opening of the receptor is mediated by direct physical coupling with the dihydropyridine receptor (DHPR) or Ca$^{2+}$ binding. Several lethal human diseases have been associated with aberrant SR Ca$^{2+}$ release caused by RyR mutations. I have determined the crystal structure of the distal N terminus (10-210) of RyR1, referred to as RyR1A. The overall fold is strikingly similar to the suppressor domain of the inositol 1,4,5-trisphosphate receptor (IP$_3$R-sup). Mapping of several disease-associated mutations on the structure of RyR1A revealed a cluster in and around a loop connecting β8 and β9, referred to as the hot spot (HS)-loop. The effect on structural fold and stability of several mutations was negligible as monitored by circular dichroism, urea unfolding and nuclear magnetic resonance (NMR) spectroscopy. The concentration of mutations and charged residues in the HS-loop region suggest it may be a site of allosteric interaction with downstream regions of RyR1 or other binding partners.
2.1 Introduction

In skeletal muscle cells, the release of \( \text{Ca}^{2+} \) into the cytosol from stores in the sarcoplasmic reticulum (SR) couples surface membrane depolarization to sarcomere shortening, in a process known as excitation-contraction (E-C) coupling [180]. Membrane depolarization is sensed by the \( \alpha_1 \)-subunit of the dihydropyridine receptor (DHPR), causing it to undergo voltage-induced conformational changes that activate the SR \( \text{Ca}^{2+} \) release channel, the ryanodine receptor (RyR).

Three isoforms of RyR have been characterized: RyR1, associated with skeletal muscle; RyR2, associated with cardiac muscle; and RyR3, which is expressed more ubiquitously. RyRs are the largest known ion channels, each composed of four identical subunits that contain \( \sim 5000 \) residues depending on the isoform [181]. Each subunit consists of a cytoplasmic region that accounts for 80-90% of the protein and a transmembrane (TM) domain that is thought to consist of 6 TM helices [182]. The cytoplasmic domain contains regions involved in protein-protein interactions, E-C coupling, ionic sensitivity and interdomain interactions that together decode cellular signals and subsequently relay this information down to the \( \text{Ca}^{2+} \) release region located within the TM domain [183].

Several human diseases arise from mutations in RyR1 and RyR2 [184, 185]. Most RyR1 mutations are clustered into three distinct regions: cytosolic N-terminal (1-614); cytosolic middle (2117-2458); and C-terminal membrane (4136-4973)(8). Extensive \textit{in vitro} experiments carried out on disease-causing mutations of RyR1 and RyR2 have revealed alterations in channel open probability \( (P_0) \) [77, 186, 187], but the influence of disease mutations on the precise molecular function and structure of RyR remains nebulous.
The 1,4,5-trisphosphate receptors (IP$_3$Rs) are responsible for releasing Ca$^{2+}$ in non-excitable cells. RyR and IP$_3$R constitute a family of tetrameric Ca$^{2+}$ channels that share the same architecture (largely cytoplasmic, small TM domain) and bell shaped Ca$^{2+}$ dependence with respect to CICR. Previous work by Bosanac et al. [188, 189], determined the crystal structures for two regions of the N-terminal domain of type I IP$_3$R: IP$_3$R-sup (1-223) and IP$_3$R-core (224-604). While previous bioinformatics analysis predicted conserved repeats designated mannosyltransferase, IP$_3$R and RyR (MIR) domains in the N-terminal structures of IP$_3$R and RyR (14), no high-resolution structure was available for this region of RyR. I now present the crystal structure of an N-terminal domain of rabbit RyR1 (RyR1A), which enabled me to identify a disease-associated hot spot loop (HS-loop). Selected mutations within and outside the HS-loop, were investigated for their structural effects. The results are also extensively compared with IP$_3$R-sup, demonstrating the importance of this N-terminal region in channel function.

2.2 Materials and Methods

2.2.1 Cloning and expression

Constructs for rabbit RyR1 were subcloned and amplified into a pET32a expression vector (Novagen, Inc.) using BamHI and XhoI restriction enzyme sites. The resultant vector was expressed as a thioredoxin (Trx)-fusion protein with a N-terminal poly-His-tag in BL21 (DE3) Escheria coli (E. coli) cells (Stratagene) at 15°C for approximately 12hr using 0.4mM isopropyl β-D-thiogalactopyranoside (IPTG) induction. Cells were harvested by centrifugation and stored at -70°C. Amino acid mutations (C36R, R164C and R178C) were introduced using the QuikChange site-directed
mutagenesis kit (Stratagene). Mutant constructs were expressed and purified as outlined above.

2.2.2 Expression of $^2$H, $^{15}$N and $^{13}$C-labeled protein

*E. coli* cells expressing recombinant protein were gradually introduced to D$_2$O. A 100 mL starter culture containing Luria-Bertani (LB) media in 25% D$_2$O (v/v) was grown overnight. Cells were spun down at 2000 rpm for 10 min and re-suspended with LB media in 75% D$_2$O (v/v) three times before being transferred to a 100 mL culture containing LB in 75% D$_2$O (v/v). After overnight growth, cells were washed as before with M9 minimal media containing 100% D$_2$O (v/v) before being transferred to a flask containing 2L of M9 media. Expression was carried out in a similar manner for unlabeled protein with the addition of 1 g/L $^{15}$N-ammonium chloride and 2 g/L $^{13}$C-glucose (Cambridge Isotope Laboratories). Incorporation of $^2$H, $^{15}$N and $^{13}$C was confirmed by electrospray mass spectrometry.

2.2.3 Protein Purification

The entire purification was carried out at 4°C with all buffers precooled to this temperature. Harvested cells were re-suspended in 125 mL of lysis buffer (20 mM Tris-HCl pH 8.0, 20 mM Imidazole, 100 mM NaCl, 10% (v/v) glycerol, 0.4% nonidet P40 (NP40), 10 mM 2-betamercaptoethanol (BME), 10 µg/mL DNase I and 1 mM phenylmethanesulfonylfluoride (PMSF). Cells were lysed by sonication using six 5-minute pulses on a Branson Sonifier 450. Supernatant, isolated by centrifugating cell lysate for 30 minutes at 20,000xg, was incubated with Ni-NTA resin (Qiagen) for 1 hr at 4°C. The resin was washed with 250 mL of washing buffer (20 mM Tris-HCl pH 8.0, 20
mM Imidazole, 500 mM NaCl and 10 mM BME). The fusion protein was eluted from the column with 50 mL of elution buffer (20 mM Tris-HCl pH 8.0, 250 mM Imidazole, 300 mM NaCl and 10 mM BME). Thrombin (Sigma) digestion and dialysis was carried out overnight in 20 mM Tris-HCl pH 7.0 and 5 mM dithiothreitol (DTT).

The cleaved protein was further purified by anion exchange chromatography using a HiTrap Q HP column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.0 and 5 mM dithiothreitol (DTT). The target protein was eluted from the column by gradually increasing the salt concentration using 20 mM Tris-HCl pH 7.0, 500 mM NaCl and 5 mM dithiothreitol (DTT). Fractions containing the target protein were collected and run on a Superdex 75 (GE Healthcare) size exclusion column, equilibrated either with 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM DTT and 2 mM Tris (2-carboxyethyl)phosphine (TCEP) or 20 mM sodium phosphate pH 7.0, 300 mM NaCl, 5 mM DTT and 2 mM TCEP, depending on which structural elucidation method was to be used, X-ray crystallography or NMR.

2.2.4 Crystallization, data collection, structure determination and refinement

RyR1A was concentrated in 20mM Tris-Cl pH 8.0, 100mM NaCl, 5mM DTT and 2mM TCEP to a final concentration of 7.5 mg/mL. Initial crystals of RyR1A were grown by the hanging-drop vapor diffusion method at 25°C by combining 1.5µL of protein solution with 1.5µL of well solution (100mM MES, 100mM MgCl₂, 24% w/v PEG 3350 and 5mM DTT). Crystals grew as clusters of thin plates after 3-4 days. A series of microseedings were required to obtain single plate crystals with dimensions of 0.1x0.4x0.05 mm³. Crystals were transferred to a cryo-protectant solution containing
20% v/v glycerol before a native data set was collected to 2.5 Å at the Advanced Photon Source Synchrotron facility (Argonne, IL). This was done at 100K on the 19-BM beam line at X-ray wavelength 0.9793. Data processing and reduction were carried out with HKL2000 [190]. Crystals belonged to the space group C2 with cell dimensions $a = 140.6$ Å, $b = 35.3$ Å and $c = 78.9$ Å with the angle $\beta = 99.4^\circ$. Two molecules were present in the asymmetric unit.

A poly-alanine model of the IP$_3$R-sup structure, (1XZZ), was used for Molecular Replacement to obtain phase information using PHASER [191]. Model building was performed with COOT [192] followed by iterative rounds of refinement using simulated annealing and positional refinement in CNS [193]. The final model was validated using PROCHECK [194], with 88.2% in the most favored and 11.8% in allowed regions.

2.2.5 NMR Spectroscopy

Protein samples for NMR backbone analyses contained 0.6mM uniformly $^{15}$N/$^{13}$C/$^2$H-labeled RyR1A in 20mM sodium phosphate pH 7.0, 300mM NaCl, 5mM DTT, 2mM TECP and 10% (v/v) D$_2$O. Experiments were carried out at 15°C on an 800MHz Bruker spectrometer equipped with a cryogenically-cooled triple resonance probe. A $^{15}$N transverse relaxation-optimized spectroscopy heteronuclear single-quantum coherence (TROSY-HSQC) experiment was performed on the triple labeled sample. Sequential backbone assignments were carried out next using the following suite of TROSY-based three-dimensional experiments: HNCO, HNCA, HN(CO)CA, HN(COCA)CB and HNCACB. The two- and three-dimensional spectra were processed,
and resonance assignments were made using NMRPipe [195] and XEASY [196], respectively.

\^{15}N-labeled RyR1A mutants were concentrated as described above. Experiments were carried out at 15°C on a 600MHz Bruker spectrometer equipped with a cryogenic, triple resonance probe. TROSY-HSQC spectra were processed as above and visualized using NMRView [197].

2.2.6 Optical Spectroscopy

Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-815 CD Spectrometer (Jasco, Inc.). Data were collected in 1-nm increments using a 0.1-cm-path length (l) cell, 10-s averaging time, and 1-nm bandwidth. Spectra were corrected for buffer contributions. Fluorescence measurements were made on a Shimadzu RF-5301PC fluorimeter (Shimadzu Corp.), and data were collected in 1ml (l=1cm) cuvettes using excitation and emission slit widths of 3.0 and 10nm, respectively.

2.3 Results and Discussion

2.3.1 Overall structure in crystal and solution

The crystal structure of RyR1A was solved to 2.5Å (Table 2.1). It is comprised of twelve β-strands and one α-helix (Figure 2.1A). These elements adopt a β-trefoil fold structure, similarly observed in IP_{3}R-sup. This fold is found among proteins with distinct functions and diverse primary structures [198]. The fold consists of three trefoils coming together to form a barrel and cap [199]. Each trefoil consists of four β-strands: two for the cap and two for the barrel (Figure 2.1A). In addition to containing the β-trefoil fold, a
Table 2.1 Data collection and refinement statistics for RyR1A

**Data collection**
- **Space group**: C2
- **Cell dimensions**
  - a, b, c (Å): 104.6, 35.3, 78.9
  - α, β, γ (°): 90, 99.37, 90
- **Resolution (Å)**: 50.0-2.5 (2.59-2.50)
- **Rsym**: 0.074(0.251)
- **<I/σI>**: 14.9(3.4)
- **Completeness (%)**: 98.4(94.4)
- **Redundancy**: 3.3(3.1)

**Refinement**
- **Resolution (Å)**: 50-2.5
- **No. reflections**: 12,895
- **Rwork/Rfree**: 0.229/0.276
- **No. atoms**
  - Protein: 2536
  - Water: 39
- **B-factors (Å²)**
  - Protein: 46.2
  - Water: 43.2
- **R.m.s. deviations**
  - Bond lengths (Å): 0.007
  - Bond angles (°): 1.352

*Values in parentheses are for highest-resolution shell.*
short protrusion in between β4 and β5 is present in the structure, which we call the ‘arm’
domain, in keeping with the

![Figure 2.1 Features of the RyR1A structure.](image)

(A) Ribbon diagram of rabbit RyR1A. The β-trefoil structure is separated into barrel
(blue strands) and cap (green strands). Dotted lines represent missing residues. A
top down view is shown on the right side (B) Sequence alignment of the distal N-
terminal residues of RyR and IP3R isoforms. Residues highlighted in teal, yellow
and magenta denote conservation in the different layers of the barrel in both RyR1A and
IP3Rsup. Residues in red text correspond to mutations sites in RyR1 that lead to MH
and CCD, as well as catecholaminergic polymorphic ventricular tachycardia (CPVT)
and arrhythmogenic right ventricular dysplasia (ARVD2) for RyR2.

nomenclature of IP3R-sup. This is the second example after IP3R-sup, of a β-trefoil
structure containing a long insertion in its architecture [189]. Several loop regions were
not built into the model due to poorly defined electron density, including a region (V86 to
G97) predicted to form the second helix (α3) in the arm domain.
Nuclear Magnetic Resonance (NMR) experiments were carried out in order to probe the structure of RyR1A in solution. A construct consisting of RyR1 10-210 was used to assign the protein backbone of this region (Figure 2.2A and B). A suite of TROSY-based 3D experiments were employed to achieve ~86% assignment completion of the protein backbone in the $^1$H-$^{15}$N TROSY-HSQC spectrum of RyR1A. A chemical shift index (CSI) was generated from the assignment data that agreed with the location and number of secondary structure elements determined from the crystal structure (Figure 2.3A). The presence of a second helix in the arm domain of RyR1A was not supported by the NMR data. Firstly, CSI values of residues residing in the proposed helix agree more with an unstructured region and secondly, peak intensities from the HSQC spectrum of these residues are two times larger than the average for the entire structure, suggesting that they reside in an unstructured region (Figure 2.3B).
Figure 2.2 NMR assignment of RyR1 10-210 (RyR1A). 
(A) \textsuperscript{1}H-\textsuperscript{15}N TROSY HSQC spectrum of \textsuperscript{15}N/\textsuperscript{2}H- labeled RyR1 10-210. (B) An enlarged view of the crowded region of the HSQC. Cross peaks colored in green come from residues in the highly flexible loop (res 85 to 97) immediately following the \( \alpha_2 \) helix.

![Figure 2.2](image)

Figure 2.3 Secondary structural analyses reveals only one \( \alpha \)-helix in RyR1A. A Chemical Shift Index (CSI) is shown in (A). The CSI was calculated using \( C\alpha \) and \( C\beta \) chemical shifts of assigned residues. \( \beta \) strands, \( \alpha \) helices and random coils are indicated by large negative, positive and small CSI values respectively. Relative peak intensity versus residue number is shown in (B).

2.3.2 Structural comparison of RyR1A and IP\(_3\)R-sup

The primary sequence identity between RyR1A and IP\(_3\)R-sup is relatively low (30\%). However, the backbone conformation of RyR1A superimposes very well (Figure 2.4A) with IP\(_3\)R-sup. The topology diagrams of RyR1A and IP\(_3\)R-sup (Figure 2.4B) illustrate the structural similarity of their \( \beta \)-trefoil fold as well as its three-fold symmetry. Key residues residing in \( \beta \)-strands making up the barrel are conserved in RyR1A (Figure 2.1B) and remarkably stack into discernable layers in the barrel with similar orientations.
as seen in IP$_3$R-sup (Figure 2.4C). However, this high similarity breaks at the unconventional helical segment found in the arm domain of IP3R-sup. In RyR1, this segment lacks a long $\alpha$-helix and is significantly shortened relative to IP3R-sup. Another striking difference between RyR1A and IP$_3$Rsup is evident when comparing surface charge representations of both structures (Figure 2.4D). RyR1A contains a large patch of positive charge at the top

![Figure 2.4 Figure 3 Comparison of RyR1A (RyRNTD) and IP$_3$Rsup structures.](image)

(A) Structural alignment of RyR1A (purple) and IP$_3$R$_{sup}$ (grey) structures with a C$\alpha$ RMSD of 1.34Å. Topology diagram for both structures are shown in (B). The three-fold symmetry of the $\beta$-trefoil is evident, as well as difference in the arm domain. The layering of residues in the barrel is shown in (C) with the same color scheme as in (Figure 2.1B). Electrostatic surface representation is represented for IP$_3$R$_{sup}$ and RyR1A in (D). A positive patch where mutations cluster is outlined in yellow. Residues with basic side groups found within and around the HS-loop are labeled. The structure is oriented in the top down view described in (A).
of the β-trefoil structure that corresponds with the location of many disease-causing mutations in RyR1 we designate the hot spot HS-loop.

### 2.3.3 MH- and CCD-associated mutations

In MH and CCD patients, 30 mutations have been identified thus far that lie within the first 614 amino acids of RyR1. Of these, 37% map onto the structure of RyR1A (Figure 2.5A). Most of the mutation sites are concentrated on the HS-loop between β8 and β9 (Q156, R157, E161, R164, G166, D167) that is located on top of the barrel (Figure 2.1A and Figure 2.5B).

To examine the structural influences of RyR1A mutations, we chose three well-studied MH-causing mutations: C35R, R163C and R177C (C36R, R164C and R178C in rabbit). R164 is present in the HS-loop, while C36 and R178 are found within the β-strands that form part of the ‘cap’ of the β-trefoil. Circular dichroism and chemical denaturation experiments showed no appreciable effect on structural stability and integrity due to the point mutations (Figure 2.6A and B). These data were consistent with NMR studies using $^1$H-$^{15}$N TROSY-HSQC experiments showing conservation of the overall fold. Comparison of R164C mutant and wild type spectra revealed negligible chemical shift perturbations (CSPs) (Figure 2.5C middle). This is not surprising, since this mutation is present on a surface-exposed loop in isolation from most of the structure.
Figure 2.5 Mapping and analysis of mutants on RyR1A structure.

(A) Mapping of residues known to be mutated in MH and CCD. 
(B) Close up view of HS-loop where mutations are concentrated. L14 is in close proximity to this hot spot of residues. Positively charged residues residing in the loop are shown in grey.

(C) Overlay of mutant (red peaks) and wild-type (black, green and blue) $^1$H-$^{15}$N TROSY-HSQC spectra in the downfield region. Arrows indicate peaks showing significant chemical shift perturbations.
Figure 2.6 Effect of C36R, R164C and R178C mutants on RyR1A structural stability and fold.
(A) Far-UV CD spectrum of mutants and wild type RyR1A.
(B) Urea unfolding of mutant and wild type RyR1A monitored by intrinsic fluorescence (excitation = 280 nm, emission = 315 nm) at 10°C.
On the other hand, the C36R and R178C mutations produced more notable CSPs. However, close inspection of the changes indicated that these shifts were localized to residues in close proximity to the mutation site and suggest little alteration in the protein fold itself (Figure 2.5C top and bottom). These results demonstrate that the point mutations (C36R, R164C and R178C) do not perturb the structural integrity of RyR1A; their role in a malfunctioning receptor may be to disrupt interactions with ligands or interdomain interactions with other regions of RyR1.

2.3.4 CPVT and ARVD mutations in type 2 RyR (RyR2)

Sequence identity among the three isoforms of RyR is high: 67% between full length mammalian RyR1 and RyR2, and 78% when the N-terminal domain is considered (RyR1 1-210 & RyR2 1-223). In light of this high sequence identity, we employed a homology model to build a structural model of RyR2 N-terminal domain (1-223) using MODELLER 6.2. We excluded 12 residues located in the hypothetical ‘arm’ domain of RyR2. Interestingly, these residues, which are missing in RyR1, may form the second helix in the helix-loop-helix motif seen in IP₃R-sup. Studies have shown that a mutation in RyR2 (R176Q) causes arrhythmogenic right ventricular dysplasia (ARVD2) in humans and ventricular tachycardia and cardiomyopathy in a mouse model of ARVD2 [200]. This mutation corresponds to the R163C MH mutation in human RyR1, illustrating a conservation of function in this region between RyR1 and RyR2. Mapping of this and other RyR2 mutations onto the homology model of RyR2 revealed a similar clustering to
RyR1 mutations (Figure 2.7), supporting a similar disease-causing mechanism between the two isoforms at the molecular level.

Figure 2.7 Mapping of mutations onto a homology model of rabbit RyR2 1-223. A modeled structure of RyR2 1-223 based on the sequence alignment in Figure 2.1B is shown on the right panel. Only residues conserved between rRyR1 and rRyR2 were used to generate the model. CPVT and ARVD mutations are mapped onto the model. MH- and CCD-associated mutations are mapped onto the structure of RyRNTD for comparison on the left panel. The dashed line indicates the HS-loop.

2.3.5 Functional consequences between RyR1A and IP₃R-sup structures

The three-dimensional clustering of disease-associated mutations within the highly localized distal N-terminal region of RyRs (i.e. the HS-loop), strongly suggests a functional significance for the β-trefoil domain structure determined in this study.

Interestingly, the same structural architecture is found in IP₃R1, whose functions are better understood compared to RyRs. In IP₃R1, this domain plays an essential role in
both the regulation of IP$_3$ binding affinity and coupling to the channel domain, hence referred to as the suppressor/coupling domain [201, 202]. In one study [203], a mutant IP$_3$R1 lacking the suppressor domain (1-223) was unable to exhibit any measurable Ca$^{2+}$ release, yet its affinity for IP$_3$ was 10-fold higher than wild type IP$_3$R1. The authors postulated a mechanism whereby the ligand-binding signal is detected at the N terminus and transmitted to the channel domain via the regulatory domain; deletion of IP$_3$R-sup seems to abrogate this coupling. Recent work however [204] has proposed that IP$_3$R-sup directly couples to the channel via a cytosol-exposed loop between transmembrane segment 4 and 5 (S4-S5 linker). Together with previous GST pull down and cross linking data [205], the authors suggest that the interaction is direct and restricted to amino acids 1-340; leading them to hypothesize that the site of interaction may be contained within IP$_3$R-sup.

In RyRs, the corresponding residues to IP$_3$R-sup do not bind IP$_3$ or any other ligand. However, it is possible that the coupling function of this domain may be retained. In a related mechanism to the proposed domain coupling of IP$_3$R, Ikemoto et. al. have proposed a model whereby the N-, middle and C-terminal regions of RyR interact with each other to gate the channel [206]. Several studies have demonstrated that domain peptides (DPs) - synthetic peptides comprising the three mutation regions - are capable of increasing the P$_0$ of single RyR channels [206] and even inducing Ca$^{2+}$ release from skinned fibers of rat muscle [207]; in essence mimicking the effect of MH mutations. Furthermore, by fluorescently labeling DPs with methylcoumarin acetate (MCA), they have shown that DP4 (Leu2442 – Pro2477) binds to amino acids 1-450 of the N-terminal domain [208]. In the same study, the authors demonstrated the ability of the skeletal
muscle relaxant, dantrolene, to stabilize interdomain interactions. Interactions involving
the C terminus have also been demonstrated with DP15 (Val4820 – Val4841), which
includes the homologous S6-S7 linker in RyR1. It was shown to elicit the same response
as other DPs and its site of interaction was localized to a 96kDa C-terminal fragment that
contains a Calmodulin-like domain (CaMLD). Recently, DP studies extended to RyR2
have shown similar results to those of RyR1. A DP encompassing RyR2 163-195 was
capable of disrupting interdomain interactions and increasing Ca²⁺ leak and spark
frequency in canine ventricular myocytes [88]. This DP includes the homologous HS-
loop region of RyR2, lending support that this region in RyR1 may be critical for
interdomain interactions. Together, these DP studies support a homologous domain
coupling model in RyR, although evidence for direct coupling between the N- and C-
termini has not been demonstrated. Importantly, both IP₃R-sup and RyR1A are found
within their respective putative regions responsible for coupling. Our discovery of a
distinct charged patch surrounding the HS-loop in RyR1A (Figure 2.4D), together with
the recent demonstration that MH-associated mutations in DP4 - three of which involve
Arg residues - cluster to a confined area in an NMR model, suggests that electrostatic
interactions may play a role in interdomain interactions. A similar hypothesis has been
suggested for IP₃R [204].

2.4 Structure of the N-terminal region of RyR1 (RyR1ABC)

Shortly after our structure was published, the lab of Dr. Filip Van Petegem was
successful in solving the structure of RyR2A and the entire N-terminal region, referred to
as RyR1ABC [209] for the three domains (A, B and C) making up this region (Figure
2.8). Domains A and B fold into β-trefoil cores and domain C is made of a five-helix
bundle. All three domains are stabilized by hydrophilic interactions. They were able to map the location of 56 disease-associated mutations on its three-dimensional architecture (Figure 2.9A). The mapping of disease-associated mutations revealed that most are located at intra-molecular interfaces between the three domains or at interfaces between ABC subunits in the tetrameric channel modeled by docking the high resolution ABC structures into the cryo-EM map [209]. An interface concentrated with mutations is located between domains A and C from the same subunit (Figure 2.9B). Two salt bridges (Arg45 Domain A and Asp447 domain C) and (Asp61 and Glu40 Domain A and Arg402 Domain C) stabilize this interface [75]. Interestingly, close to a third of all RyR1ABC mutants (19 out of 56) map to an interface between domain A of one ABC subunit and domain B of the adjacent subunit (Figure 2.9C). This interface is made up of the HS-loop

**Figure 2.8 Arrangement of domains in RyR1ABC.**
(A) Boundaries for domains A in blue (1-205), B in green (206-394) and C in red (395-532) are shown. (B) Shows a different view of A. (C) Omit map of randomly selected region, contoured at 3σ. Figure taken from (Tung et al., 2010)
found in domain A and two flexible loops in domain B. A recent study solved the structure of several RyR1ABC disease-associated mutants and probed their affect on structural stability and fold [210]. The introduction of mutations mainly resulted in local effects that did not have a drastic effect on the fold and thermal stability of RyR1ABC, in most cases the mutations affect the

![Figure 2.9](image)

**Figure 2.9 Mapping RyR1 and RyR2 disease-associated mutations on RyR1ABC.** (A) RyR1 mutations (red) and RyR2 mutations (blue) are mapped onto the structure of RyR1ABC. For clarity, mutations in flexible loops are not shown. (B) Salt bridges at the A–C interface. Domain A (blue), domain B (green) and domain C (red) are shown. Underlined residues are targets for disease-associated mutations. (C), Interface 1 across ABC subunits, according to docking in the 10.3 Å RyR1 cryo-EM. The inset shows the relative view. Figure taken from (Tung et al., 2010)

domain interfaces that are thought to stabilize this region. Taken together, it appears as though disease-associated mutations could weaken domain interactions that act as a ‘break’ on channel gating. By doing so, the energy barrier for channel opening is lowered, resulting in SR Ca\(^{2+}\) leak.
2.5 Conclusion

Our study revealed structural conservation among the distal N-terminal residues of IP$_3$R and RyR, while also highlighting differences between them (i.e. the ‘arm’ domain). It also demonstrated that structurally, MH- and CCD-associated mutations have little to no local effect, suggesting that at the molecular level, mutations may perturb interactions with RyR auxiliary proteins as FKBP12 or other regions of the protein responsible for channel gating. The present study provides the first atomic-level insight into the effect of disease-associated mutations on RyR function. Together with the recent studies on RyR1ABC, the first clues into the complex structural mechanism of SR Ca$^{2+}$-release and its perturbation by RyR mutations are being elucidated.
Chapter 3

RYANODINE RECEPTOR TYPE 2 DOMAIN A CONTAINS A DYNAMIC $\alpha$-HELIX THAT TRANSITIONS TO A $\beta$-STRAND IN A MUTANT LINKED WITH A HERITABLE CARDIOMYOPATHY

Abstract

Cardiac ryanodine receptors (RyR2s) are Ca\(^{2+}\)-release channels found on the sarcoplasmic reticulum (SR) that respond to dihydropyridine receptor (DHPR) activity through indirect Ca\(^{2+}\) sensitivity. Several debilitating diseases are linked to heritable mutations in RyR2 including catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2). Despite the recent appreciation that many disease-associated mutations within the N-terminal RyR1-ABC domains (i.e. residues 1-559) are located in the putative interfaces mediating tetrameric channel assembly; the precise structural and dynamical consequences of the mutations are not well understood. We used solution nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography to examine the effect of ARVD2- (i.e. R176Q) and CPVT- (i.e. P164S, R169Q and delta exon 3) associated mutations on the structure and dynamics of RyR2A. Our solution NMR data revealed a previously unresolved \(\alpha\)-helix in wild-type RyR2A; further, this \(\alpha\)-helix rescues the \(\beta\)-strand lost in RyR2A exon \(\Delta3\), but remains dynamic in the hot-spot (HS)-loop P164S, R169Q and R176Q mutant proteins. Docking of our x-ray crystal/NMR hybrid structure into the RyR1 cryo-electron microscopy map revealed that this RyR2A \(\alpha\)-helix is in close proximity to dense “columns” projecting toward the channel pore. Taken together, our data suggest that ARVD2 and CPVT mutations have at least two distinct structural consequences linked to channel dysfunction: perturbation of the HS-loop (i.e. domain A); domain B inter-subunit interface, and disruption of the communication between the N-terminal region and the channel domain.
3.1 Introduction

Calcium (Ca\(^{2+}\)) is a universal signaling messenger, controlling myriad cellular activities in all mammalian cells. Cells compartmentalize most intracellular Ca\(^{2+}\) in the sarco-/endoplasmic reticulum (S/ER), where it is spatio-temporally released into the cytosol during signaling cascades. S/ER receptor operated channels, responsible for moving Ca\(^{2+}\) from the lumen to the cytosol include the inositol 1,4,5 trisphosphate receptors (IP\(_3\)Rs), abundant in non-excitable cells and the ryanodine receptors (RyRs), found more exclusively in electrically excitable cells. Dysfunction of Ca\(^{2+}\) release through these receptor operated channels can have devastating physiological consequences as several heritable human diseases are associated with mutations of the three RyR genes. These include malignant hyperthermia (MH) [59, 69] and central core disease (CCD) [60, 61] that are associated with RyR1 mutations or catecholaminergic polymorphic ventricular tachycardia (CPVT) [71, 72] and arrhythmogenic right ventricular dysplasia type 2 (ARVD2) [73] which are associated with RyR2 mutations.

RyRs form tetrameric complexes that are the largest known ion channels at ~2.2MDa. They reside on the SR in close apposition to plasma membrane voltage-gated dihydropyridine receptor (DHPRs). RyRs play an integral role in excitation-contraction (E-C) coupling in skeletal cells and cardiomyocytes [25, 117], as they propagate Ca\(^{2+}\) release from the SR in response to increased cytosolic Ca\(^{2+}\) levels caused by DHPR activity or through direct sensing of a voltage-dependent conformational change in DHPR. RyR1 is found primarily in skeletal muscle [37], RyR2 in cardiac muscle [39] and RyR3 which is found in several cell types, but was originally discovered in brain cells.
Most disease-associated mutations render the channel hypersensitive to activating stimuli such as Ca^{2+} either on the luminal or cytosolic side of the receptor.

Structural investigations of the tetrameric channel by cryo-electron microscopy (cryo-EM) [97-102] and folded domains by x-ray crystallography [209, 211-215] have provided insight into how RyRs function and how disease-associated mutations might affect this function. A study by Tung et al., showed that the majority of mutations found within the first 539 residues of RyR1 (RyR1ABC) are clustered at inter- and intra-subunit interfaces rather than being buried within the folded domain cores [209]. The ‘hot-spot’ loop (HS-loop) is the location of over half the mutations found within the A domain of the RyR1 and forms an interface with domain B from an adjacent subunit. An investigation of the position of the RyR1ABC region in open and closed cryo-EM maps suggests that this interface is severely altered during channel opening, and acts as a brake on channel opening [210]. Recently, Lobo et al., investigated a mutant associated with a severe form of CPVT that employs a remarkable structural adaptation [212]. The mutant consists of a deletion encompassing the entire third exon of RyR2 that encodes a 35-residue stretch made up of a β-strand and α-helix in RyR2A. Rather than this invasive deletion mutant inducing misfolding, the structural fold of RyR2A is rescued by an insertion into the β-trefoil domain by a flexible loop immediately downstream from the deletion. This RyR2A Δ exon 3 mutant shows a significant increase in thermal stability which is incongruent with the severe nature of the disease state [213]. Therefore, it seems likely that this mutation disrupts inter- and/or intra-molecular interactions that gate channel function, while enhancing the structural stability of the individual domain. Superimposing RyR2A Δ exon 3 with RyR1ABC allowed for docking of this domain
onto the cryo-EM map [212]. The docking revealed that the α-helix from RyR2A is at an interface with electron dense columns that point toward the TM domain. Presumably, deletion of this helix in RyR2A Δ exon 3 would disrupt this interface and lead to aberrant channel activity.

Despite progress in locating disease-associated mutations within the structurally resolved N-terminal RyR domains and relative to the tetrameric assembly of the channel, the structural and dynamical consequences of the mutations are not well understood. Here, I present solution nuclear magnetic resonance (NMR) spectroscopy and x-ray crystallography that examines the effect of ARVD2- (i.e. R176Q) and CPVT- (i.e. P164S, R169Q and delta exon 3) associated mutations on the structure and dynamics of RyR2A. Remarkably, the solution NMR data elucidated the presence of a previously unresolved α-helix in wild-type RyR2A; further, docking of our x-ray crystal/NMR hybrid structure into the cryo-EM map of RyR1 revealed this helix resides in close proximity to electron dense “columns” projecting toward the channel pore. In solution, this α-helix rescues the β-strand deleted in RyR2A Δ3, but remains dynamic in HS-loop mutant proteins (i.e. P164S, R169Q and R176Q). Mutations in the HS-loop cause structural changes within the A domain largely localized to the loop itself, which presumably cause dysfunction via perturbation of inter-subunit HS-loop:domain B interfaces. This study shows that ARVD2 and CPVT mutations found in the A domain result in at least two different structural consequences which are presumably linked to the functional defects found in the mutated channels [200, 216].
3.2 Materials and Methods

3.2.1 Cloning and expression

Dr. Filip Van Petegem generously provided a mouse RyR2 Δ exon 3 construct (residues 1-51,91-217) that was subsequently truncated (residues 10-51,91-217) and engineered with BamHI and XhoI restriction enzyme sites. Both RyR2A Δ exon 3 and mouse RyR2A (residues 10-224) were subcloned into pET32a. Amino acid mutations (P164S, R169Q and R176Q) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). *E. coli* cells expressing recombinant protein were gradually introduced to D₂O. A 100 mL starter culture containing Luria-Bertani (LB) media in 25% D₂O (v/v) was grown overnight. Cells were spun down at 2000 rpm for 10 min and re-suspended with LB media in 75% D₂O (v/v) three times before being transferred to a 100 mL culture containing LB in 75% D₂O (v/v). After overnight growth, cells were washed as before with M9 minimal media containing 100% D₂O (v/v). Expression was induced with 0.4 mM IPTG and carried out at 18°C for 16 hrs. ¹⁵N-ammonium chloride (1 g/L) and ¹³C-glucose (2 g/L) (Cambridge Isotope Laboratories) were the sole nitrogen and carbon source. Cells were harvested and stored at -70°C. Incorporation of ²H, ¹⁵N and ¹³C was confirmed by electrospray mass spectrometry.

3.2.2 NMR Spectroscopy

Protein samples for NMR backbone analyses contained 0.4 mM and 0.6 mM uniformly ¹⁵N/¹³C/²H-labeled wild-type and disease-associated mutant RyR2A, respectively. NMR buffers consisted of 20 mM sodium phosphate, pH 7.0, 300 mM NaCl, 5 mM DTT, 2 mM TCEP for wild-type and HS-loop mutant RyR2A, and 20 mM sodium
phosphate, pH 7.4, 300 mM NaCl, 5 mM DTT, 2 mM TCEP for RyR2A Δ exon 3. All samples contained 10% (v/v) D2O. All experiments were carried out at 15°C on an 800 MHz Bruker spectrometer equipped with a cryogenically cooled triple resonance probe. A 15N transverse relaxation-optimized spectroscopy heteronuclear single-quantum coherence (TROSY-HSQC) experiment was performed on all samples. Sequential backbone assignments were carried out next using the following suite of TROSY-based three-dimensional experiments: HNCO, HNCA, HN(CO)CA, HN(COCA)CB and HNCACB. In addition, 15N-edited 3D NOESY-TROSY [217] spectra were recorded for P164S and R169Q to aid with backbone assignment. The two- and three-dimensional spectra were processed, and resonance assignments were made using NMRPipe [195] and XEASY [196], respectively.

3.2.3 X-ray crystallography-NMR hybrid model

Restraints for backbone φ and ψ torsion angles and hydrogen bonds for the mobile α2 region (i.e. residues 85-109) were derived from the backbone chemical shifts using TALOS [218]. Coordinates from the RyR2A crystal structure (PDB ID: 3IM5) were used to generate restraints for the remaining residues of RyR2A. Both sets of restraints were combined and used to anneal an extended polypeptide chain, which was water-refined using the RECOORD scripts [219] in CNS [193]. The 20 lowest energy models out of 200 were chosen as a representative ensemble.

3.2.4 15N Relaxation analysis

15N relaxation data were collected on all samples at 9°C, employing TROSY versions of the T1, T2 and 15N-{1H} NOE pulse sequences [220]. Relaxation delays for
RyR2A and the HS-loop mutants were as follows: T1: 10, 100, 400, 800 × 2, 1200, and 1600 ms; T2: 0, 15.84 × 2, 31.68, 47.52, 63.36, 79.2 and 95.04 ms. Delays used for RyR2A Δ exon 3 were as follows: T1: 10, 100 × 2, 200, 500, 1000, 1200 and 1600 ms; T2: 0, 15.84 × 2, 31.68, 47.52, 63.36 and 79.2 ms. Duplicate spectra were used to estimate experimental error. Peak intensities were measured using SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). \(^{15}\text{N}\)^{\text{1H}} NOE experiments were carried out in the presence and absence of a 3 s proton saturation period before the \(^{15}\text{N}\) excitation pulse. Peak intensity uncertainties were estimated from the noise level of the two spectra. Relaxation rates and heteronuclear NOE ratios were determined using the program Relax [221, 222]. The relaxation data were analyzed according to the model-free approach of Lipari and Szabo [223, 224] using TENSOR2 [225].

### 3.2.5 Cryo-EM Docking

The lowest energy structure of the RyR2A hybrid ensemble was superimposed onto the A domain of RyR1ABC docked onto three different RyR1 cryo-EM maps (EMDB 1275, 1606 and 1607). To perform the docking, extensive six-dimensional searches with Laplacian filtering were implemented in the programs Situs [226] and ADP_EM [227] as previously described in Tung et al., [209].
3.3 Results and Discussion

3.3.1 RyR2A forms a dynamic α2 helix in solution

A construct encompassing residues 10-224 in mouse RyR2 was used in solution NMR experiments to assign the protein backbone of RyR2A (Figure 3.1A and B) (BMRB accession number 19199). Deuteration and transverse relaxation-optimized spectroscopy (TROSY)-based experiments were implemented to ameliorate spectral crowding and broadened signals undergoing chemical exchange. Assignment of the $^1$H-$^{15}$N TROSY-heteronuclear single-quantum coherence (TROSY-HSQC) spectrum was achieved for approximately 88% of observable cross-peaks. A chemical shift index (CSI) plot derived from the Cα and Cβ chemical shifts of RyR2A revealed a new α2 helix, which is absent in the crystal structure of RyR2A (Figure 3.2). This new helix spans a 10-residue stretch from V95 to T104 within a 12-residue insertion specific to the RyR2 sequence compared to RyR1 and RyR3 (Figure 3.3A). All other secondary structural elements extracted from the CSI of RyR2A were in excellent agreement with the previously solved crystal structure [213].
Figure 3.1 Amide backbone assignment of RyR2A. 
(A). $^1$H-$^{15}$N TROSY-HSQC spectrum of 15N/13C/2H- labeled RyR2A 10-224. Assignment was achieved for 88% of the protein backbone. (B) An enlarged view of the crowded region of the HSQC. Cross peaks colored in orange correspond to some residues undergoing chemical exchange. Peaks (L133, G155, H193 and W212) that were analyzed after zz-exchange spectroscopy experiments are in larger font and designated by an asterisk.

Figure 3.2 Solution-NMR evidence of a second α-helix in RyR2A. 
The CSI was generated using Cα and Cβ chemical shifts of assigned residues. β-strands, α-helices and random coils are indicated by large negative, positive and small CSI values respectively. Residues encompassing the newly discovered α2 helix are colored in red. Secondary structure elements derived from the CSI are shown above in cartoon form.
Figure 3.3 X-ray crystallography-NMR hybrid model of RyR2A.

(A) A sequence alignment for residues 10-217 demonstrates a high sequence identity between RyR1A, RyR2A and RyR3A, except for a 12-residue insertion, only present in RyR2. (B) The 20 lowest energy backbone structures are shown for RyR2A. The newly identified α2 helix is shown in red.

Conservation of the number and location of secondary structural elements between RyR2A in solution and in the crystalline state allowed us to generate an x-ray crystallography-NMR hybrid model that includes the new α2 helix (Figure 3.3B) (PDB submission under review). The hybrid structure was calculated using hydrogen bond, torsion angle and distance restraints for the core RyR2A domain extracted from the crystal structure and combined with hydrogen bond and torsion angle restraints derived from the chemical shift data of RyR2A in solution. Alignment of the conserved secondary structure components of the 20 lowest energy hybrid structures revealed that
the location of the α2 helix exhibits a greater backbone root mean square deviation (RMSD) compared to the remaining secondary structure elements (Figure 3.3B). This increased variability is consistent with the assessment of dynamics for this region (see below).

In a previous study, I observed that a number of peaks in the RyR1A domain exhibited peak doubling in $^1$H-$^{15}$N HSQC spectra [211]. In this study, I consistently observed peak doubling for a large number of cross peaks in $^1$H-$^{15}$N TROSY-HSQC spectra of wild-type and the disease-related mutants, P164S, R169Q, R175Q and Δ3 (Figure 3.1). To further test whether the peak doubling was due to chemical exchange caused by multiple protein conformations I carried out a zz-exchange experiment on RyR2A which showed a buildup of exchange peak intensity and simultaneous decrease in auto peak intensity as a function of mixing time, thereby confirming the relatively slow chemical exchange phenomenon (Figure 3.4A and B). I also observed a negligible change from low to moderately high temperatures on peak doubling (i.e. ~9 to 30°C) (Figure 3.5A), suggesting that each conformation is moderately stable. Ultimately, I quantified the peak doubling by measuring the difference in the $^1$H and $^{15}$N chemical shifts between each doublet to obtain a delta omega ($\Delta \omega$) value (Figure 3.5B). Plotting a gradient of $\Delta \omega$ values on the hybrid structure revealed that the highest values were concentrated on the face of the structure where the newly identified α2 helix is located (Figure 3.5C). Taken together, these data suggest that RyR2A slowly exchanges between two moderately stable conformations in solution and α2 undergoes the greatest change in chemical environment.
Figure 3.4 Monitoring chemical exchange of L133 in RyR2A.

(A) Plot of buildup and decay curves for cross (blue and green) and autopeaks (red and orange) as a function of mixing time. Intensities are normalized to the major autopeak (red). (B) Close up of L133 peak region at different mixing times. Mixing times are indicated for each spectrum.
Figure 3.5 Observation and quantification of peak doubling in RyR2A.

(A) Temperature dependence on peak doubling. A series of TROSY-HSQCs were collected at increasing temperatures from 9 to 30°C to follow the change in delta omega values for peaks experiencing peak doubling. Two representative residues (V68 and G109) with distinct delta omega values were followed. Subscripts denote major and minor peaks based on intensity (1 = major, 2 = minor). Intensities were measured using the program SPARKY.  

(B) Plot of measured delta omega values for residues experiencing peak doubling.  

(C) Mapping of delta omega values in (B) onto the lowest energy hybrid RyR2A model. The face closest to the mobile α2 helix is concentrated with large delta omega values.
3.3.2 HS-loop mutations in RyR2A cause local perturbations in RyR2A structure

In order to investigate the structural impact of disease-associated mutations located within the HS-loop of RyR2, the Van Petegem lab solved the crystal structures of RyR2A mutants, P164S, R169Q, and R176Q to resolutions between ~2.15 and 2.55Å (Table 3.1). Because the conformation of the loop might be affected by crystal contacts, and the wild type RyR2A structure was solved in a different space group (C2), they compared these structures with the previously published A77V mutant [213], which crystallized in the same space group as the three HS mutants. A77V is located far away from the HS-loop, and is therefore, not expected to impact the conformation of the loop.

Pro164 is located at the beginning of the HS-loop and presumably provides a degree of conformational rigidity to this loop. Mutating this residue to Ser likely results in partial flexibility, as a portion of the loop exhibits weak electron density (Figure 6A and B). The sidechain of Arg169 also appears inherently flexible with poorly defined electron density and was not modeled in the A77V structure. In the R169Q mutant structure several residues, including R169Q, were not visible in the electron density, consistent with the higher degree of flexibility (Figure 6C and D). The R176Q mutation facilitates the formation of a hydrogen bond between Gln176 and Arg169. This interaction also positions Arg169 for a salt bridge with Asp179; however, several neighboring residues still showed missing electron density (Figure 6E and F).

Although the HS-loops in the mutant proteins appear partially flexible, as indicated by poor electron density, crystal contacts may have affected the resolution of the loops. For example, in the wild type RyR2A structure, which was crystallized in a
### Table 3.1 Data Collection and Refinement Statistics for RyR2A HS-loop mutants

<table>
<thead>
<tr>
<th></th>
<th>P164S</th>
<th>R169Q</th>
<th>R176Q</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>$I_4$</td>
<td>$I_4$</td>
<td>$I_4$</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$a=b, c$ (Å)</td>
<td>111.75, 37.82</td>
<td>112.86, 38.09</td>
<td>100.27, 37.99</td>
</tr>
<tr>
<td>$\alpha=\beta=\gamma$ (°)</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50-2.40 (2.49-2.40)</td>
<td>50-2.55 (2.64-2.55)</td>
<td>50-2.15 (2.19-2.15)</td>
</tr>
<tr>
<td>$R_{sym}$ or $R_{merge}$ (%)</td>
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<td>9.3 (49.3)</td>
<td>6.1 (43.1)</td>
</tr>
<tr>
<td>$I/\sigma$</td>
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<td>24.9 (2.3)</td>
<td>33.3 (2.1)</td>
</tr>
<tr>
<td>Completeness (%)</td>
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<td>98.8 (90.0)</td>
<td>98.1 (86.0)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.2 (5.5)</td>
<td>6.6 (3.3)</td>
<td>4.9 (2.4)</td>
</tr>
</tbody>
</table>

| **Refinement**   |       |       |       |
| Resolution (Å)   | 50-2.4 | 50-2.55 | 50-2.15 |
| No. reflections  | 8805  | 7484  | 9885  |
| $R_{work}$ / $R_{free}$ (%) | 24.2/27.3 | 23.6/29.7 | 25.0/29.0 |
| No. atoms        |       |       |       |
| Protein          | 1256  | 1264  | 1233  |
| Ligand/ion       | 0     | 0     | 5     |
| Water            | 15    | 19    | 14    |
| B-factors (Å²)   |       |       |       |
| Protein          | 61.6  | 47.3  | 51.1  |
| Ligand/ion       | NA    | NA    | 61.8  |
| Water            | 52.2  | 37.8  | 47.8  |
| R.m.s. deviations|       |       |       |
| Bond lengths (Å) | 0.002 | 0.003 | 0.002 |
| Bond angles (°)  | 0.554 | 0.696 | 0.649 |

*Values in parentheses are for highest-resolution shell.
Figure 3.6 RyR2A HS-loop mutant structures.
Structures of mutant (colors) are superimposed onto the RyR2A A77V structure (gray) (PDB ID 3IM7). The HS-loop (residues 164-179) is highlighted in orange (respective mutant) and light orange (A77V). The site of mutation is shown in black. (A) and (B) P164S. (C) and (D) R169Q. (E) and (F) R176Q.

different space group, the loop position is different than in these mutant structures.

Although crystal contacts do not impart large structural changes, the conformations of loops that are inherently flexible can be particularly sensitive. Therefore, I performed
more detailed solution NMR experiments to circumvent any affects by crystal contacts on the HS-loop properties.

The spectra of the P164S, R169Q and R176Q mutants were assigned by transference from wild-type RyR2A and using $^{15}$N-resolved nuclear Overhauser enhancement spectroscopy (NOESY)-TROSY data for peaks that did not coincide sufficiently between spectra. I examined the effect of the three HS-loop mutations on the structural fold of RyR2A, including the $\alpha$2-helix by monitoring chemical shift perturbations (CSPs) (Figure 3.7A).

**Figure 3.7 Chemical shift perturbations of RyR2A TROSY-HSQC.**

(A) Chemical shift perturbation (CSP) values (in Hz) are plotted for each mutant. Colored arrows indicate the site of mutation in the sequence. Secondary structural elements for RyR2A are shown above the plot. Regions outside the HS-loop showing significant CSPs are the N-terminal region and $\beta$12, colored in brown and pink respectively. (B) Close-up view of HS-loop region showing the site of mutations as well as perturbed structural elements including Tyr115 which fits into a cleft created by the HS-loop.
Plotting the CSPs on the hybrid structure of RyR2A revealed distinct magnitudes of structural perturbations caused by each mutation (Figure 3.8). The P164S mutation resulted in the largest CSPs for residues throughout HS-loop as well as residues immediately adjacent to the HS-loop. The R169Q mutation had a moderate conformational effect on the HS-loop and the core structure, while the perturbation of

![Figure 3.8](chemical-shift-perturbations-ryr2a.png)

**Figure 3.8 Chemical shift perturbations on RyR2A caused by the disease-associated P164S, R169Q and R176Q mutations.**

CSPs generated by each HS-loop mutant are mapped onto the hybrid model of RyR2A. The P164S, R169Q and R176Q perturbations are shown in blue, red and green, respectively, and the HS-loop is outlined in yellow. An asterisk shows the site of each mutation.
R176Q was limited to the immediate vicinity of the substituted residue. It is noteworthy that no mutation-induced structural perturbations were observed in α2 despite the close proximity of the C-terminal region of α2 to the HS-loop (Figure 3.3A and Figure 3.7B). Taken together, the data suggest that P164S, R169Q and R176Q cause local structural perturbations within or near the HS-loop, overall consistent with the crystal structure analyses.

3.3.3 Residues in the α2 region undergo fast internal motion

We acquired $^{15}$N-$^1$H backbone relaxation data to probe the dynamics of RyR2A. Plots of the longitudinal (R1) and transverse relaxation (R2) rates as well as steady state $^{15}$N-$^1$H NOE reveal several areas of increased mobility. In particular, the N- and C-terminal regions as well as the loops flanking the α2 helix exhibit increased R1, decreased R2 and attenuated $^{15}$N-$^1$H-heteronuclear NOE ratios, indicative of increased mobility (Figure 3.9). The increased R1 and decreased $^{15}$N-$^1$H NOEs suggest that these regions are undergoing fast dynamics on a ∼ps-ns timescale (Figure 3.9A and C). The P164S, R169Q and R176Q HS-loop mutants all exhibited very similar relaxation parameter profiles as the wild-type protein (Figure 3.10, Figure 3.11 and Figure 3.12).

I subjected the relaxation data for wild-type and the P164S mutant which showed the greatest CSPs among the HS-loop mutants, to a model-free analysis in order to obtain parameters describing the internal motion of the individual NH bond vectors (Fig. 3a (3.14A). The generalized order parameter, S$^2$, suggests that the majority of RyR2A is rigid with an average S$^2$ value of 0.86 ± 0.03. Values range from 0, complete mobility, to 1, complete rigidity. However, the two loops flanking α2 demonstrate high mobility on a
~ps-ns timescale as evidenced by their lower $S^2$ values. The internal correlation time ($\tau_c$) extracted for residues 85-109 (ave. 15 ns), which correspond to $\alpha_2$ and the two loops flanking the helix, demonstrate that this entire region of RyR2A is undergoing fast internal motions on a ~ps-ns timescale compared to the overall rotational motion of the protein (Fig. 3b(3.14B)). The higher internal mobility of this region is consistent with the lack of electron density observed for $\alpha_2$ in any crystal structure of RyR2A and the variability observed in the position of $\alpha_2$ in our hybrid structure. Surprisingly, the P164S HS-loop mutation did not significantly alter the model free parameters within the HS-loop or any other part of the structure compared to wild-type (Figure 3.14A and B).
Figure 3.9 Measured $^{15}$N relaxation rates and $^{15}$N-{$^1$H} hetNOEs for RyR2A. Longitudinal (R1) and Transverse (R2) rates are plotted versus residue number in panel (A) and (B), respectively. (C) $^{15}$N-{$^1$H} hetNOE values are plotted versus residue number. Lower values indicate increased backbone dynamics. The α2-helix and flanking loops are highlighted by a dashed box. The α2-helix and HS-loop are colored red and yellow, respectively.
Figure 3.10 Measured $^{15}$N relaxation rates and $^{15}$N-$^{1}$H hetNOEs for RyR2A P164S.

Longitudinal (R1) and Transverse (R2) rates are plotted versus residue number in panel (A) and (B), respectively. (C) $^{15}$N-$^{1}$H hetNOE values are plotted versus residue number. Lower values indicate increased backbone dynamics. The α2-helix and flanking loops are highlighted by a dashed box. The α2-helix and HS-loop are colored red and yellow, respectively.
Figure 3.11 Measured $^{15}$N relaxation rates and $^{15}$N-$^1$H hetNOEs for RyR2A R169Q.

Longitudinal (R1) and Transverse (R2) rates are plotted versus residue number in panel (A) and (B), respectively. (C) $^{15}$N-$^1$H hetNOE values are plotted versus residue number. Lower values indicate increased backbone dynamics. The $\alpha$2-helix and flanking loops are highlighted by a dashed box. The $\alpha$2-helix and HS-loop are colored red and yellow, respectively.
Figure 3.12 Measured $^{15}$N relaxation rates and $^{15}$N-$^1$H hetNOEs for RyR2A R176Q.

Longitudinal (R1) and Transverse (R2) rates are plotted versus residue number in panel (A) and (B), respectively. (C) $^{15}$N-$^1$H hetNOE values are plotted versus residue number. Lower values indicate increased backbone dynamics. The $\alpha_2$-helix and flanking loops are highlighted by a dashed box. The $\alpha_2$-helix and HS-loop are colored red and yellow, respectively.
Figure 3.13 Backbone dynamics of wild-type and P164S RyR2A.

(A) Plot of generalized order parameter, $S^2$, derived from model-free analysis. Lower values indicate dynamic regions of the protein on a fast time-scale (ps-ns). (B) A plot of the internal correlation time, $\tau_e$, outlines regions undergoing faster internal motions than the average on a ps-ns timescale. (C) $R_{ex}$ contributions are shown for residues undergoing slower internal motions (µs-ms). Secondary structure elements for RyR2A are shown above. The mobile $\alpha_2$ region is outlined by a red dotted line.
Overall, the NMR backbone relaxation data suggest that the globular domain of RyR2A is relatively rigid with the newly identified α2 helix and associated loop residues undergoing fast internal motion.

3.3.4 Exon 3 deletion induces an α2-helix-to-β4 transformation in RyR2A

The $^1$H-$^{15}$N TROSY-HSQC for the RyR2A Δ exon 3 protein was ~92% assigned using conventional 3D backbone experiments and sequential assignment as per wild-type (Figure 3.14) (BMRB accession number 19199). The CSI-derived secondary structure components were in excellent agreement with the previously solved crystal structure [212]. Comparison of the wild-type RyR2A with the RyR2A 3 CSI data provides evidence in solution for the rescue of the β-trefoil fold of RyR2A via the remarkable α-helix-to-β-strand switch (Figure 3.15). CSPs of RyR2A Δ exon 3 relative to the wild-type spectrum are mapped as a gradient on the wild-type structure, demonstrating that the conformational changes are localized to the region involved in the switch mechanism (Figure 3.16). Taken together, the RyR2A Δ exon 3 solution NMR data suggest the core β-trefoil fold remains largely unaltered despite the drastic change in primary structure.

Backbone dynamics parameters were also obtained for RyR2A Δ exon 3 (Figure 3.17). RyR2A Δ exon 3 remains a relatively rigid protein with an average $S^2$ of 0.87 ± 0.2. However, in the case of this deletion mutant, the loops flanking the rescued β4 strand exhibited increased dynamics on fast timescales (i.e. elevated R1 and decreased $^{15}$N-$^1$H-NOE). Increased mobility of residues in the loop immediately after β4 (i.e. residues 104-109) as indicated by systematically lower $S^2$ values compared to RyR2A was observed. The consistently fitted $\tau_c$ values for residues 104-109 show that
Figure 3.14 Amide backbone assignment of RyR2A Δ exon 3.
(A) $^1$H-$^{15}$N TROSY-HSQC spectrum of $^{15}$N/$^{13}$C/$^2$H- labeled RyR2A Δ exon 3. Assignment was achieved for 92% of the protein backbone. (B) An enlarged view of the crowded region of the HSQC. Cross peaks colored in orange correspond to some residues undergoing chemical exchange.

Figure 3.15 Rescue of β-trefoil fold in solution by the α2-to-β4 switch in RyR2A Δ exon 3.
(A) Solution NMR evidence of an α2-helix-to-β-strand switch as a consequence of exon 3 deletion. Residues in exon 3 are colored black in the sequence. Cartoon representation of the secondary structural elements are derived from x-ray crystallography structures and solution-based NMR methods. CSI values are plotted for both wild-type RyR2A and RyR2A Δ3. (B) Important structural elements involved in the α2-helix-to-β-strand switch are highlighted in the hybrid model of RyR2A (right panel). Rescued β4 strand is shown in the crystal structure of RyR2A (left panel) (PDB ID: 3QR5).
Figure 3.16 Structural consequences of Δ exon 3 mutation in solution. Mapping of CSPs on RyR2A hybrid model. The region encoded by exon three is colored black. N- and C-terminal residues for this region are also highlighted.
Figure 3.17 Measured $^{15}\text{N}$ relaxation rates and calculated backbone dynamic parameters for RyR2A Δ exon 3.

Longitudinal (R1) and Transverse (R2) rates are plotted versus residue number in panel (A) and (B), respectively. $^{15}\text{N}$-{$^{1}\text{H}$} hetNOE values are plotted versus residue number (C). Lower values indicated increased backbone dynamics. The α2-helix and flanking loops are highlighted by a dashed box. The α2-helix and HS-loop are colored red and yellow, respectively. (D) A plot of the generalized order parameter, $S^2$, shows regions with increased backbone dynamics (lower values) at ps-ns timescales. (E) Regions undergoing increased mobility are indicated by elevated $\tau_e$ values. (F) Residues undergoing slow time-scale motions (μs-ms) exhibit $R_{ex}$ terms.

this loop also undergoes fast internal motions relative to the global tumbling; further, the lack of fast internal mobility for the rescued β4 is consistent with this strand robustly stabilizing the trefoil structure as a result of the exon 3 deletion.

3.3.5 The dynamic α2-helix is in close proximity to electron dense ‘columns’ connected to the pore

In order to find the location of the RyR2A α2-helix in the tetrameric RyR structure, I first docked the RyR1-ABC structure onto the map of tetrameric RyR1 using the same unbiased protocol applied by Tung et. al [209]. Subsequently, I superimposed our hybrid structure onto the A domain of the docked RyR1-ABC structure (Figure 3.16A and B). In this orientation, the α2-helix projects away from the cytosol and is in close apposition to the electron dense region, previously designated a ‘column’ that extends toward the transmembrane region [100, 101]. The α2-helix, which is adjacent to α1, is the closest structural element to the electron dense column connected to the pore (Figure 3.16A). Interestingly, the α2-helix of the IP$_3$R1 suppressor domain also projects toward the transmembrane region in cryo-EM docking studies [228, 229]. Thus, it is tempting to speculate that α2 interacts with a downstream RyR2 segment in the electron dense region.
in one of several interactions, including those mediated by α1, that connect the pore to the N-terminal domain. The α2 helix interaction may be specific for RyR2, conferring a distinct mode of channel regulation compared to RyR1 or RyR3.

Previous docking studies highlighted two intermolecular interfaces involving domain A [209, 212]. In the first interface, the HS-loop of domain A is modeled in close apposition to domain B of an adjacent subunit (Figure 3.16B) [212]. The exact position of
**Figure 3.18 Location of the RyR2A hybrid structure in the tetrameric cryo-EM map of full-length RyR1.**

(A) Side view of the RyR2A hybrid model (blue) superimposed with the RyR1-ABC structure (PDB ID: 2XOA) docked on the 9.6Å cryo-EM map of ‘closed’ RyR1 (EMDB code 1275). The B (green) and C (pink) domains from the N-terminal of RyR1-ABC are also shown. Black arrows indicate the location of the α1-helix and α2-helix, which is colored in red for clarity. The electron dense column is outlined by a dashed oval. (B) Top view of the docked RyR2A hybrid model. Small black arrows indicate the location of the HS-loop region, which is shown in yellow. The interface between domains A and B is indicated by a dotted line.

the domains across this inter-subunit interface differs between open and closed RyRs, and it is likely that the interactions are significantly altered during channel opening [209, 210].

The precise conformation of the HS-loop would, therefore, play a critical role in defining this interface. The CSPs observed for the RyR2A HS-loop mutants are primarily localized near the site of the mutation, and only marginal global structure, stability and backbone dynamics changes are observed for these mutant proteins, suggesting that local mutation-induced alterations to the HS-loop perturb this important interface in disease-associated dysfunction.

In the RyR2A Δ exon 3 mutant protein, the α2 helix is transformed into a β-strand and sequestered in the β-trefoil fold. This structural conversion preserves wild-type-like rigidity in the β-trefoil core, consistent with an energetically favorable α2-to-β4 transition [212]. When the RyR2A Δ exon 3 is studied in isolation, structural perturbations associated with this deletion mutation are limited to the regions involved in the motif conversion. However, the cryo-EM docking revealed that the location of the α2, which is omitted in the exon 3 deletion mutant, coincides with the interface between the mutation-rich N-terminal region and the ‘column’ density immediately above the transmembrane channel region. These observations strongly argue that the exon 3 deletion may affect the
communication between the N-terminal region and the channel domain via the ‘column’ density identified in the cryo-EM structure. Consistent with this notion, a recent study revealed the functional consequences of removing the 35-residues in exon 3 on Ca\textsuperscript{2+} release termination [216], which is a critical step in E-C coupling and controlling cytosolic Ca\textsuperscript{2+} transients. The authors demonstrated that RyR2 with the exon 3 deletion has a reduced luminal Ca\textsuperscript{2+} threshold for Ca\textsuperscript{2+} release termination and increased fractional Ca\textsuperscript{2+} release [216]. Considering our aforementioned structural analyses, we suggest that these functional observations are due to the uncoupling of a conformational linkage between the luminal Ca\textsuperscript{2+} sensing [77, 230] function and the cytoplasmic regulatory function, part of which is played by the N-terminal region where numerous mutations including the exon 3 deletion have been identified.
3.4 Conclusion

Taken together, our data suggest that the HS-loop and the RyR2A Δ3 mutations are detrimental through a disturbance of the interfaces facilitated by the formation of the tetrameric receptor and/or regions involved in the allosteric regulation of the receptor, rather than a global conformational destabilization or structural change of domain A. Consistent with this notion, the mutant RyR2A proteins show a conservation in the backbone dynamics with wild-type and the structural perturbations are limited to the sites of mutation, modeled in the interfaces through docking experiments. Further, high-resolution structural analyses are needed to elucidate the precise mechanisms of interface perturbation and the allosteric dysfunction they cause in disease states.
Chapter 4

THE ‘AEN DOMAIN’ IN THE REGULATORY REGION OF RYR IS AMMENABLE TO HIGH-RESOLUTION STRUCTURAL STUDIES
Abstract

The SR-associated protein ryanodine receptor (RyR) mediates Ca\(^{2+}\) release from SR stores. In mammals, there are three isoforms of RyR: RyR1, which is primarily found in skeletal muscle; RyR2, which is found in cardiomyocytes; and RyR3, which is found in several cell types. All three isoforms contain roughly 5000 amino acids, with the majority (80-90\%) of the primary structure comprising the cytosolic region. Many binding partners have been shown to bind to this region and regulate the function of RyR. The region containing the first two SPRY domains (residues 655-1205 in RyR1) has been implicated as a site of interaction with the dihydropyridine receptor (DHPR) and FK506-binding protein (FKBP12) \[\text{122, 231}\]. DHPR is the voltage sensor located on the T-tubule membrane that makes a physical connection with RyR1, causing the latter to open and release Ca\(^{2+}\) into the myoplasm. FKBP12 binds tightly to RyR and stabilizes its closed state. Recently, the binding sites for both DHPR and FKBP have been localized to the clamp region of RyR \[\text{232, 233}\]. Furthermore, docking of a homology model for the region flanked by SPRY1 and SPRY2 has been localized to the FKBP binding site \[\text{231}\].

I present preliminary structural information for the SPRY region and demonstrate that the domain flanked by SPRY1 and SPRY2, which I named the AEN domain, is amenable to high-resolution structural studies. I demonstrate using size exclusion chromatography that the inclusion of SPRY1 and/or SPRY2 induces oligomerization. Furthermore, the AEN domain is monomeric based on MALS analysis, folded as shown by far-UV CD and amenable to high-resolution structural studies as indicated by its HSQC spectrum.
4.1 Introduction

Ryanodine receptors (RyRs) are Ca$^{2+}$ release channels found on the SR membrane of myocytes. They release Ca$^{2+}$ from SR stores in response to physical or indirect interaction with the T-tubule voltage sensor, the dihydropyridine receptor (DHPR). Three RyR isoforms exist in mammals: type 1, primarily found in skeletal muscle; type 2, associated with cardiac muscle; and type 3, which is found in several cell types. All three share ~70% identity in their amino acid sequence, with three divergent regions: 4254-4631 (D1), 1342-1403 (D2) and 1872-1923 (D3) in RyR1 and 4210-4562 (D1), 1353-1397 (D2) and 1852-1890 (D3) in RyR2. RyRs are tetrameric receptors composed of four identical subunits with a similar structural architecture: large cytoplasmic N-terminal domain (80-90%) and C-terminal transmembrane domain that contains the ion conducting pore.

Mutations in all three RyR isoforms have been linked with lethal human diseases. Malignant hyperthermia (MH) \[56-59\] and central core disease (CCD) \[60-62\] are associated with RyR1 mutations; catecholaminergic polymorphic ventricular tachycardia (CPVT) \[71\] and arrhythmogenic right ventricular dysplasia (ARVD2) \[73\] are associated with RyR2 mutations; while more recently RyR3 mutations have been linked with neurodegenerative disorders such as Alzheimer’s Disease (AD) \[54, 55\].

RyRs are also a target for many regulators either on the cytoplasmic or luminal side. These include proteins such as DHPR, FKBP and calsequestrin; small molecules like Ca$^{2+}$, Mg$^{2+}$ and ATP; posttranslational modifications such as phosphorylation, nitrosylation and oxidation; and pharmacological agents such as caffeine, ryanodine and dantrolene \[105\].
The cytoplasmic domain of RyR contains many of the regulator binding sites and also harbors the majority of mutations linked with human diseases. This is not surprising since the cytoplasmic domain is ~four-fifths of RyR and it contains many cavities, as revealed by cryo-EM studies [100, 101]. How RyRs integrate the combined effect of regulators and modifications to control SR Ca\(^{2+}\)-release is still not well understood at the molecular level. Furthermore, disease-associated mutations add another layer to this complex web of interactions and modifications.

Recent structural information derived from cryo-EM studies of RyR in both the closed and open state, fluorescence resonance energy transfer (FRET) experiments and the publication of several atomic resolution structures of folded RyR domains have provided the first insights into how these giant molecules work at the molecular level. These three techniques provide information at different scales. Cryo-EM studies have revealed the global yet subtle changes in RyR structure for open and closed states [102]. It has also revealed the location of several binding partners such as FKBP and CaM [128, 233]. FRET-based experiments have provided medium range information by focusing in on specific regions of RyR [234-236]. Recently, a study used FRET-based methods to examine the ligand-dependent conformational changes in the clamp region of RyR2 [237], which is known to undergo large movements during channel gating. The study showed that different ligands such as Ca\(^{2+}\), ATP, 4-chloro-\textit{m}-cresol and aminophylline induce different conformational changes in the clamp region, suggesting that RyR possesses multiple ligand-dependent gating mechanisms. Atomic resolution studies have revealed the molecular architecture of individual domains and allowed mapping of disease-associated mutations on their three-dimensional structures [209, 211, 214]. Most of these
studies have focused on the N terminus, but recent work has revealed detail about the central region of the protein, which contains the phosphorylation domain [214, 215].

The phosphorylation domain from rabbit RyR1 (2734-2940) [214, 215], mouse RyR2 (2699-2904) [215], human RyR3 (2597-2800) [215] and several disease-associated mutants [215] were solved to resolutions ranging from 1.6 to 2.2 Å. In both RyR1 and RyR2, the domain consists of a two-fold symmetrical structure in which each motif consists of two α-helices, one or more 3₁₀ helix and a β-strand. A long and flexible loop separates each motif and contains the previously determined phosphorylation targets of S2843 (RyR1) and S2808/2814 (RyR2).

There are 11 disease-associated mutations that can be mapped onto the phosphorylation domain structures. These can be divided into three groups: the first group contains seven mutants all found on the same face of the structure near the S2843 phosphorylation site, the second contains three mutants that are on the opposite face, while the last group contains the L2867G mutant. Most mutants have a minimal affect on the stability of the domain except for L2867G which demonstrated a melting temperature 13°C lower than the wild type and readily aggregated and precipitated at room temperature [215].

This novel structural information allowed another group to generate a pseudo-atomic model of a homologous region in RyR1 (850-1056) and RyR2 (861-1067) [231]. Using rigid body docking and molecular dynamics, they were able to dock this model into the cryo-EM map of RyR1 and located it to the FKBP binding interface on the clamp region. Docking of FKBP onto the EM map revealed a high occurrence of charged
residues between the two proteins, suggesting that electrostatic interactions could play an important role in FKBP binding to RyR [231]. Furthermore, previous cryo-EM work revealed that the DHPR binding site, the SPRY2 region, also co-localizes to the clamp region of RyR1 [232], reinforcing the importance of this region for channel function.

After publishing the RyR1A structure, I began work on a region I call the ‘SPRY region’ (residues 655-1205 in rabbit RyR1). I chose this region because of its functional importance and because some structural information was known about SPRY2 [122]. My intent was not to examine SPRY2 on its own since it would be too small to dock into the RyR cryo-EM map, but to determine the structure of the entire SPRY region. This proved challenging, but the region flanked by the two SPRY domains was amenable to high-resolution structural studies. This region was previously termed the ‘RyR motif/domain/fragment’ since analysis of the primary sequence revealed a repeating sequence motif (average identity of 28%) that occurred four times in two doublets (841-954 & 955-1068 and 2725-2844 & 2845-2958) [37]. The latter region corresponds to the phosphorylation domain and the former to the recently proposed FKBP binding domain [231]. The term ‘RyR motif/domain/fragment’ seems arbitrary for a protein the size of RyR, therefore I decided to use the term ‘AEN domain’ since this three amino acid repeat was the longest in this region [37].

4.2 Material and Methods

4.2.1 Cloning and expression of SPRY region

    Constructs encompassing residues 655-1205 in rabbit RyR1 were design based on secondary structure prediction using PSIPRED v3.3 and from previous functional studies
delineating the boundaries of the SPRY domains [122]. Altogether, 8 constructs were chosen that contained one, two or no SPRY domains (Figure 4.1 and Table 4.1).

![Figure 4.1 Design of SPRY region constructs.](image)

Schematic diagram showing the boundaries of SPRY region constructs used in preliminary structural experiments. The NTD (domains A, B and C) is shown for reference.

**Table 4.1** SPRY region constructs and their physical properties

<table>
<thead>
<tr>
<th>Construct</th>
<th>Pre-thrombin cleavage</th>
<th>Post-thrombin cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MW (Da)</td>
<td>pl</td>
</tr>
<tr>
<td>655-1205</td>
<td>76512</td>
<td>5.86</td>
</tr>
<tr>
<td>655-1086</td>
<td>63329</td>
<td>6.38</td>
</tr>
<tr>
<td>655-1068</td>
<td>61148</td>
<td>6.23</td>
</tr>
<tr>
<td>798-1205</td>
<td>61117</td>
<td>6.04</td>
</tr>
<tr>
<td>841-1205</td>
<td>56126</td>
<td>5.83</td>
</tr>
<tr>
<td>798-1086</td>
<td>47934</td>
<td>7.1</td>
</tr>
<tr>
<td>841-1086</td>
<td>42944</td>
<td>6.63</td>
</tr>
<tr>
<td>841-1068</td>
<td>40763</td>
<td>6.36</td>
</tr>
</tbody>
</table>

Theoretical molecular weights (MW) and isoelectric points were calculated using the ProtParam service from the ExPASy webserver.

These constructs were subcloned and amplified into a pET32a expression vector (Novagen, Inc.) using *BamHI* and *XhoI* restriction enzyme sites. The resultant vectors were expressed with a N-terminal thioredoxin (Trx)- and poly-His-tag in BL21 (DE3) *Escheria coli* (*E. coli*) cells (Stratagene) grown at 18°C in 50 mL Luria-Bertani (LB)
media for approximately 16 hr using 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) induction. Cells were harvested by centrifugation and stored at -70°C.

4.2.2 Expression of $^{15}$N-labeled protein

A 50 mL starter culture containing E. coli cells expressing recombinant protein was grown overnight in LB media. Cells were spun down at 2000 rpm for 10 min and re-suspended with M9 minimal media three times before being transferred to a 2 L culture containing M9 minimal media and 1 g/L $^{15}$N-ammonium chloride (Cambridge Isotope Laboratories). Incorporation of $^{15}$N and was confirmed by electrospray mass spectrometry.

4.2.3 Protein Purification

Purification was carried out at 4°C with all buffers precooled to this temperature. Cells were resuspended in 15 mL of lysis buffer (20 mM Tris-HCl pH 8.0, 20 mM Imidazole, 100 mM NaCl, 10% (v/v) glycerol, 0.4% nonidet P40 (NP40), 10 mM 2-betamercaptoethanol (BME), 10 µg/mL DNase I and 1 mM phenylmethanesulfonylfluoride (PMSF). Cells were lysed by sonication using six 1-minute pulses on a Branson Sonifier 450. Supernatant, isolated by centrifuging cell lysate for 30 minutes at 20,000x g, was incubated with Ni-NTA resin (Qiagen) for 1 hr at 4°C. The resin was washed with 50 mL of washing buffer (20 mM Tris-HCl pH 8.0, 20 mM imidazole, 500 mM NaCl and 10 mM BME). The fusion protein was eluted from the column with 5 mL of elution buffer (20 mM Tris-HCl pH 8.0, 250 mM imidazole, 300 mM NaCl and 10 mM BME). Overnight thrombin cleavage was carried out in 20 mM Tris-HCl pH 8.0, 300 mM NaCl and 5 mM DTT for SPRY-containing constructs.
Due to the large theoretical isoelectric point (pI) difference for the AEN constructs before and after thrombin cleavage, the following buffer was used instead: 20 mM HEPES pH 7.0, 300 mM NaCl and 5 mM DTT.

Samples were concentrated down to 1 mL and loaded directly onto an analytical Superdex 75 10/30 (GE Healthcare) sizing column. Buffers used were: 20 mM Tris-HCl pH 8.0, 300 mM NaCl and 5 mM DTT for SRPY-containing constructs and 20 mM Bis-Tris pH 6.0, 300 mM NaCl and 5 mM DTT for AEN constructs. Additionally buffer containing 20 mM HEPES pH. 7.0, 300 mM NaCl and 5 mM DTT was used for NMR samples.

4.2.4 Size exclusion chromatography (SEC) and Light Scattering

All buffers used were passed through a 0.22 µm filter, degassed and pre-chilled to 4°C. SEC was performed on a Superdex 200 10/300 GL column (GE Healthcare). Multi-angle light scattering (MALS) measurements were performed in-line using a three-angle (45, 90, and 135°) miniDawn static light-scattering instrument with a 690-nm laser (Wyatt Technologies, Inc.). Molecular weight was calculated using ASTRA software (Wyatt Technologies, Inc.) based on Zimm plot analysis using a refractive index increment, \( dn/dc = 0.186 \ g\cdot1\ ) [238]

4.2.5 Optical Spectroscopy

Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-815 CD spectrometer (Jasco, Inc.) at 25°C. Data were collected in 1-nm increments using a 0.1-cm-path length (l) cell, 10-s averaging time, and 1-nm bandwidth. Spectra were corrected for buffer contributions.
4.2.6 NMR Spectroscopy

NMR experiments were carried out on $^{15}$N-labeled RyR1 841-1068. Two concentrations were used: low (0.3 mM) and high (1 mM). Buffer consisted of 20 mM HEPES pH 7.0, 5 mM DTT and 10% (v/v) D$_2$O containing either 100 mM or 300 mM NaCl. Experiments were carried out at 20°C and 30°C on a 600 MHz Bruker spectrometer and at 30°C on an 800 MHz spectrometer, both equipped with a cryogenically-cooled triple resonance probe. A $^{15}$N heteronuclear single-quantum coherence (HSQC) experiment was performed on the $^{15}$N-labeled sample.

4.3 Results and Discussion

4.3.1 Expression and purification

SPRY region constructs can be separated into four groups: full-length, SPRY1-containing, SPRY2-containing, and AEN constructs. AEN constructs showed robust expression, while those containing SPRY2 had lower expression, SPRY1-containing constructs showed minimal expression and the full-length construct did not express (Figure 4.1). Some precipitation was observed for SPRY-containing constructs independent of buffer, temperature, salt concentration and pH (data not shown). AEN constructs remained soluble and stable at room temperature. All constructs were amenable to thrombin cleavage and did not show degradation after removal of the Trx fusion protein and 6X-His-tag (Figure 4.2).
Figure 4.2 Expression of RyR1 SPRY region constructs in *E. coli*.
Molecular weight marker is shown on the left side of the 15% SDS-PAGE gel stained with Coomassie Blue. Ni-NTA flow-through and thrombin cleaved protein are shown in each set of lanes. Constructs are labeled above. Lower molecular weight band seen in the thrombin cleaved lanes corresponds to thioredoxin.

Size exclusion chromatography revealed that both SPRY domains induced oligomerization. In contrast, AEN constructs showed a single monomer peak at the expected elution volume for a protein of ~30 kDa (Figure 4.3). Although, recombinant SPRY2 has been purified to homogeneity in a folded state, the purification protocol required refolding conditions [124]. Therefore SPRY1 and/or SPRY2 seem to induce oligomerization of this region and future structural studies containing either one might require refolding conditions. I therefore focused my attention on the AEN constructs (798-1086, 841-1068 and 841-1086) for further biophysical assays and high-resolution studies.
Thrombin-cleaved samples were loaded directly onto an S75 10/30 sizing column (Bottom four). RyR1 841-1068 was loaded onto a S75 26/60 Prep-grade column. Single peak at ~13 mL for 798-1086 and 841-1068 reveals a monomeric AEN protein. The peak at ~15 mL corresponds to thioredoxin. A symmetric single peak is observed for 841-1068 at ~180 mL, indicative of a monomeric protein. SPRY2 induces oligomerization as evidenced by a large amount of protein present in the void volume.
4.3.2 RyR1 constructs are monomeric and folded

In order to determine the molecularity of the AEN constructs, I carried out SEC in-line with MALS to obtain their molar mass in solution. This requires extremely clean samples with no impurities, therefore fractions containing RyR1 protein without Trx and 6x-His were pooled together and concentrated down to 100 µL and passed through a 0.22 µm filter before being injected into the column. All buffers were passed through a 0.22 µm filter, degassed and pre-chilled to 4°C. SEC showed a single peak for each sample (Figure 4.4). SEC in-line MALS gave Zimm plot based molecular masses of ~ 25, 28 and 34 kDa for RyR1 841-1068, 841-1086 and 798-1086 respectively, which are very close to the expected mass of the monomers (26.0, 28.2 and 33.2 kDa).

The secondary structure of the recombinant AEN constructs was assessed using far-UV CD. The spectrum of all three constructs was typical of an α-helical protein, with two intense minima at ~ 208 and 225 nm and a maximum at < 195 nm (Figure 4.5). The relatively high intensity of the bands suggests that all three constructs are well folded at room temperature. This experimentally determined structural data agrees well with the recently generated homology model for this region in rabbit RyR1 (residues 850-1056) and mouse RyR2 (861-1067).
Figure 4.4 Molar mass of AEN constructs in solution is consistent with a monomer. SEC (solid lines) with in-line MALS (dots) at 4°C. MALS molecular weights of 841-1068 (red), 841-1086 (blue) and 798-1086 (green) were consistent with a monomer. Height of peaks is dependent on injected sample concentrations.
Figure 4.5 AEN constructs are well folded and stable at room temperature. Far-UV CD. The spectrum of all three constructs are consistent with an \( \alpha \)-helical protein, with two intense minima at \(~208\) and \(225\) nm and a maximum at \(<195\) nm.

4.3.3 RyR1 fragments are amenable to high-resolution structural studies using NMR

The monomeric and well-folded state of the AEN fragments made them good targets for high-resolution structural studies using NMR. The smallest construct (841-1068) was chosen to carry out these studies. In general, a well-dispersed HSQC spectrum is a pre-requisite for further NMR-based structural studies. Therefore several factors required optimization to obtain such a spectrum. The first factor tested was protein concentration; typically a more concentrated sample gives rise to more and/or stronger peaks since NMR is an inherently sensitive method. Interestingly, a lower concentrated sample (0.3 mM) produced a more dispersed spectrum compared to the concentrated (1
mM) sample. This may be due to the formation of dimers or higher-order oligomers at high concentrations, which increase the apparent molecular weight of the molecules and hence increases relaxation rates, causing peaks to be broadened out. Normally, NMR buffers containing low salt concentrations are preferred because they decrease the 90° pulse width, which results in two beneficial outcomes: shorter experimental times but more importantly, reduced heat generation in the sample tube. Fortunately, high-salt samples can be routinely run using shaped tubes (Bruker Biospin, Inc) that increase the signal-to-noise ratio (SNR) and shorten pulse widths. The AEN fragment gave a more dispersed HSQC spectrum in a high-salt (300 mM) buffer, which may arise from the high salt environment disrupting transient molecular interactions. Lastly, experiments were carried out at a higher temperature (30°C) as another means to increase SNR and the dispersion of peaks. This had a beneficial effect as more peaks appeared and the spectrum was more dispersed. Importantly, no evidence of sample degradation was noted either in the HSQC or in the sample tube.

The final spectrum of RyR1 841-1068 therefore was obtained using a shaped tube at 30°C and 300 mM NaCl. It contains ~170 peaks out of an expected 228 (Figure 4.6). This apparent lack of peaks is most likely due to the size of the protein (~26 kDa). NMR spectra of large proteins (>20 kDa) suffer from two challenges: the low SNR (due to increased relaxation rates caused by the slower overall tumbling of larger proteins in solution) and the lack of spectral resolution, to resolve overlapping peaks, due to the large number of peaks [239]. This will be overcome by utilizing deuterium labeling methods.
Preliminary NMR data suggests that the AEN domain is amenable to structural studies at the atomic level. Furthermore, the recent homology model study showed a direct interaction between the pseudo-atomic model of RyR1 residues 850-1056 (AEN domain) and FKBP in a docked model. NMR may be a useful tool in studying this interaction. In order to carry this out, backbone assignments of both proteins, AEN domain and FKBP12, are required. The former should be attainable with deuterium labeling methods and TROSY-based experiments. The latter has already been published.
along with detailed multi-timescale dynamics including rapamycin and FK506 binding effects [240]. The authors of the homology study were able to map two mutations: hRyR1 R1043C (MH) and hRyR2 R1051P (CPVT), that were localized to a helix that could interact directly with FKBP. NMR is a powerful tool to study site-specific effects of mutations, by probing the structural stability and fold of wt versus mutant protein and by examining the perturbations, if any, to FKBP-RyR interaction. It should be stressed that several domains seem to come together to form the FKBP binding pocket as revealed by cryo-EM [233, 241, 242], suggesting that the AEN-FKBP interaction may be weak in isolation. Therefore, NMR may be at the limit of detecting this interaction. Another mutation (hRyR2 R1013Q) was mapped to an interface between the docked AEN domain and phosphorylation domain. This suggests the likelihood of a direct domain-domain interaction between the homologous domains. Interestingly, a loop harboring seven disease-associated mutations in the phosphorylation domain makes direct contact with a loop in the AEN domain that contains the R1013Q mutation. Again, NMR could be a powerful tool to probe this interaction and any effects that mutations and phosphorylation may have.
4.4 Conclusion

Structural studies were carried out on the SPRY region (RyR1 655-1205) containing SPRY1, AEN domain and SPRY2. Both SPRY1 and SPRY2 induce oligomerization under native purification conditions, suggesting refolding methods should be used in their future studies. AEN domains, which are flanked by the SPRY domains are soluble, monomeric and stable at room temperature, making them amenable for high-resolution structural studies. Preliminary NMR studies reveal a well-dispersed HSQC spectrum. Deuterium labeling and TROSY-based experiments should improve the quality of the spectrum and allow for backbone assignment. This would allow two distinct interactions with the AEN domain to be probed: (1) the phosphorylation domain and (2) FKBP. Furthermore, the location of three disease-associated mutations at the interface of these two domains and the dynamic nature of the ‘clamp’ region they reside in makes this RyR region structurally compelling to study.
Chapter 5

Discussion
5.1 Summary

Since the discovery and purification of RyRs [32-34], a great deal has been learned about its function and structure. Extensive biochemical and functional analyses have revealed the complex network of post-translational modifications; interactions with small molecules and proteins; and mutational effects that together regulate the release of Ca$^{2+}$ from the SR.

Several fatal human diseases have been linked with mutations in RyR1 and RyR2. A large amount of time and effort has been spent identifying novel mutations associated with human disease, resulting in over 500 mutations discovered to date. The functional consequences of these mutations on either RyR1 or RyR2 have been examined and several theories such as inter-molecular domain-domain interactions [243], FKBP disruption by PKA phosphorylation [244] and store overload-induced Ca$^{2+}$ release (SOICR) [83] have been put forth. Although they differ in the mechanism by which the mutations affect the function of RyR, all essentially describe a receptor that opens prematurely in response to stimuli. Recent structural studies have started to answer some of the mechanistic questions regarding channel function and disease-associated mutations.

A number of cryo-EM reconstructions of RyR isoforms have been carried out over the past 20 years, with recent maps reaching a resolution of 9.6 Å. Two morphologically distinctive regions, one being the cytoplasmic region and the other the channel region, are distinguishable in all reconstructions. Studies of the open and closed states at higher resolutions have demonstrated large-scale movements in the cytoplasmic region and rotations in the pore region as a consequence of channel gating. FRET studies have also demonstrated the dynamic nature of cytoplasmic regions (e.g. clamp) during
channel gating. More recently, high-resolution structures from several RyR regions have highlighted the structural architecture of individual domains and the location of mutations within those domains. These studies along with my NMR work and recently published homology model of the AEN region have revealed inter- and intra-domain interfaces, some concentrated with mutations, that are involved in allosteric movements in RyR. Although these high-resolution studies provide evidence for Ikemoto’s domain-domain interaction hypothesis, they do not rule out regulation via SOICR since no structures have been solved from the luminal side of the receptor, and the mechanism of FKBP-RyR interaction may yet be determined by focusing on the AEN region of RyR.

Alongside these advances in the RyR field, another Ca^{2+}-release channel, the inositol 1,4,5-trisphosphate receptor (IP^{3}R) has been extensively studied. Recent high-resolution structural studies of the N-terminal domain (NTD) of IP^{3}R reveal a striking similar with RyR in its domain architecture and localization in each respective tetrameric structure [228, 245]. Furthermore, the HS-loop in both proteins is located and oriented in a similar fashion that juxtaposes it to a neighboring NTD. Presumably, disruption of this interface (and others) would allow for channel gating. Perhaps, most strikingly, these structural similarities allow for swapping of functional domains between the two receptors resulting in functional chimeras that mimic the native proteins [228]. These functional studies agree with our genomic analysis showing that the NTD of IP^{3}R seems to have evolved from a lower organism RyR [246].

5.2 Future Directions

5.2.1 Studies of an extended NTD construct (RyR1 1-630)
The first high-resolution structure of RyR was of the distal N terminus (RyR1 1-210), this was extended to include the B and C domains in the RyR1ABC structure by Tung et. al [209]. All together, 56 disease-associated mutations were mapped to this latter structure, highlighting several interfaces thought to be involved in stabilizing the closed state of the receptor. This study however, did not extend to the end of the N-terminal mutation hotspot at R614. This left out several mutations including the most functionally studied, R614C. Furthermore, a study has localized the dantrolene binding site to residues 590-609 in RyR1 [67].

With this information in mind, I decided to make extended NTD constructs (rabbit RyR1 1-662, 1-630, and 1-639). All three expressed well and purified with high purity as monomers. Crystals for RyR1 1-639 grew after one week and subsequent microseeding experiments resulted in single crystals that diffracted to 7Å.

These initial results are encouraging; optimizing the cryo-protectant protocol and trying different additives during crystallization should improve diffraction. Lastly, crystal trials with the addition of dantrolene are underway.

5.2.2 Dynamic Studies of RyR fragments using NMR

My NMR study of RyR2A revealed the importance of carrying out structural studies in solution as well as measuring protein backbone dynamics. I was able to detect a new α-helix that was dynamic and not visible in any crystal structure of RyR2A. This helix appears to be located at an interface with electron dense ‘columns’ that connect to the channel region of the receptor. On another note, what my dynamics study showed was that overall, RyR2A and surprisingly, RyR2A Δ exon 3, are fairly rigid. One can imagine
the B and C domains of NTD having similar properties. The dynamic regions may be the linkers connecting each domain, thereby giving the entire NTD some flexibility as has been observed [210].

The method used in my study of probing amide backbone dynamics is too cumbersome for a protein the size of RyR1ABC. However, another approach that focuses on the methyl (CH$_3$)- containing sidechains of specific amino acids has been successful at studying the structure and dynamics of large proteins and complexes (reviewed in [247]). Using the methyl moiety as a structural probe has several advantages; they are usually located at protein-protein interfaces [248] and their resonance peaks are stronger and sharper because there are three protons per methyl group and they rotate rapidly about their three-fold axis, respectively [249].

5.2.3 Probing the AEN-FKBP and AEN-phosphorylation domain interactions

Out of the three future directions, the study of AEN domains seems like the most feasible in a short period of time. The demonstration that the AEN domain gives a dispersed HSQC spectrum was a positive development for further NMR studies. NMR backbone assignments for proteins this size (~ 25 kDa) are routine and now that I have carried out dynamic studies on RyR2A, it should be straightforward to transfer this protocol to the AEN domain.

One advantage of NMR is that we can choose which binding partner is NMR active, generating structural information from both protein and its ligand. For instance, this could allow us to study the AEN-FKBP interaction from either side since FKBP12’s amide backbone has already been assigned. Similarly, a postdoctoral fellow in the lab,
Masahiro Enomoto, has begun NMR studies on the phosphorylation domain of RyR. Therefore both interfaces with AEN could be analyzed in a site-specific manner.

5.3 Thesis limitations

One of the limitations with my thesis is trying to infer structural consequences from small domains (~ 200 residues), especially in relation to the overall size of RyRs (~ 5,000 amino acids). This was demonstrated when we did not detect any perturbation to the structure or stability of RyR1A due to the C36R mutation. However, when this mutation was examined in the context of the much larger RyR1ABC construct, it caused a dramatic decrease in thermal stability of this domain. Furthermore, our structural studies did not provide us with insight into how we might probe the function of RyR, since most of the mutants in the first 200 residues had already been functionally examined.

Due to the difficulty of expressing, purifying and crystallizing membrane proteins, we have left out a critical region of the receptor that harbors many disease-associated mutations, is thought to contain a \( \text{Ca}^{2+} \) binding site and is critical for the function of RyRs. Hopefully with the use of techniques such as T4-lysozyme fusion proteins, removal of flexible loops and micro-crystallography, we will soon know the high-resolution structural workings of the transmembrane region.

Lastly, all of my studies used high-resolution methods, which are critical to better understanding RyR function. However, we must not forget lower-resolution methods such as FRET-probes. These have been useful in showing the dynamic nature of RyR during \( \text{Ca}^{2+} \)-release and would complement NMR-based dynamic studies of larger RyR fragments.
5.4 Conclusions

The work on RyR has been challenging over the years, especially in between my first and second publication. I started out working on a small 200-residue region, which accounts for less than 4% of the primary sequence of RyR. Recent x-ray crystal structures from other groups have increased this number to 15%. Obviously, much more work remains in solving the atomic-level structural mechanics of RyR. The combination of structural data from different techniques (e.g. docking crystal structures into cryo-EM maps) has been of great benefit. More recently I have shown the complementarity between NMR and X-ray crystallography, which I believe will become more important as we begin to study the dynamics of larger fragments of RyR. My initial structure of RyR1A hinted at the importance of structural features that harbored mutations, which we are only now beginning to understand as crucial sites of allosteric interactions. All in all, the work I have done, with the help of many, should serve as a starting point for continuing structural studies on RyRs.
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