Towards Understanding the Formation of an SR Luminal Ca-Sensor

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2012

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School of Medicine
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<th>Description</th>
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<tr>
<td>∆</td>
<td>Delta</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>f</td>
<td>Frequency</td>
</tr>
<tr>
<td>³H</td>
<td>Tritium</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
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<tr>
<td>AMP-PCP</td>
<td>β,γ-methyleneadenosine 5’-triphosphate</td>
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<td>BCIP-NBT</td>
<td>5-Bromo-4-chloro-3-indolyl phosphate-Nitro blue tetrazolium</td>
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<td>Ca</td>
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<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate</td>
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<td>CICR</td>
<td>Calcium induced calcium release</td>
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<tr>
<td>CPM</td>
<td>Count per minute</td>
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<td>CPVT</td>
<td>Catecholaminergic polymorphic ventricular tachycardia</td>
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<tr>
<td>CSQ</td>
<td>calsequestrin</td>
</tr>
<tr>
<td>CTF</td>
<td>Contrast transfer function</td>
</tr>
<tr>
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<td>dissipation</td>
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<td>DADs</td>
<td>Delayed afterdepolarisation</td>
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<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ECC</td>
<td>Excitation contraction coupling</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EMAN</td>
<td>Electron Micrograph ANalysis</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>RyR</td>
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<td>soyabean lecithin</td>
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<td>Triton X 100</td>
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Abstract

The University of Manchester

Ahmed Handhle

Doctor of Philosophy

Towards understanding the formation of an SR luminal Ca-sensor

29/05/2012

Calcium induced calcium release (CICR) is the process mediating cardiac excitation contraction coupling (ECC). In brief, depolarisation of the plasma membrane of the cardiac myocyte leads to an influx of calcium ($\text{Ca}^{2+}$) into the cytosol via the L-type voltage gated $\text{Ca}^{2+}$ channels. The raised level of cytosolic $\text{Ca}^{2+}$ initiates $\text{Ca}^{2+}$ release from the junctional cisternae of sarcoplasmic reticulum (SR) through the opening of the ryanodine receptor 2 (RyR2). The exact mechanism of termination of CICR remains to be elucidated. It has been proposed that a drop in the luminal $[\text{Ca}^{2+}]$ reduces the open probability of RyR2 thereby leading to termination of CICR. It is also believed that RyR2 senses the luminal $[\text{Ca}^{2+}]$ through the formation of a quaternary complex with the SR proteins; calsequestrin (CSQ), triadin and junctin. However, the mechanism governing the assembly of this SR ‘luminal $\text{Ca}^{2+}$ sensing complex’ is still far from being fully understood. A thorough knowledge of how this protein network is assembled is not only required for a robust understanding of the normal physiology of ECC but also for understanding the pathogenesis of disease, since disruption in luminal $\text{Ca}^{2+}$ sensing is reported to lead to diastolic $\text{Ca}^{2+}$ leak resulting in delayed after depolarisations (DADs), the precursor of premature beats and tachyarrhythmias.

The primary focus of this thesis research was to investigate the structural basis for the formation of the luminal $\text{Ca}^{2+}$ sensing complex with an emphasis on RyR, CSQ and triadin interactions. In order to achieve this goal, a protocol was developed to purify RyR2 from bovine heart employing a variety of techniques. Unfortunately this work resulted in only a partial purification of RyR2 with very low yields. However, more success was achieved with the isolation of the skeletal muscle ryanodine receptor isoform, RyR1, from sheep skeletal muscle employing sucrose gradient fractionation. The second aim of this study was to purify calsequestrin to enable investigations into its mode of interaction with the RyR. A molecular biology approach was taken and human cardiac calsequestrin (hCSQ2) was expressed as a GST tagged fusion protein and purified from E.coli BL21 (DE3) cells. A similar strategy was taken to express and purify the full-length and C-terminal luminal domain of mouse cardiac triadin isoform 1 (Trd1). However, this proved unsuccessful.
A range of biochemical and biophysical techniques was next employed to examine whether the ryanodine receptor associated with hCSQ2 in the absence of triadin. It was found that purified RyR1 bound to immobilised GST-hCSQ2 through co-precipitation experiments suggesting a direct interaction between the two proteins. Further studies using surface plasmon resonance (SPR) also showed that immobilised hCSQ2 bound both RyR1 and RyR2. These findings were then developed by experiments employing quartz crystal microbalance and dissipation (QCM-D) monitoring. The data from QCM-D was also found to support a direct interaction of RyR1 (closed state) with both Ca$^{2+}$ free and Ca$^{2+}$ bound hCSQ2. Isolation of a RyR1 (closed state) and hCSQ2 complex (in the absence of Ca$^{2+}$) was achieved using a sucrose cushion with an aliquot of the sample examined by transmission electron microscopy (TEM) using both negative staining and cryo-electron microscopy methods. The raw images of the complex suggested a direct interaction between RyR1 and CSQ2 in agreement with the data described above. However, intriguingly the hCSQ2 appeared to form strands of protein linking adjacent RyR molecules. These images, therefore, may suggest a possible role for hCSQ2 in a putative RyR coupled-gating mechanism.

Another aspect of this research work was to optimise and employ a [$^3$H] ryanodine binding assay to investigate how the channel activity of RyR1 and RyR2 within the SR preparations was regulated by hCSQ2 and triadin. Neither removal of endogenous CSQ from the SR membranes nor the addition of the recombinant hCSQ2, after removal of endogenous protein, modified the channel activity. However, interestingly, a synthesized domain of triadin (Trd KEKE motif) was found to enhance the channel activity as indicated by an increased [$^3$H] ryanodine binding to both RyR1 and RyR2.

In conclusion, the results from this thesis work provide evidence for a direct interaction between RyR and hCSQ2 and suggest a stimulatory role of a domain of triadin upon the activity of both isoforms of the ryanodine receptor.
IV Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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VI Acknowledgement

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VII The Author

In 2000 I completed a 6 year M.B.B.Ch in “Medicine & Surgery” at Faculty of Medicine, Mansoura University, Egypt. By 2003, I completed my foundation year and the compulsory military service as a medical doctor. From 2003-2008 I worked as demonstrator and assistant lecturer of medical biochemistry at Faculty of Medicine, Mansoura University, Egypt; during this period I got my MSc in medical biochemistry in 2006. I was awarded a governmental PhD scholarship and I began my PhD at the University of Manchester in the Faculty of Medical and Human Sciences in January 2008.
Chapter 1: Introduction

Sudden cardiac death (SCD) is defined as an unexpected death due to cardiac causes within 1 hour from the onset of symptoms in a person without any prior condition that would appear fatal (Siddiqui and Kowey 2006; Arshad, Mandava et al. 2008). Cardiologists define sudden cardiac death as the occurrence of a malignant ventricular arrhythmia (ventricular tachycardia (VT) deteriorating into ventricular fibrillation (VF)) (Mehta, Curwin et al. 1997; Zipes and Wellens 1998; Turakhia and Tseng 2007), with little or no alarm resulting in sudden unexpected collapse and death (Smith and Cain 2006). SCD is a major public health problem in the developed world. In the United States, cases range from less than 200,000 to 450,000 per year (Myerburg and Wallens 2006; Smith and Cain 2006; Arshad, Mandava et al. 2008). The wide gap between estimates is due to different inclusion criteria. For example, the higher figure (450,000 per year) includes all deaths with underlying cardiac disease occurring either out-of-hospital, in an emergency department or “dead on arrival” (Zheng, Croft et al. 2001; Smith and Cain 2006; Arshad, Mandava et al. 2008). However, with a more conservative approach, an estimate of 300,000 deaths per year was reported in the United States (Myerburg, Kessler et al. 1992; Myerburg, Kessler et al. 1993; Smith and Cain 2006; Arshad, Mandava et al. 2008). Therefore, if the US population is assumed to be about 300 million, the incidence of SCD is 1/1000 per year. Europe seems to have a similar incidence of SCD (Priori, Aliot et al. 2001; Smith and Cain 2006; Arshad, Mandava et al. 2008). A SCD rate of 1 per 1000 per year was reported in a review of cardiac arrests (not sudden deaths) in patients aging between 25 and 75 years in Maastricht (de Vreede-Swagemakers, Gorgels et al. 1997; Smith and Cain 2006; Arshad, Mandava et al. 2008). In Japan, the incidence of sudden death ranges from 1-2 per 1000 (with a definition of sudden death as that occurring within 24 h of the onset of symptoms) (Toyoshima, Hayashi et al. 1996; Kawamura, Kondo et al. 1999) (Kawakubo and Lee 2005; Smith and Cain 2006; Arshad, Mandava et al. 2008). The primary aetiology of SCD can be either structural or electrical. Acute myocardial infarction (MI), post-MI, heart failure, left ventricular hypertrophy (LVH), inflammatory or infiltrative myocardial disease and arrhythmogenic right ventricular dysplasia (ARVD) are the most common primary structural causes of SCD. However,
the primary electrical aetiology of SCD include long QT syndrome (LQTS) (congenital or acquired), short QT, Brugada syndrome, idiopathic VF and catecholaminergic polymorphic ventricular tachycardia (CPVT) (an extensive review can be found in (Turakhia and Tseng 2007)).

CPVT is primarily an electrical myocardial disease in which a sudden increase in sympathetic tone following physical or emotional stress leads to adrenergically mediated ventricular arrhythmias causing syncope, cardiac arrest and sudden death in young individuals with structurally normal hearts (Leenhardt, Lucet et al. 1995; Viskin and Belhassen 1998; Cerrone, Napolitano et al. 2009; Katz, Arad et al. 2009). In some CPVT patients, minor abnormalities can be detected in the resting electrocardiogram (ECG) (sinus bradycardia, prominent U waves, borderline QT interval prolongation). However, diagnosis can not be established before eliciting the typical pattern of arrhythmia (CPVT-related arrhythmias) (Fig. 1.1) which is triggered by exercise or emotional stress. During an exercise stress test the reproduction of bidirectional ventricular tachycardia (BVT), characterised by a beat-to-beat 180° rotation of the QRS axis, or polymorphic ventricular tachycardia (PVT) with a continuously varying QRS morphology is the key for diagnosis of CPVT (Liu, Colombi et al. 2007; Cerrone, Napolitano et al. 2009).
Figure 1.1: Exercise stress test in a catecholaminergic polymorphic ventricular tachycardia (CPVT). Runs of polymorphic ventricular tachycardia (PVT) are observed with a progressive worsening during exercise with QRS complexes of continuously varying morphology (Bruce Stage I & II). Typical bidirectional ventricular tachycardia (BVT) with the characteristic beat-to-beat 180° rotation of the QRS axis develops after 6 minutes of exercise (Bruce Stage III) at a sinus heart rate of approximately 120-130 beat per minute (bpm). Arrhythmias rapidly resolve in the recovery phase. Taken from (Napolitano and Priori 2004).

CPVT, also called familial polymorphic VT, exhibits a familial transmission in about 30% of cases (Leenhardt, Lucet et al. 1995; Liu, Colombi et al. 2007; Turakhia and Tseng 2007). CPVT has a heterogeneous genetic basis with mutations in the cardiac ryanodine receptor channel (RyR2) gene identified in 55% to 60% of the clinically affected patients. More than 70 different RyR2 mutations have been reported (Cerrone, Napolitano et al. 2009) responsible for CPVT1 (an autosomal dominant disease) (Katz, Arad et al. 2009). However, mutations in the cardiac calsequestrin gene (CSQ2) found in 1% to 2% of the patients (di Barletta, Viatchenko-Karpinski et al. 2006; Cerrone, Napolitano et al. 2009) are responsible for CPVT2 (an autosomal recessive disease) (Katz, Arad et al. 2009). CPVT has also been, also, reported in families with mutations
encoding the ankyrin B protein (Mohler, Schott et al. 2003; Mohler, Splawski et al. 2004; Turakhia and Tseng 2007).

Delayed afterdepolarizations (DADs) have been hypothesized as the mechanism of arrhythmias in CPVT since the bidirectional morphology of VT that is observed in CPVT patients is also the characteristic VT associated with intracellular calcium overload and the DADs seen during digitalis toxicity (Leenhardt, Lucet et al. 1995; Liu, Colombi et al. 2007; Liu, Ruan et al. 2008). Moreover, data from in vitro studies show that CPVT-associated RyR2 mutations and CSQ2 mutations produce “gain of function” and cause a Ca\(^{2+}\) “leakage” from the sarcoplasmic reticulum (SR) (Jiang, Xiao et al. 2002; George, Higgs et al. 2003; Lehnart, Wehrens et al. 2004; Liu, Ruan et al. 2008). In CPVT, this diastolic Ca\(^{2+}\) leak is not solely dictated by RyR2 and/or CSQ2 mutations; however, an increased SR Ca\(^{2+}\) content plays a crucial role and this is consistent with the clinical finding that arrhythmias in CPVT patients occur under stress where β-adrenergic stimulation increases the SR Ca\(^{2+}\) content to the threshold required for the development of the “Ca\(^{2+}\) leak” (Kashimura, Briston et al. 2010). The induced cytosolic calcium overload leads to activation of the sodium-calcium exchanger that can result in a net inward current (the so-called “transient inward”, ITi current) that underlies diastolic membrane depolarisations, DADs, that may be large enough to reach the threshold for sodium current activation and trigger premature ventricular beats and sustained tachyarrhythmias (Liu, Colombi et al. 2007; Liu, Ruan et al. 2008).

In order to understand how single mutations and how changes to the function of cardiac myocyte proteins can lead to such catastrophic events a brief overview describing the ultrastructure of striated muscles and the mechanisms governing muscle contraction and relaxation will be presented in the following sections.

1.1 Structural aspects of striated muscles

The sarcomere is the fundamental repeating contractile unit of striated muscle, for an overview see (Craig and Padron 2004). A characteristic feature of the sarcomere is the presence of thin and thick filaments composed of polymers of actin and myosin respectively. In addition to actin and myosin, there are at least 26 other different proteins. For example, proteins C, H and X are integral components of the thick
filaments binding to myosin. However, actin, the thin filament main protein, associates with a complex of troponin and tropomyosin which is the key to trigger the act of muscle contraction. Microscopically, as illustrated in Fig. 1.2, the sarcomeres have boundaries called Z-lines. The Z-line is composed mainly of α-actinin together with at least 10 other proteins, and can be described as a dark narrow line (~0.1µm) dividing a lighter area shared between adjacent sarcomeres known as I-band. At the centre of the sarcomere, next to I-band, is a dark area called the A-band, the middle part of which is occupied by a less dense H-zone. There is also a region termed the M-line, composed of myomesin, M-protein and creatine kinase.

Figure 1.2: Electron microscope image of sections of rabbit psoas muscle stained with uranyl acetate and lead citrate, 26,000 X. The image illustrates adjacent sarcomeres separated by Z-lines (Z). M-line (M) bisects the H-zone (H) which divides the A-band (A) occupying the centre of the sarcomere. The I-band (I) is the area shared between adjacent sarcomeres. These lines and bands are of varying densities and are different in their protein composition. Taken from (Stromer, Hartshorne et al. 1967).

The myofibrils of striated muscles are surrounded by an intracellular Ca\(^{2+}\) storage organelle, the sarcoplasmic reticulum (SR) that provides most of the Ca\(^{2+}\) needed for contraction. The SR is divided into segments containing a longitudinal part and a region termed the terminal cisternae. The principle SR membrane proteins are the SR calcium release channel (RyR) and the calcium pump (SERCA). Whereas RyR is localised to the terminal cisternae, SERCA is housed in the longitudinal SR. SERCA is
the most abundant protein in the SR membrane accounting for ~35-40% of total protein. Within the SR, Ca^{2+} is buffered by calreticulin, in the longitudinal SR, and by calsequestrin (CSQ) in the terminal cisternae (Ter Keurs and Boyden 2007; Katz, Arad et al. 2009). T tubules are transverse invaginations of the sarcolemma near the Z lines of the myofibrils housing a series of membrane proteins including the dihydropyridine-sensitive Ca^{2+} channels (DHPR) and Na^{+}/Ca^{2+} exchange proteins. Triads, dyads or peripheral couplings are the names of the intracellular junctions formed between the plasma membrane, or T tubules, and the SR. The nomenclature depends on the number and configuration of their membrane constituents. If the junction consists of two SR cisternae in contact with one T tubule, it is called a triad (see Fig. 1.3). Dyads consist of one SR cisternae in contact with one T tubule. However, SR cisternae make direct contact with the plasma membrane in peripheral coupling (Flucher and Franzini-Armstrong 1996).

Figure 1.3: (A): Cartoon of a skeletal muscle triad structure where two SR cisternae are in contact one T-tubule. The junctional feet (RyR) are in close proximity to DHPR which exhibits a tetrad organisation. Taken from (Flucher and Franzini-Armstrong 1996). (B): Cardiac muscle Ca^{2+} release units. Junctional SR, JSR, (containing CSQ and bearing feet (RyR)) associate either with the surface membrane (peripheral coupling) or membrane of t-tubule invagination (dyads). However, the corbular SR (also containing RyR and CSQ) does not associate with the cell membrane. Taken from (Ter Keurs and Boyden 2007).
There are important ultrastructural differences between the junctional SR in heart and skeletal muscle. The Ca\(^{2+}\) channels (DHPR), within the t-tubules of skeletal muscles, are organised into groups of four know as tetrads. The triadic junction of skeletal muscles shows a close association between tetrads and the cytoplasmic domain of ryanodine receptor (RyR) (junctional foot) (Fig. 1.3 A) so that there appears to be a DHPR tetrad for every two RyRs (Block, Imagawa et al. 1988; Wasserstrom 1998). In contrast, the cardiac muscle shows a higher ratio of RyR to Ca\(^{2+}\) channels (approaching 8:1) as well as a more random association between the two proteins (Bers and Stiffel 1993; Wasserstrom 1998). The well organised Ca\(^{2+}\) channels (arranged in tetrads) identified in skeletal muscle is not observed in the heart (Sun, Protasi et al. 1995; Wasserstrom 1998). This different anatomy of the calcium release units at the triadic junction between cardiac and skeletal muscles is consistent with the finding that the excitation contraction coupling (ECC) mechanism differs between the two muscle types.

1.2 Cardiac excitation-contraction coupling

Cardiac ECC is the mechanism by which electrical excitation of the myocyte is linked to contraction of the heart. Upon depolarisation of the plasma membrane of the cardiac myocyte during the action potential, DHPR (L-type voltage gated Ca\(^{2+}\)) channels rapidly open allowing extracellular Ca\(^{2+}\) to flow into the cell generating an inward Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) which contributes to the action potential plateau. The influx of Ca\(^{2+}\) into the cytosol triggers Ca\(^{2+}\) release from the junctional sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR) in a process known as “Ca\(^{2+}\)-induced Ca\(^{2+}\) release” (CICR) (Fig. 1.4) (Lamb 2000; Bers 2002). However, in skeletal muscle the structural arrangement of the DHPR and the RyR favours a direct physical coupling between the two proteins that mediates direct signal transduction of voltage-induced Ca\(^{2+}\) release (VICR) (Rios, Ma et al. 1991; Rios, Pizarro et al. 1992; Schneider 1994; Weisleder, Takeshima et al. 2008).
Figure 1.4: Schematic representation of cardiac excitation contraction coupling. Ca\(^{2+}\) entry into the cytosol via L-type voltage gated Ca\(^{2+}\) channel (DHPR) triggers Ca\(^{2+}\) release from junctional sarcoplasmic reticulum (SR) through ryanodine receptor (RyR). The resulting increase in the free cytosolic Ca\(^{2+}\) permits binding of Ca\(^{2+}\) to troponin C (a component of the thin filament). This triggers sliding of the myofilaments and muscle contraction. The reduction of the free cytosolic Ca\(^{2+}\) which is required for relaxation of the muscle is achieved, mainly, by the SR Ca\(^{2+}\)-ATPase pump (SERCA) the activity of which is regulated by phospholamban (PLB) and the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) which extrudes the Ca\(^{2+}\) from the cell. The plasmalemmal Ca\(^{2+}\)-ATPase (PMCA) and mitochondrial Ca\(^{2+}\) uniport play a minor role for removal of Ca\(^{2+}\) from the cytosol.

The elevation of free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\textsubscript{i}]) and the subsequent Ca\(^{2+}\) binding to the thin myofilament protein troponin C is the key event for muscle contraction (Bers 2002). The binding of Ca\(^{2+}\) to troponin C results in allostERIC modulation of tropomyosin relieving steric hindrance of myosin allowing movement of myosin heads along the actin filament and cross-bridge cycling. The contraction results from sliding of the filaments (the actin and myosin filaments) over each other producing an increased overlap of the filaments without shortening them. This sliding-filament
model has replaced the previous theory that contraction resulted from the large scale shortening (by some sort of internal folding) of actomyosin filaments which were believed to form a single set of continuous filament in each sarcomere (Craig and Padron 2004). In order for the muscle to relax, there must be a drop in \([\text{Ca}^{2+}]_i\) in order for \(\text{Ca}^{2+}\) to dissociate from from troponin. This occurs during the diastole with SR reuptake of \(\text{Ca}^{2+}\) by the SR \(\text{Ca}^{2+}\)-ATPase (SERCA) (accounting for removal of up to two thirds of cytosolic \(\text{Ca}^{2+}\)), the activity of which is regulated by phospholamban (PLB). The other three pathways responsible for \(\text{Ca}^{2+}\) transport out of the cytosol involve \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX) and to a lesser extent the plasmalemmal \(\text{Ca}^{2+}\)-ATPase (PMCA) and mitochondrial \(\text{Ca}^{2+}\) uniport (Bers 2002; Ter Keurs and Boyden 2007).

### 1.3 Ryanodine Receptor (RyR)

#### 1.3.1 RyR; molecular mass, topology, function & isoforms

The RyRs also referred to as the SR calcium release channels are so named after ryanodine, a plant alkaloid used as an insecticide, which was found to bind to the channel with high affinity and specificity. Ryanodine binding has been extensively exploited as a means to evaluate the functional state of the channel as it shows preferential interactions with the open state of the channel (Imagawa, Smith et al. 1987; Inui, Saito et al. 1987; Lai, Erickson et al. 1988; Chu, Diaz-Munoz et al. 1990; Needleman and Hamilton 1997; Fleischer 2008; Lanner, Georgiou et al. 2011). Ryanodine receptors are composed of four identical subunits (i.e. homotetramers) with a total molecular mass of ~2.3MDa; each subunit is ~565kDa comprising ~5000 amino acids (Marks, Tempst et al. 1989; Takeshima, Nishimura et al. 1989; Otsu, Willard et al. 1990). Hydropathy profiles of RyR have revealed that the N-terminal domain, which constitutes approximately four-fifths of the protein, is cytoplasmic, and the C-terminal domain corresponding to one-fifth forms the luminal and membrane spanning domains that anchor the protein in the terminal SR (Serysheva 2004; Rossi and Dirksen 2006).

Ryanodine receptors (SR-Ca\(^{2+}\) release channels) are considered the largest integral membrane channels mediating \(\text{Ca}^{2+}\) release from the SR intracellular store. In addition to \(\text{Ca}^{2+}\), to which a high conductance is exhibited, the channel can also conduct other divalent cations such as \(\text{Ba}^{2+}\) and \(\text{Mg}^{2+}\) as well as monovalent ions in the absence
of Ca\(^{2+}\). The SR-Ca\(^{2+}\) release channel has a lower selectivity for Ca\(^{2+}\) and 10-fold higher conductance when compared with the sarcolemmal Ca\(^{2+}\) channel (Ca\(_v\)1.2) under similar conditions. This high conductance permits rapid and substantial calcium release from the terminal SR during EC coupling (Rossi and Dirksen 2006; Ter Keurs and Boyden 2007). In addition to their crucial role in EC coupling in both skeletal and cardiac muscle, RyRs are part of signal transduction pathways in the nervous system and in osteoclasts where they participate in many physiological processes and are involved in apoptosis (Zaidi, Shankar et al. 1992; Chavis, Fagni et al. 1996; Schwab, Mouton et al. 2001; Lanner, Georgiou et al. 2011).

There are three isoforms of RyR that have been identified in mammals: RyR1, RyR2 and RyR3. RyR1, first detected and primarily expressed in skeletal muscles (Takeshima, Nishimura et al. 1989; Zorzato, Fujii et al. 1990), is encoded, in humans, by a gene located on chromosome 19q13.2 having 104 exons (Lanner, Georgiou et al. 2011). RyR2 is the predominant form of RyR in cardiac muscle in which it was identified (Nakai, Imagawa et al. 1990; Otsu, Willard et al. 1990). In humans, the gene encoding RyR2 is located on chromosome 1q43 and has 102 exons (Lanner, Georgiou et al. 2011). RyR3 is expressed, primarily, in the diaphragm (Jeyakumar, Copello et al. 1998), smooth muscle and brain (Furuichi, Furutama et al. 1994; Gianinni, Conti et al. 1995). The RyR3 gene with 103 exons is housed on chromosome 15q13.3-14 (Lanner, Georgiou et al. 2011). A high degree of homology exists among the three isoforms with an overall amino acid sequence identity of ~65%. The three RyR isoforms share more than 90% of homology in some regions; however, in the so-named divergent regions (D1-D3), the homology drops significantly. Regions D1, D2 and D3 span amino acids: 4254-4631, 1342-1403 and 1872-1923 respectively (with reference to RyR1 sequence); and 4210-4562, 1353-1397 and 1852-1890 respectively (with reference to RyR2 sequence). Isoform related functional differences may be explained by the presence of the divergent regions (Hamilton and Serysheva 2009; Lanner, Georgiou et al. 2011).
1.3.2 RyR; mechanism of channel activation

The domain-domain interaction concept was proposed by (Ikemoto and Yamamoto 2002) to explain the mechanism of RyR channel activation. Ikemoto and Yamamoto suggested that the N-terminal domain (~600 amino acid residues) and a region termed the central domain (~500 amino acid residues) facilitate “domain zipping” (i.e. close contact at several sub-domains) in the resting or non-activated state of RyR. The zipped configuration of these two domains imparts the conformational constraints which stabilize and maintain the closed state of the Ca^{2+} channel. In other words, the tight ‘zipping’ or association between the interacting domains serves as a stabilization mechanism. These critical inter-domain contacts are weakened on excitation contraction coupling resulting in loss of conformational constraints (domain unzipping) and thus lowering the energy barrier for Ca^{2+} channel opening (Fig. 1.5) (Yano 2008; Yano, Yamamoto et al. 2009; Uchinoumi, Yano et al. 2010). This model of domain-domain interaction does not only provide the mechanism of RyR channel opening and closing during excitation contraction coupling, but also may provide a possible explanation as to the “leaky” nature of the mutated RyR. Mutations of RyR (either isoform 1 or 2) result in hyperactivation of the Ca^{2+} channel and intriguingly many of these mutations (as discussed in the section below) are clustered in the N-terminal and central domains of RyR and thus may contribute to a weakening of the stabilizing domain-domain interaction.
1.3.3 RyR; mutations

RyR1 mutations have been linked to malignant hyperthermia (MH) and central core disease (CCD) (McCarthy, Quane et al. 2000). RyR2 mutations have also been identified in patients with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVC2) (Tiso, Stephan et al. 2001) or catecholaminergic polymorphic ventricular tachycardia (CPVT) (Priori, Napolitano et al. 2001). Interestingly, the mutations identified in RyR1 and RyR2 are not randomly located but are clustered into three specific hotspots as shown in Fig. 1.6; the N-terminal domain (amino acids 1-600), the central domain (amino acids 2,100-2,500) and the channel region (amino acids 4,100-5,000). Therefore, mutations in the N-terminal and central domains which are proposed (as indicated in the section above) to underlie channel opening during excitation contraction coupling may be responsible, at least in part, for defective RyR channels.
(Yamamoto, El-Hayek et al. 2000; Yano 2008; Uchinoumi, Yano et al. 2010). Furthermore, cryo-EM structural studies have revealed that the N-terminal and central mutation sites are close to each other in the three dimensional structure of the RyR (see Fig. 1.7) and so adds further support to the the zipping and unzipping concept proposed by Ikemeto and Yamamoto underlying the mechanism of RyR channel activation (Liu, Wang et al. 2005).

Mutations in the channel region are also described in the literature to alter the channel activity of RyR. For example, Zhao et al (Zhao, Li et al. 1999) have proposed a pore segment motif (amino acids 4820-4829; corresponding to mouse cardiac ryanodine receptor) and reported that single point mutations in this region greatly diminishes the open probability of the channel as shown by a reduction in $[^3]$H ryanodine binding and single channel conductance. Therefore, conservation of this motif seems to be crucial for RyR channel function, and so mutations in this segment are unlikely to contribute to the development of CPVT in which an increased RyR2 open probability is a feature as reported in previous studies (Jiang, Xiao et al. 2004). A question yet to be answered and of interest to this research project is whether RyR2 CPVT mutations alter the interaction of the receptor with luminal proteins (calsequestrin, triadin and junctin) which would then contribute to the increased RyR2 open probability. Lee et al (Lee, Rho et al. 2004) reported that single mutations of the second intraluminal loop of RyR1 (amino acids 4860-4917) interfere with a direct interaction between the ryanodine receptor and triadin. Another group (Goonasekera, Beard et al. 2007) showed that a combination of these mutations is required for reducing triadin binding to RyR. However, similar research conducted with the cardiac isoform of the ryanodine receptor is still required.
Figure 1.6: Schematic diagram showing some MH, CCD mutation sites identified in RyR1 & ARVC2, CPVT mutation sites in RyR2. The figure illustrates that there are 3 hotspot regions (N-terminal domain, amino acids 0-600; central domain, amino acids 2,100-2,500; channel region, amino acids 4,100-5,000) and allows comparison of the RyR1&2 mutations found in these regions. ARVC2, arrhythmogenic right ventricular cardiomyopathy type 2; CPVT, catecholaminergic polymorphic ventricular tachycardia; MH, malignant hyperthermia; CCD, central core disease. There are a large number of mutations in both the N-terminal and central domain sites that may alter the “Domain-Domain” zipping that is proposed to stabilize the receptor (Ikemoto and Yamamoto 2002). Adapted from (Yano 2008).

1.3.4 RyR; structure & arrangement

1.3.4.1 Structure of RyR

To date the principal structural data pertaining to the architecture of the RyR isoforms have been delivered by cryo-electron microscopy (cryo-EM) methods. Due to the large size and highly flexible nature of the cytoplasmic domain the RyR has not been, to date, amenable to study by techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) which have the potential to yield structural data at higher resolution than electron microscopy methods.

The first 3D structure of RyR1 was published some 20 years ago (Radermacher, Wagenknecht et al. 1992) from cryo-EM and single particle analysis methods.
then many further studies of the RyR structure have been conducted using similar approaches. The majority of these studies have focused upon the skeletal muscle isoform, RyR1, probably because of its higher expression level and the relative ease of purification from skeletal muscle. However, there are some reports describing the 3D structure of the cardiac isoform, RyR2 e.g. (Sharma, Penczek et al. 1998; Liu, Zhang et al. 2002) and the brain isoform, RyR3 e.g. (Sharma, Jeyakumar et al. 2000; Liu, Zhang et al. 2001) but at lower resolutions than those for RyR1. Consistent with the high sequence identity (~65%), the overall 3D molecular architecture for each of the RyR isoforms has been shown to be very similar at medium resolution. However, discrete structural differences have been identified between the isoforms which are likely to account for the specialised function of each isoform (Lanner, Georgiou et al. 2011).

RyRs assemble to form homotetramers with cryo-EM revealing that they adopt a mushroom shaped complex composed of two principal domains; a cytoplasmic and transmembrane (TM) assembly; reviews can be found in (Radermacher, Rao et al. 1994; Serysheva 2004; Lanner, Georgiou et al. 2011). As can be seen in the cartoon in Fig. 1.7 A, the cytoplasmic view of the RyR homotetramer reveals the square-shaped organization of the four monomer units, with four distinct regions at the corners of the cytoplasmic assembly, termed the “clamp” domains which are connected through the “handle” domains surrounding the central rim. Rotation of this view around the x-axis shows the side view of the RyR tetramer (i.e. perpendicular to the membrane plane) illustrating the “column” region connecting the cytoplasmic region with the much smaller transmembrane segment. Panel B of the figure shows the EM volume for RyR1 (Serysheva, Ludtke et al. 2008) at 9.6 Å resolution determined using cryo-EM and single particle analysis providing details of the tetramer structure. As can be seen from Fig. 1.7 B (I), the cytoplasmic assembly is a square-shaped assembly with overall dimensions of 280×280×120 Å. The cytoplasmic assembly, extending into the gap between the SR membrane and the t-tubule, is very complex with cavities and microstructures that facilitate interactions with small molecules and protein modulators. The TM region is smaller than the cytoplasmic assembly and is a square prism with overall dimensions of 120×120×60 Å constituting approximately one-fifth of the channel. The membrane part is localised to the carboxy terminal of the protein and forms the ion
conducting pore. The transmembrane domain is proposed to traverse the SR membrane permitting the distal portion to protrude into the lumen of the SR as shown in Fig. 1.7 B (II). This luminal part is believed to contribute to the formation of a quaternary luminal \( \text{Ca}^{2+} \) sensor complex, which is of particular interest to this research project, with accessory proteins calsequestrin, triadin and junctin. Panel (III) of the figure shows the luminal view of the complex with the dashed circle highlighting the luminal portion of RyR.

**Figure 1.7:** (A): Schematic drawing of Ryanodine Receptor (RyR); (I): Cytoplasmic view, (II): Side view. The drawing shows the tetrameric organization of the RyR homo-oligomer and structural features. (B): RyR1 3D structure (cryo-EM reconstruction) at 9.6 Å resolution (Serysheva, Ludtke et al. 2008). The EM map (accession no. 1275) was downloaded from European Molecular Biology Laboratory European Bioinformatics Institute database (http://www.ebi.ac.uk/pdbe-srv/emsearch/atlas/1275_summary.html). The map was modelled using the UCSF Chimera software (Pettersen, Goddard et al. 2004) to illustrate the different orientations and structural features of RyR1; (I): Cytoplasmic View, (II): Side view, (III): Luminal view. These orientations show that RyR1 is composed of a large cytoplasmic assembly (~ 29 x 29nm). The cytoplasmic assembly is composed of “clamp” regions connected through “handle” domains surrounding the “central rim”. These structural units / features are common to all RyR isoforms. The dashed circle represents the luminal portion of RyR which is proposed to form a quaternary complex with triadin, junctin and calsequestrin. Scale bar, 10 nm.
A subnanometer resolution 3D structure of RyR1 has also been achieved by another group (Samso, Wagenknecht et al. 2005) allowing delineation of secondary structure elements in various regions. Serysheva et al have identified 36 α-helices with various orientations and seven β-sheets within the cytoplasmic assembly of each RyR1 subunit (Fig. 1.8) (Serysheva, Ludtke et al. 2008).

Figure 1.8: RyR1 secondary structure elements in the closed state (9.6 Å resolution). The resolution of this EM volume allowed secondary structure elements to be mapped to the densities using SSEHunter, a feature detection program. Only two RyR1 monomer units from the channel homotetramer are shown perpendicular to the membrane plane for clarity. The α-helices are illustrated as cylinders coloured according to their location in the map; red (TM assembly), green (central region of cytoplasmic assembly), purple (clamp domains) and cyan (column). β-sheets are shown in orange. The cytoplasmic assembly contains 36 α-helices with different orientations and 7 β-sheets. Six α-helices are illustrated within the structure of the transmembrane part. The asterisk (*) highlights the “inner helix” proposed to be involved in channel gating by Samso and co-workers (Samso, Feng et al. 2009). Adapted from (Serysheva, Ludtke et al. 2008).

Cryo-EM has also been successfully employed to capture the different functional states of channels such as RyRs. For example, in 1999, Hamilton and co-workers calculated the 3D volume of RyR1 in the open and closed states (at 30Å resolution)
permitting conformational differences to be identified. Conformational changes to the “clamp” subdomains as well as a pronounced mass depletion from the centre of the TM region was reported when the RyRs were imaged as open channels (Serysheva, Schatz et al. 1999). Later, Samso et al (Samso, Feng et al. 2009) published a cryo-EM three dimensional reconstruction of RyR1 (open and closed states) at a nominal resolution of ~10 Å. This high resolution enabled them to identify rod-like densities suggestive of α-helical and β-sheet structures. Samso’s group described a gating mechanism for RyR1 in which three densities, given the names: inner helix, h1 and inner branch, play a crucial role by forming a mobile axial structure (see Fig. 1.9). The inner helices (which were also identified by Serysheva and co-workers (Serysheva, Ludtke et al. 2008) and indicated by “*” in Fig. 1.8) are four transmembrane densities (one contributed by each monomer) forming a tepee-like structure framing the ion pore. The h1 densities are structures existing on the cytosolic side of the SR membrane and surrounding the putative ion gate. The inner branches are rod-like structures located on the cytoplasmic side of this ion gate. The proposed gating mechanism by Samso and co-workers can be summarized as follows. In the closed state, the inner helices form a bundle tightening towards the cytosol; however, in the open state, these inner helices are kinked so that the lower halves (nearer to the lumen) become more horizontal in relation to the plane of the SR membrane with the upper portions bent outwards away from each other. Secondly, the inner branches, which are associated with each other in the closed state of the channel, become separated on the cytoplasmic side of the SR membrane and acquire a more tilted position resulting in a larger diameter of the ion gate in the RyR open conformation. Finally, upon the transition from the closed to open states, the h1 densities move outwards and thus contribute to the increased diameter of the ion gate in the open state. A schematic diagram showing the proposed movement of the helices between the open and closed state of the channel is shown in Fig. 1.9.
Figure 1.9: Schematic diagram representing the gating mechanism of RyR1 channel based on the model proposed by Samso and co-workers (Samso, Feng et al. 2009). Illustrated is two of the four subunits of RyR1 (side view). In the transition from the closed state (left panel) to the open state (right panel), the following events occur. Firstly, the inner helices, which are transmembrane structures lining the pore of the channel (indicated by the dashed circle), are kinked. Secondly, the h1 densities, which are additional structures lying on the cytosolic side of the SR membrane and surrounding the ion gate (indicated by the dashed rectangle), move outwards. Thirdly, the inner branches, which are densities existing on the cytoplasmic side of the ion gate, separate and become more tilted. These events result in an increased diameter of the ion gate and permit cytosolic Ca\(^{2+}\) efflux.

Furthermore, Samso and co-workers found that the inner helices of closed-state RyR1 resemble closed K\(^{+}\) channels rather than open K\(^{+}\) channels; and the reverse is true regarding the inner helices of the open-state RyR1. In contrast, another group, with a subnanometer resolution of cryo-EM reconstruction model of closed RyR, has docked a kinked helix in the pore lining region of closed RyR suggesting structural similarity to the open K\(^{+}\) channel whereby kinking of the pore-lining helix underlies the mechanism of channel opening, and this might indicate that kinking of the inner helix does not produce a sufficient conformational change to cause RyR channel opening (Ludtke, Serysheva et al. 2005).

Clearly, although the EM volumes have been fundamental for advancing the knowledge of RyR structure and have provided hints as to how the channel opens and
closes, atomic resolution data is required for a full understanding. However, cryo-EM and single particle analysis have been also employed to examine and to map binding of small accessory proteins to RyR, e.g. FKBP 12.6 & 12 (Fig. 1.10 A) (Wagenknecht, Grassucci et al. 1996; Samso, Shen et al. 2006; Sharma, Jeyakumar et al. 2006), calmodulin (Fig. 1.10 B) (Wagenknecht, Berkowitz et al. 1994; Samso and Wagenknecht 2002), the chloride intracellular channel 2 (CLIC2) (Meng, Wang et al. 2009) as well as to localise the RyR divergent regions (D1-D3) (Liu, Zhang et al. 2002; Zhang, Liu et al. 2003; Liu, Zhang et al. 2004), oxidoreductase-like domain (Baker, Serysheva et al. 2002), phosphoryration sites (Ser2030 and Ser2808) (Meng, Xiao et al. 2007; Jones, Meng et al. 2008) and disease-associated mutation sites (Liu, Wang et al. 2005; Wang, Chen et al. 2007).

Figure 1.10: Cryo-EM & SPA employment for location of binding of different ligands to RyR1. Panel (A) shows location of binding of FKBP12. Panel (B) shows how interaction of Ca\(^{2+}\) with calmodulin (CaM) results in a shift of the binding site on RyR1; apoCaM and Ca\(^{2+}\)-CaM bind to different areas on RyR1 with a region of overlap. Taken from (Liu and Wagenknecht 2005).

1.3.4.2 Arrangement of RyRs

Although the majority of the structural data pertaining to RyRs has been from single particle analysis of individual channels, Lai and co-workers (Yin, Blayney et al. 2005) have, also, reported the formation of two dimensional (2D) crystals of RyR1 and image processing of negatively stained 2D crystals provided structural evidence for an intimate physical coupling between RyRs. Images of the 2D crystals show a “checkboard-like” (Fig. 1.11) configuration rather than a higher density “side-by-side” model as an array for native RyRs. These results supported a previous suggestion of “coupled gating” between RyRs (Marx, Gaburjakova et al. 2001) and the theory of
intermolecular allosteric behaviour within a receptor lattice, which states that conformational changes spread from an initial point by communication between physically associated receptors (Bray and Duke 2004).

Figure 1.11: The native checkboard-like array model of physical coupling between RyRs. The 2D lattice constants are: \(a=b=31.0\) nm (centre to centre distance between adjacent particles) and the skew angle (relative to lattice vector) \(=62.5^\circ\). The numbering of the cytoplasmic domains correspond to regions of the three-dimensional reconstruction carried out by Radermacher and co-workers (Radermacher, Rao et al. 1994). These putative domains were defined on the basis of their distinctive globular shapes. The designation of these domains is dependent on the achieved resolution; for example, with higher resolution some of these domains might appear to be multidomain structure. The illustrated “P” (dashed circle) corresponds to a globular density mass in the centre of the channel which is referred to, by Radermacher and co-workers, as the “channel plug”. The figure illustrates that it is the cytoplasmic assembly that mediates crystal contacts and in particular the domain in the clamp region, numbered 6, indicated by the dashed box. Adapted from (Yin, Blayney et al. 2005).
1.3.4.3 Crystal structure of RyR

Although there are no atomic resolution structures for the full-length RyR homotetramer, Amador et al (Amador, Liu et al. 2009) have published the crystal structure of the amino-terminal domain (residues 1-210) of rabbit RyR1 (RyR1\textsubscript{NTD}). This domain is a possible location for 11 mutations. These 11 mutations are from 30 identified as responsible for MH and CCD and existing in the 600 amino-acid N-terminal domain (some of these mutations are illustrated above in Fig. 1.6). The structure reveals that RyR1\textsubscript{NTD} adopts a β-trefoil structure consisting of 3 trefoils coming together to form a barrel and cap and that each trefoil contains 4 β-strands: 2 for the cap and 2 for the barrel (Fig. 1.12). Additionally, an α-helix containing domain called the “arm domain” was found protruding from the β-trefoil, with a mutation site forming a hot spot loop (HS-loop). This loop extends between β8 and β9 and is a potential site of 6 mutations detected in MH and CCD patients. Amador and co-workers also investigated the structural effects of 3 MH-linked mutations (C36R, R164C and R178C in the rabbit sequence which correspond to C35R, R163C and R177C in the human sequence) revealing that they had no real impact on the structure stability and integrity. Another group (Lobo and Van Petegem 2009) has reported an overall similarity between the crystal structures of RyR1\textsubscript{NTD} and RyR2\textsubscript{NTD}, however a number of differences exist in the loops connecting the various β-strands. A major difference is a long loop connecting the α-helix to β5. This loop lacks a well-defined electron density suggesting that the loop has a high degree of flexibility; however, a unique feature in the loop of RyR2 is the single tryptophan residue (W98), which packs in a hydrophobic pocket lined by the conserved L78 and W159 residues next to the α-helix (Fig. 1.13). Lobo and Van Petegem have also investigated A77V, V186M and Δexon3 RyR2\textsubscript{NTD} disease mutants in terms of impact upon structure. These mutations are linked to CPVT and ARVD2. The deletion mutation results in drastic structural changes in the form of the removal of the 3\textsuperscript{10} helix, β4, the α helix and 7 residues of the next flexible loop. However, the point mutations result only in a change to the local environment without altering either the overall structure or the stability of the domain. Therefore, the effects of these point mutations can be explained by a change to the local surface of the N-terminal domain abolishing the domain-zipping mechanism (as described in section
1.3.2) with the central domain which is proposed as crucial for normal channel function (Ikemoto and Yamamoto 2002; Lobo and Van Petegem 2009).

Figure 1.12: Crystal structure of rabbit RyR1\textsubscript{NTD} (residues 1-210) (ribbon diagram). Illustrated is a β-trefoil structure divided into a barrel (blue strands) and a cap (green strands) where each trefoil has 4 β-strands; 2 related to the barrel and 2 related to the cap. The arm domain is a short protrusion between β4 and β5; however the hot spot loop (HS-loop) extends between β8 and β9. Missing residues are represented by dotted lines. In the right panel is the top-down view. “N” and “C” indicate the positions of amino- and carboxy termini respectively. Taken from (Amador, Liu et al. 2009).

Figure 1.13: Crystal structure of mouse RyR2\textsubscript{NTD} (residues 1-217) (ribbon diagram). The overall similarity with RyR1\textsubscript{NTD} is clear. The illustrated views show helices (α1 and $3_{10}h1$) in red, β strands in blue, and loops in white. Dotted lines represent the non-modelled part of the structure. The stick representation in α1-β5 loop is of W98. “N” and “C” indicate the positions of amino- and carboxy termini respectively. Taken from (Lobo and Van Petegem 2009).
1.3.5 Regulation of RyR

As alluded to above, the ryanodine receptor activity is also modulated by the binding of a number of cytosolic (e.g. calmodulin, FK-binding proteins and sorcin) and luminal (e.g. calsequestrin, triadin and junctin) accessory proteins as well as Ca\(^{2+}\), Mg\(^{2+}\), nucleotides, reactive oxygen species, reactive nitrogen species and protein kinase A (PKA) and calmodulin kinase II (CaMKII) phosphorylation (Fig. 1.14). Extensive reviews can be found in (Bers 2004; Lanner, Georgiou et al. 2011).

![Schematic drawing showing the cardiac ryanodine receptor (RyR2) as a scaffolding protein interacting with various modulators. Ca,1.2, the cardiac voltage gated Ca channel, is not in direct contact with RyR2 as excitation contraction coupling in the cardiac muscle is mediated by calcium induced calcium release (CICR). FK-binding protein (FKBP), CaM (calmodulin), protein kinase A (PKA) and calmodulin kinase II (CaMKII) regulate RyR through binding to the cytoplasmic region. However, triadin, junctin and calsequestrin (CSQ) modulate the activity of RyR by binding to its luminal side.](image-url)
As illustrated in the cartoon above, the large cytoplasmic region of RyR is a scaffold assembly binding many protein modulators; however the small segment extending into the SR lumen also binds accessory proteins. This chapter will focus on those luminal auxiliary proteins which are relevant to this research project.

1.4 Calsequestrin (CSQ)

It is perhaps surprising that a large fraction of the Ca\(^{2+}\) that triggers muscle cell contraction is stored in and released from the sarcoplasmic reticulum which has a small luminal volume compared to the extracellular space. The amount of intra-luminal Ca\(^{2+}\) that could be accumulated would be very small without an appropriate luminal Ca\(^{2+}\) buffer. Moreover, the amount of Ca\(^{2+}\) accumulated would compromise the ability of the Ca\(^{2+}\) release mechanism to deliver the signal Ca\(^{2+}\) to the cytoplasm in sufficient quantity and in a sufficiently short time (MacLennan, Abu-Abed et al. 2002). Calsequestrin (CSQ) is a soluble Ca\(^{2+}\) binding protein, ~46kDa. Two isoforms of calsequestrin have been identified; skeletal (CSQ1) and cardiac (CSQ2) which are encoded by two different genes and but share high sequence homology; cardiac and skeletal human CSQ have 84% sequence homology. High sequence homology between species, also, exists, for example, a 98% homology is revealed by sequence alignment between rabbit and human skeletal CSQ (Beard, Laver et al. 2004).

Wang and co-workers (Wang, Trumble et al. 1998) have determined the crystal structure of rabbit skeletal calsequestrin revealing that the monomeric molecule of calsequestrin is composed of three almost identical domains each with arrangement of secondary structural elements that closely resemble thioredoxin, a ubiquitous redox protein. The individual domain has a core of five-stranded mixed β-sheet, flanked by four α-helices, two on each side of the sheet. Kim and co-workers (Kim, Youn et al. 2007) published a similar overall structure for human CSQ2 (hCSQ2) which is of particular interest to this research project. The crystal structure of an hCSQ2 monomer is shown in Fig. 1.15 with these domains depicted in red, green and blue. The C- and N-termini of the protein (disordered) were not crystallised.
CSQ buffers Ca\(^{2+}\) in the terminal SR in order to maintain free Ca\(^{2+}\) concentration at levels around 1mM. Despite having a low affinity for Ca\(^{2+}\) (\(K_d = 40 \mu M\)), CSQ2 has a high Ca\(^{2+}\) binding capacity (\(~40 \text{ mol Ca}^{2+} / \text{ mol protein at pH 7.5}\)) so that is able to sequester Ca\(^{2+}\) within SR that leads to the name calsequestrin (MacLennan and Wong 2007).
CSQ is a highly acidic protein with acidic residues accounting for 33%-46% of residues within the calsequestrin monomer forming at least 21 clusters of doublets or triplets which can potentially bind Ca\textsuperscript{2+}. The C-terminus contains the highest surface density of negative charge (Fig. 1.16), and is thought to be responsible for ~26% of the Ca\textsuperscript{2+}-binding sites. CSQ2 has more acidic residues in its C-terminal region than CSQ1. However, despite the longer acidic C-terminal tail in the cardiac isoform, the number of calcium ions bound to skeletal calsequestrin is approximately double the number bound to cardiac calsequestrin (Beard, Laver et al. 2004).

![Figure 1.16: The electrostatic potential of the molecular surface of calsequestrin; (a) the front and (b) the back. The surface rendering is where red is negative, blue is positive and white is uncharged or hydrophobic. Domain III, containing the C-terminus, shows the highest electronegative surface potential. All of the electronegative patches indicate the Asp/Glu patches and are potential Ca\textsuperscript{2+} binding sites. Taken from (Beard, Laver et al. 2004).]

The presence of a high concentration of CSQ, in the lumen of the junctional terminal cisternae of the sarcoplasmic reticulum, comprising ~27% of all junctional SR proteins, provides an understanding of the efficient Ca\textsuperscript{2+} release mechanism despite the
small luminal volume of sarcoplasmic reticulum (MacLennan and Wong 1971; Campbell, MacLennan et al. 1983).

Wang et al (Wang, Trumble et al. 1998) have also suggested two types of dimers that are likely to facilitate calsequestrin polymerisation. A “back-to-back” interaction, through the C-terminal segments, forms the first type of dimer, however, a “front-to-front” contact, involving the N-termini, forms the second type. Both types of calsequestrin dimers generate negatively charged pockets that favour Ca\(^{2+}\) binding. It is proposed that Ca\(^{2+}\) binding likely creates cross-bridges that stabilize the dimer interface.

Park and co-workers (Park, Wu et al. 2003) have proposed that the relationship between Ca\(^{2+}\) and calsequestrin is an interplay among protein folding, Ca\(^{2+}\) binding and calsequestrin polymerisation (Fig. 1.17). They report that Ca\(^{2+}\) promotes the folding and polymerisation of calsequestrin. It has been proposed that with an increase in Ca\(^{2+}\) concentration, “front-to-front” interaction occurs first since domain I containing the N-terminus has fewer charged amino acid residues; however, a further increase in Ca\(^{2+}\) concentration is needed for “back-to-back” interaction in order to shield the C-terminal region within domain III which is richer in charged amino acid residues. A highly charged surface onto which Ca\(^{2+}\) can be adsorbed is provided by the growing calsequestrin polymer. An extended surface would have an attractive force with a longer range than that of an isolated molecule. Ca\(^{2+}\) dissociation and diffusion will also be more rapid with a linear structure (MacLennan, Abu-Abed et al. 2002; Park, Wu et al. 2003).
Figure 1.17: The model proposed by (Park, Wu et al. 2003) for calsequestrin folding and polymerization. (A): Calsequestrin, in the absence of ionic strength, exists in an unfolded state due to charge repulsion. (B): Calsequestrin domains begin to fold as the ionic strength is increased. (C): Calsequestrin is fully folded and the three domains come together as the charge repulsion is shielded. (D): “Front-to-front” interaction occurs first with a further increase in the divalent cation concentration since the N-termini has fewer charged amino acid residues. (E): “Back-to-back” interaction occurs last since more divalent cations are needed to shield the C-terminal region (richer in acidic amino acid residues). (F): A linear polymer of calsequestrin is finally formed with the “front-to-front” and “back-to-back” interactions.

CSQ1 and CSQ2 might differ in their polymerisation tendency. Dulhunty and co-workers have reported that CSQ2 is not significantly polymerized when physiological concentrations (100µM) of the monomer were exposed to a solution of ionic strength with 1mM Ca^{2+} (Wei, Hanna et al. 2009).

1.5 Triadin & Junctin

Triadin and junctin are integral membrane proteins that span the SR membrane and share structural and amino acid sequence similarity. At the junctional SR membrane in cardiac and skeletal muscle, triadin and junctin have been shown to co-localise with
the ryanodine receptor and calsequestrin (Guo and Campbell 1995; Zhang, Kelley et al. 1997).

The skeletal and cardiac isoforms of triadin are splice variants of one gene. The skeletal muscle triadin (95kDa) contains 706 amino acids, 47 of which comprise the N-terminal cytoplasmic part of triadin, with the following 20 residues forming a single transmembrane α-helix, and the remaining bulk of the protein is located within the SR lumen. Triadin has been reported to self-associate and oligomerise through disulphide links involving two luminal cysteine residues, Cys$^{270}$ and Cys$^{671}$ (Beard, Laver et al. 2004). Guo and co-workers (Guo, Jorgensen et al. 1996) have identified three cardiac isoforms of triadin; 92kDa (slightly smaller than the skeletal counterpart), 35kDa, and 40kDa. However, Kobayashi et al demonstrated that the band observed at 40kDa was in fact the glycosylated form of the 35kDa triadin (Kobayashi and Jones 1999).

Junctin (26kDa), in contrast to triadin, is identically expressed in skeletal and cardiac muscle, is not glycosylated, and doesn’t form disulphide linked oligomers (the protein doesn’t contain cysteine residues). Junctin is composed of a short 21 amino acid cytoplasmic N-terminus, a single transmembrane domain and a relatively long highly charged luminal C-terminal tail (Beard, Laver et al. 2004).

The multiple Lys-Glu-Lys-Glu (KEKE) motifs shared by the luminal portions of triadin and junctin are required for their specific interactions with each other, as well as with other junctional SR proteins that contain a high density of charged residues (Zhang, Kelley et al. 1997).

1.6 The formation of a luminal Ca-sensor

Termination of CICR is an outstanding question in the field of cardiac EC coupling. Terentyev and co-workers (Terentyev, Viatchenko-Karpinski et al. 2002) have shown that termination of SR Ca$^{2+}$ release and the subsequent restitution behaviour, i.e., “refractoriness,” of Ca$^{2+}$ release units in cardiac muscle is controlled by intra-SR, “luminal”, Ca$^{2+}$ regulating RyR opening. Abnormal regulation of RyRs by luminal calcium is associated with “diastolic Ca$^{2+}$ leak” which has a well-established relationship to various cardiac disease states, including Ca$^{2+}$-dependent arrhythmia and
heart failure (Gyorke, Gyorke et al. 2002; Gyorke and Terentyev 2008). Another question regarding EC coupling is the mechanism of “luminal Ca\(^{2+}\) transduction” to the RyR. It is proposed that the effects of luminal Ca\(^{2+}\) on RyR are mediated by the luminal proteins calsequestrin, triadin and junctin. There is a growing body of evidence that points to a luminal Ca\(^{2+}\) transduction machine (luminal Ca-sensor) formed by a quaternary complex composed of ryanodine receptor, calsequestrin, triadin and junctin (Zhang, Kelley et al. 1997; Beard, Wei et al. 2009). Calsequestrin is proposed to not only buffer Ca\(^{2+}\) but additionally act as a luminal Ca\(^{2+}\) sensor for RyR complex serving as an active regulator of the Ca\(^{2+}\) release process (Gyorke and Terentyev 2008; Beard, Wei et al. 2009). Triadin and junctin are also believed to play an integral part in the Ca\(^{2+}\) release process rather than just anchoring calsequestrin to the junctional face membrane and ryanodine receptor (Beard, Laver et al. 2004; Gyorke and Terentyev 2008; Beard, Wei et al. 2009).

1.7 Aims and Objectives

The overall aim of this thesis research was to investigate the assembly of the ryanodine receptor with calsequestrin and triadin to form a luminal Ca\(^{2+}\) sensor.

Aim 1: One of the primary goals was to purify both the cardiac and skeletal muscle RyRs since this protein forms the hub of the Ca\(^{2+}\) sensing complex. Chapter 3 details the development of the purification protocols for cardiac and skeletal ryanodine receptors (RyR1 and RyR2) from bovine ventricles and sheep skeletal muscle, respectively, as well as characterization of the purified proteins.

Aim 2: The subsequent aim was to purify calsequestrin (CSQ). This was achieved by expressing human cardiac CSQ2 recombinantly in \textit{E.Coli} as described in Chapter 4. Attempts were made to also express and purify mouse triadin isoform 1 (Trd1) however, this was unsuccessful and led to the use of a synthetic peptide corresponding to the region within Trd that has been shown to interact with both RyRs and CSQ isoforms 1 and 2.

Aim 3: To examine the association of RyRs with CSQ2 and the Trd peptide. Utilizing a range of biochemical, biophysical and imaging methods protein-protein
interactions were explored as described in Chapters 5 & 6. Experiments were also conducted in the presence and absence of calcium which is predicted to change the conformation of CSQ.

The data in this thesis work describes the association of the RyRs with CSQ2 and a domain of Trd. In addition, by the application of a functional assay, that is correlated to the open probability of the channel, these data are interrogated in the context of RyR function and hence have provided new insights into how a luminal calcium sensor may assemble and influence RyR activity.
Chapter 2: Materials & Methods

2.1 Purification of cardiac and skeletal ryanodine receptors, RyR1 and RyR2

Several different methodologies were explored for purification of both RyR2 and RyR1 to homogeneity suitable for structural studies and represent a considerable component of this thesis research work. For both tissue types the first stage involved subcellular fractionation, followed by solubilisation of the protein away from the membrane bilayer with a series of purification steps to yield pure RyR.

2.1.1 Isolation of heavy sarcoplasmic reticulum (SR) from bovine hearts

SR membrane vesicles were isolated according to the method of Sitsapesan (Sitsapesan and Williams 1990). The whole procedure was performed at 4°C. Approximately 200g of minced bovine heart was defrosted overnight in the cold room prior to homogenisation (3x30 seconds, allowing to stand for 1-2 minutes) using a typical domestic kitchen blender (Moulinex® Optiblend 2000) in a solution containing 1mM phenylmethyl-sulphonide fluoride (PMSF), 1µM pepstatin A, 1µM E64, 1mM benzamidine, 300mM sucrose and 20mM potassium piperazine-N’N’-bis-2-ethanesulphonic acid (PIPES), pH 7.4. The homogenate was centrifuged for 20 min at 9200 xg in a Sorvall® SLA-1500 rotor. The pellet was discarded; and the supernatant was centrifuged in a Sorvall T-865 rotor at 91,078 xg for 60 min. This step sedimented the mixed membrane population which was then resuspended in a solution containing 400mM KCl, 0.5mM MgCl2, 0.5mM CaCl2, 0.5mM EGTA, 25mM PIPES, pH7.0, complete mini EDTA-Free protease inhibitors cocktail tablets plus 10% (w/v) Sucrose. The membrane suspension was layered onto sucrose gradient of 20, 30 and 40% (w/v) sucrose steps and was subjected to centrifugation for 120 minutes at 113,246 xg in a Sorvall® Surespin 630 rotor. The 30-40% interface containing junctional SR membrane vesicles was collected and diluted in 400 mM KCl. The diluent was then centrifuged for 60 min at 78,702 xg in a Sorvall® T-865 rotor and the pellet was resuspended in a solution containing 400 mM sucrose, 5mM N’-2-hydroxyethylpiperazine-N’-2-ethanesuphonic acid (HEPES), pH 7.2, complete mini EDTA-Free protease inhibitors cocktail tablets, quick frozen in liquid nitrogen and stored at -80°C.
2.1.2 Isolation of sarcoplasmic reticulum junctional terminal cisternae (JTC) from sheep skeletal muscle (Chu, Dixon et al. 1988; Meng, Wang et al. 2009)

Approximately 200 grams of sheep skeletal muscles were homogenised (3x30 seconds, allowing to stand for 1-2 minutes) using a typical domestic kitchen blender (Moulinex® Optiblend 2000) in 1L of buffer containing 0.3M sucrose, 10mM Hepes, pH 7.0, 0.5mM EDTA, 2mM PMSF, 1µM pepstatin A, 1µM E64, 1mM benzamidine. The homogenate was centrifuged for 15 minutes at 11,000 xg in a Sorvall® SLA-1500 rotor. The pellet was discarded; and the supernatant was centrifuged in a Sorvall® T-865 rotor at 110,000 xg for 60 minutes. This step sedimented the mixed membrane population which was resuspended in 200 ml of solution containing 0.65M KCl, 0.3M sucrose, 10mM Hepes, pH 7.0, 0.5mM EDTA, 2mM PMSF, 1µM pepstatin A, 1µM E64, 1mM benzamidine. The resuspension was stirred in the cold room for 60 minutes and then re-pelleted by centrifugation in a Sorvall® T-865 at 200,000 xg for 60 minutes. This was followed by re-homogenisation in 30ml of a buffer containing 0.65M KCl, 0.3M sucrose, 10mM Hepes, pH 7.0, 0.5mM EDTA and complete mini EDTA-Free protease inhibitors cocktail tablets. The re-homogenate was layered onto a sucrose gradient of 45%, 38%, 32% and 27% (w/v) sucrose steps and was centrifuged at 100,000 xg in Sorvall® Surespin 630 rotor for 16 hours. The 38-45% interface containing sarcoplasmic reticulum junctional terminal cisternae (JTC) was collected, diluted twofold with buffer containing 0.3M sucrose, 10mM Hepes, pH 7.0, 0.5mM EDTA, 2mM PMSF, 1µM pepstatin A, 1µM E64, 1mM benzamidine and re-centrifuged in Sorvall® T-865 at 110,000 for 60 minutes. The pellets were resuspended in buffer containing 0.3M sucrose, 10mM Hepes, pH 7.0, and complete mini EDTA-Free protease inhibitors cocktail tablets, quick frozen in liquid nitrogen and stored at -80°C.

2.1.3 Isolation of skeletal & cardiac junctional face membrane (JFM)

A method described by (Costello, Chadwick et al. 1986) was followed with some modifications., The isolated skeletal and cardiac SR (~ 6mg protein) was adjusted to 3.3mg protein/ml and 1mM CaCl₂ using a buffer containing 0.3M sucrose, 10mM Hepes, pH 7.0, and complete mini EDTA-Free protease inhibitors cocktail tablets and then kept on ice for 10 minutes. This was followed by the addition of TX-100 for a final concentration of 0.5% (v/v). The sample was vortexed and left on ice for 20 minutes and
then centrifuged using Sorvall® T890 or Beckman® TLA-100.3 for 60 minutes at 110,000 xg. After separation of the supernatant, consisting mainly of solubilised calcium pump membrane, the pellet corresponding to the junctional face membrane with compartmental complex (JFM-CC) was resuspended and adjusted to 1.0 mg protein/ml with a buffer containing 0.3M sucrose, 10mM Hepes, pH 7.0, 0.5M NaCl and complete mini EDTA-Free protease inhibitors cocktail tablets. The resuspension was left on ice for 10 minutes and then centrifuged as described above to separate the supernatant, containing compartmental complex (CC), from the pellet, consisting of JFM. The CC was decanted and JFM was rinsed with and resuspended in a buffer containing 0.3M sucrose, 10mM Hepes, pH 7.0, and complete mini EDTA-Free protease inhibitors cocktail tablets and then rapidly frozen in liquid nitrogen and stored at -80°C.

2.1.4 Functional characterisation of isolated membrane vesicles

Functional assays were employed to assess the binding of $[^3\text{H}]$ ryanodine, a plant alkaloid shown to bind RyR in the open state. A method described by Hamilton and co-workers (Needleman and Hamilton 1997) to specify [9,21(n)-$[^3\text{H}]$ ryanodine association kinetics was employed with some modifications. In a total volume of 0.9 ml, SR membranes (0.03 mg/ml final protein concentration) were added to a buffer containing 0.3M KCl, 50mM 3-[N-morpholino]propanesulfonic acid (Mops) pH 7.4, 100µM CaCl$_2$, 10nM $[^3\text{H}]$ ryanodine, 5mM β,γ-methyleneadenosine 5'-triphosphate (AMP-PCP), 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) and complete mini EDTA-Free protease inhibitors cocktail tablets. At 25 minutes intervals, a 90µl aliquot was taken and filtered through a Millipore vacuum filtration apparatus equipped with Whatman® GF/F glass fibre filters (pre-soaked for 15 minutes in 2% (v/v) polyethyleneimine). Then, the filters were washed 5 times with 3 ml of ice-cold buffer (0.3M KCl, 100µM CaCl$_2$ and 10mM Mops, pH 7.4). The filters were transferred to scintillation vials (Pony vials, Packard Instruments, USA) and 4ml of scintillation fluid (Ecoscint™ A, National Diagnostics) added. Samples were shaken for 60 minutes after which time the amount of $[^3\text{H}]$ radioactivity was measured by liquid scintillation counting using liquid scintillation analyser (1900 TR or 2500 TR, Packard instruments, USA). The background radiation was subtracted from each measurement.
[\textsuperscript{3}H] ryanodine binding at different [Ca\textsuperscript{2+}] was explored as following. In 10 aliquots of 125\(\mu\)l, SR membranes (0.03mg/ml) were added to a buffer containing 0.3M KCl, 50mM Mops, pH 7.4, 5mM AMP-PCP, 0.1% (w/v) CHAPS complete and mini EDTA-Free protease inhibitors cocktail tablets. Using 1mM stock, final CaCl\textsubscript{2} concentrations were adjusted to be 0.05\(\mu\)M, 0.1\(\mu\)M, 0.5\(\mu\)M, 1\(\mu\)M, 5\(\mu\)M, 10\(\mu\)M, 50\(\mu\)M, 100\(\mu\)M, 0.5mM, 1mM and 10mM. [\textsuperscript{3}H] ryanodine (10nM final concentration) was added to the tubes which were then incubated at room temperature with gentle shaking for 100 minutes. 100\(\mu\)l aliquots were filtered through a Millipore vacuum filtration apparatus equipped with Whatman® GF/F glass fibre filters (pre-soaked for 15 minutes in 2% (v/v) polyethyleneimine). The assay was then performed as described above.

To determine the amount of [\textsuperscript{3}H] ryanodine bound non-specifically to the SR membranes 15\(\mu\)M of cold ryanodine was added to 125\(\mu\)l; SR membranes (0.03mg/ml) in a buffer containing 0.3M KCl, 50mM Mops pH 7.4, 50\(\mu\)M CaCl\textsubscript{2}, 10nM [\textsuperscript{3}H] ryanodine, 5mM AMP-PCP, 0.1% (w/v) CHAPS and complete mini EDTA-Free protease inhibitors cocktail tablets. After an incubation of 100 minutes, a 100\(\mu\)L aliquot was filtered through a Millipore vacuum filtration apparatus equipped with Whatman® GF/F glass fibre filters (pre-soaked for 15 minutes in 2% (v/v) polyethyleneimine). The assay was then performed as described above.

2.1.5 Purification of RyR2

2.1.5.1 Method 1: Immunoaffinity purification

This method was applied according to (Sharma, Jeyakumar et al. 2006). Using a synthetic peptide amino-acid sequence “EKFQEQKTKEEE”, an isoform-specific RyR2 polyclonal antibody was developed commercially (AMS Biotechnology).

Prebound RyR2 specific antibody was prepared by incubating and gently mixing the antibody for 30 minutes at room temperature with protein A/G matrix which was equilibrated in phosphate buffered saline (PBS) containing 137mM NaCl, 10mM Na\textsubscript{2}HPO\textsubscript{4} pH 7.4 and 2.7mM KCl. The RyR2 specific antibody was already in PBS solution. Excess, unbound, antibody was removed by washing the column with PBS. Isolated SR membranes (~5mg) were solubilised at 6.0mg/ml in buffer A (20mM Na\textsubscript{2}PIPES, pH 7.2, 0.6M NaCl, 0.1mM (ethylene glycol bis (2-aminoethylether)-N-N-
N’-N’-tetra acetic acid (EGTA), 5.0mM di-sodium adenosine monophosphate (Na$_2$AMP), 0.2mM CaCl$_2$, 2.0mM dithiothreitol (DTT), 0.6% (w/v) CHAPS/0.3%(w/v) soyabean lecithin (SBL) and complete mini EDTA-Free protease inhibitors cocktail tablets) for 90 minutes at 4°C with gentle mixing. To remove the insoluble material, the mixture was then centrifuged at 50,000 xg in a Sorvall T890 for 30 minutes at 4°C. The supernatant was diluted three fold in buffer B containing 20.0mM Na$_2$Pipes, pH 7.2, 0.1mM EGTA, 0.2mM CaCl$_2$, 5.0mM Na$_2$AMP, 2.0mM DTT and 0.3M sucrose. This was followed by pre-absorption with 20µl of the pre-equilibrated protein A/G matrix for 15 minutes at 4°C with gentle mixing to remove non-specifically bound components. Low speed sedimentation allowed removal of the matrix and the supernatant was transferred to another container where it was incubated with the A/G-anti RyR2 matrix overnight at 4°C with gentle mixing. The antibody was essentially in three- to four-fold molar excess in relation to RyR2 in the cardiac microsomes (a figure determined by the previously described [$^3$H] ryanodine binding experiments). After overnight incubation, a low speed centrifugation was applied to sediment the column matrix and separate out any unbound protein. The column matrix was then washed sequentially, first with Buffer B containing 0.15M NaCl, and 0.5% (w/v) CHAPS / 0.25% (w/v) SBL, followed by Buffer B containing 0.5M NaCl and 0.5% (w/v) CHAPS / 0.25% (w/v) SBL and then Buffer B containing 0.1M NaCl and 0.5% (w/v) CHAPS / 0.25% w/v SBL. Protein was eluted by gently mixing the column matrix for 60 minutes at 4°C with Buffer B containing 20mM PIPES pH 7.2, 0.4M NaCl, 2.0M DTT, 0.6% (w/v) CHAPS / 0.3% (w/v) SBL, 0.1mM EGTA, 0.2mM CaCl$_2$, 5.0mM Na$_2$AMP and excess of epitope peptide (3.0mg/ml). Then, the eluate was separated and a posteluate was collected after washing the pellet again with the elution buffer without epitope peptide. SDS-PAGE followed by silver staining and western blotting were performed for aliquots taken at each step of the immunoaffinity purification to assess RyR2 purification.

2.1.5.2 Method 2: Size-exclusion chromatography

Approximately 1mg of cardiac SR membranes were labelled with [$^3$H] ryanodine and then added to the unlabelled membranes (~ 33mg) to permit monitoring of RyR2 isolation. The SR membranes were pelleted (by ultracentrifugation using Sorvall T 865
rotor for 30 minutes at 90,000 xg) and then resuspended in solubilisation buffer (20mM Na$_2$Pipes, pH 7.2, 0.6M NaCl, 0.1mM (ethylene glycol bis (2-aminoethylether)-N-N'-N'-N'-tetra acetic acid (EGTA), 5.0mM di-sodium adenosine monophosphate (Na$_2$AMP), 0.2mM CaCl$_2$, 2.0mM dithiothreitol (DTT), 0.6% (w/v) CHAPS /0.3% (w/v) soyabean lecithin (SBL) and complete mini EDTA-Free protease inhibitors cocktail tablets) for a protein concentration of 2 mg/ml on ice for 90 minutes. The solubilised protein was separated from membrane fragments by centrifugation at 50,000 xg in a Sorvall® T890 for 30 minutes at 4°C. The supernatant was collected and concentrated using VIVASPIN 20 (Sartorius Biolab Products).

Size exclusion chromatography was carried out according to West and co-workers (West, Smith et al. 2002). A Bio-Rad chromatography Econo-Column was packed with Sephacryl S-300 HR gel filtration media (GE Healthcare) to create a bed volume of ~20ml. The packed column was then connected to a peristaltic pump (Amersham Bioscience) and a fraction collector (Bio-Rad®). The packed column was equilibrated with several column volumes of (solubilisation buffer + 15% glycerol). Then 60ml of (solubilisation buffer + 15% glycerol) was used to elute the loaded concentrated solubilised SR at a flow rate of 0.75ml/min. Fractions were collected at 1 minute intervals and aliquots taken for scintillation counting and SDS-PAGE and western blot analysis.

2.1.5.3 Method 3: Combining sucrose gradient fractionation and column chromatography

2.1.5.3.1 Linear sucrose gradient fractionation

Isolated cardiac SR membrane vesicles (~12mg), 1mg of which had been pre-labeled with [³H] ryanodine, were solubilised as described above at a final protein concentration of 2mg/ml. The solubilised SR membrane proteins were layered on the top of 5-25% linear sucrose gradient containing 20mM Na$_2$Pipes, pH 7.2, 0.6M NaCl, 0.1mM EGTA, 5.0mM Na$_2$AMP, 0.2mM CaCl$_2$, 2.0mM DTT and 0.6% (w/v) CHAPS/0.3% (w/v) SBL. The gradients were then centrifuged in a Sorvall® Surespin 630 rotor at 125,000 xg for 16 h. This was followed by fractionation into ~0.5ml portions. 100µl of every other portion was reserved for liquid scintillation counting.
Another 100µl was reserved for protein assay and SDS-PAGE. All fractions were quick frozen in liquid N\textsubscript{2} and stored at -80°C.

2.1.5.3.2 Step-wise sucrose gradient fractionation

Following the method described by (Lindsay and Williams 1991), solubilised SR membrane proteins, aliquot of which had been pre-labeled with [\textsuperscript{3}H] ryanodine, in a buffer containing 25mM PIPES, pH 7.2, 0.15mM CaCl\textsubscript{2}, 1M NaCl, 5mM Na\textsubscript{2}AMP, 0.5% (w/v) CHAPS, 0.25% (w/v) SBL, and complete mini EDTA-Free protease inhibitors cocktail tablets was layered onto step-wise sucrose gradient (5-25%) on top of 35% sucrose cushion. Sucrose gradient solutions were prepared in buffer containing 25mM PIPES, pH 7.2, 0.15mM CaCl\textsubscript{2}, 1M NaCl, 5mM Na\textsubscript{2}AMP, 0.5% (w/v) CHAPS, 0.25% (w/v) SBL and complete mini EDTA-Free protease inhibitors cocktail tablets. The gradient was then centrifuged in a Sorvall\textsuperscript{®} Surespin 630 rotor at 125,000 xg for 16 hours. This was followed by fractionation into ~0.5ml portions. 100µl of every other portion was reserved for liquid scintillation counting. Another 100µl was reserved for protein assay and SDS-PAGE. All fractions were then quick frozen and stored at -80°C.

2.1.5.3.3 Heparin agarose chromatography

Heparin-agarose resin was equilibrated with buffer containing 25mM PIPES, pH 7.2, 5mM Na\textsubscript{2}AMP 0.15mM CaCl\textsubscript{2}, 0.5% (w/v) CHAPS, 0.25% (w/v) SBL and 0.2M NaCl. Sucrose gradient fractions corresponding to the radioactive peaks from the previous purification step were pooled together diluted with a buffer containing 25mM PIPES, pH 7.2, 5mM Na\textsubscript{2}AMP, 0.15mM CaCl\textsubscript{2}, 0.5% (w/v) CHAPS, 0.25% (w/v) SBL and complete mini EDTA-Free protease inhibitors cocktail tablets for a final salt concentration of 0.2M NaCl. The diluted sample was then incubated with the equilibrated heparin-agarose resin (5ml) for 1 hour at 4°C. The resin was then washed with 3 column volumes of buffer containing 25mM PIPES, pH 7.2, 5mM Na\textsubscript{2}AMP, 0.15mM CaCl\textsubscript{2}, 0.5 % CHAPS, 0.25% SBL, 0.2M NaCl and complete mini EDTA-Free protease inhibitors cocktail tablets. This was followed by step-wise salt elution using ascending NaCl concentrations of the above buffer; 0.25M, 0.3M, 0.4M, 0.5M, 0.55M, 0.6M, 0.65M, 0.7M and 1M. 0.5ml fractions were collected and aliquots taken for scintillation counting, SDS-PAGE, dot blot and protein assay.

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2.1.6 Purification of RyR1

2.1.6.1 Sucrose gradient fractionation of solubilised sarcoplasmic reticulum JTC

A modified method described by Serysheva and co-workers was developed (Serysheva, Schatz et al. 1999). Approximately 20 mg sarcoplasmic reticulum JTC and ~1.5 mg membranes which had been labeled with $[^3]$H ryanodine, were separately solubilised at 2.0 mg/ml in buffer containing (50mM MOPS, pH 7.4, 0.185M NaCl, 0.1M ethylene glycol bis (2-aminoethylether)-N-N’-N’-tetra acetic acid (EGTA), 2.0mM dithiothreitol (DTT), 2% (w/v) CHAPS/1% (w/v) soyabean lecithin (SBL) and complete mini EDTA-Free protease inhibitors cocktail tablets) for 60 minutes on ice. To remove the insoluble material, the mixture was then centrifuged at 100,000 x g in a Sorvall® T890 for 30 minutes at 4°C. The solubilised skeletal SR was layered onto step-wise sucrose gradient (5-25% (w/v)) on top of 35% (w/v) sucrose cushion. Sucrose gradient solutions were prepared in buffer containing 50mM MOPS, pH 7.4, 0.185M NaCl, 0.1M EGTA, 2.0mM DTT, 0.5% (w/v) CHAPS and complete mini EDTA-Free protease inhibitors cocktail tablets. The labelled sample was loaded into one tube and the unlabelled solubilised fraction distributed over the remainder of the centrifuge tubes. The gradients were centrifuged in a Sorvall® Surespin 630 rotor at 125,000 x g for 16 hours. This was followed by fractionation into ~0.5 ml portions. Fractions from the tube containing the labelled solubilised SR membranes were analyzed by liquid scintillation counting. Aliquots were also reserved for protein assay and SDS-PAGE. All fractions were then quick frozen in liquid nitrogen and stored at -80°C.

2.2 Expression & purification of cardiac recombinant calsequestrin

Clones of full length human CSQ2 (hCSQ2) inserted between restriction sites SalI and NotI of a pGEX-6P-3 vector containing an N-terminal GST tag were generated by another member in Kitmitto laboratory. The starting point in this thesis work was glycerol stocks of these clones that had been previously sequenced. Calsequestrin was expressed as a GST fusion protein in E. coli strain BL21 (DE3) colonies. A single colony was grown at 37°C into lysogeny broth (LB) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.2M NaCl, 200µg/ml ampicillin) to an optical density at 600nm of ~0.7, and expression was induced by 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 28°C for 5 hours. Bacteria were pelleted by centrifugation in Sorvall® SLA-1500 at 2500
xg for 45 minutes at 4°C. The cell membrane of harvested bacteria was disrupted by re-suspension and incubation in phosphate buffered saline (PBS) buffer containing 137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄·2H₂O, 1.76mM KH₂PO₄, pH 7.4, 100µg/ml lysozyme, 10µg/ml Deoxy-ribonuclease (DNase I) and complete mini EDTA-Free protease inhibitors cocktail tablets for 45 minutes at 4°C with shaking. Cell debris was pelleted by centrifugation using a Sorvall® SS-34 rotor at 47,000 xg for 40 minutes. The supernatant was collected, filtered (0.45µm filters, Millipore) and aliquots added to 1ml Microspin™ GST (GE Healthcare) columns packed with Glutathione Sepharose™ 4B (GE Healthcare) resin which had been equilibrated with PBS buffer. This was followed by incubation with shaking at 4°C for 2 hours. The columns were centrifuged in an Eppendorf®, Mini Spin Plus centrifuge at ~600 xg to remove the lysate buffer. The resin was washed with PBS buffer and then equilibrated with a cleavage buffer (10mM Hepes, pH 7.4, 150mM NaCl, 1mM DTT and 1mM EDTA). The resin was then incubated for 16 hours with the cleavage buffer containing PreScission Protease (2 units per 100µg of protein) at 4°C. The columns were then centrifuged to collect the cleaved human CSQ2. However, to elute hCSQ2 with its GST fusion tag, the resin was incubated with the elution buffer (10mM Hepes, pH 7.4, 150mM NaCl, 1mM DTT and 1mM EDTA and 40mM reduced glutathione) for 30 minutes at 4°C.

2.3 Circular dichroism spectroscopy
Circular dichroism, using a Jasco-810 spectropolarimeter, was employed to examine the secondary structure of the recombinant hCSQ2. hCSQ2 (3µM) was examined in a buffer composed of 10mM Tris-Base, 150 mM NaCl, pH 7.4. Data was collected at a scan rate of 0.5nm per 0.5 sec and over the 190-260nm scan range and spectra were acquired at 25°C. Eight scans were averaged for each spectrum. Buffer scans were recorded under the same conditions and subtracted from the sample spectra.

2.4 Expression of cardiac triadin isoform 1 (Trd1); full length and luminal domain
Clones of full length and C-terminal (detailed in the results and discussion section) mouse cardiac triadin isoform 1 in a pTrcHis2A vector were made and transformed in E. coli BL21 (DE3) by another member of the Kitmitto laboratory. The starting point in this thesis work was glycerol stocks of these clones. A single colony was grown at 37°C in LB supplemented with 200 µg/ml ampicillin to an optical density
at 600nm of ~0.7. Different permutations were employed to try to express the recombinant protein; IPTG concentrations 0.5 - 2mM, induction temperatures of 37°C, 28°C and 20°C as well as a range of induction times (1h, 2h, 3h, 4h and 5h). Bacteria were pelleted by centrifugation in Sigma® 11180 at 2500 xg for 45 minutes at 4°C. The cell membrane of the harvested bacteria was disrupted by re-suspension and incubation in PBS buffer plus 100µg/ml lysozyme, 1% (w/v) CHAPS, 10µg/ml Deoxy-ribonuclease (DNase I) and complete mini EDTA-Free protease inhibitors cocktail tablets for 45 minutes at 4°C with shaking. Cell debris was pelleted by centrifugation in Beckman® TLA-100.3 at 47,000 xg for 40 minutes and the supernatants were collected. SDS-PAGE, Coomassie blue staining and western blotting were employed to assess the expression trials.

2.5 Biochemical characterisation methods

2.5.1 Protein assay

2.5.1.1 Protein assay of membrane samples

To a 20µl of SR membranes, 1 ml of acetone was added to 20µl aliquots of SR membrane vesicles. The mixture was vortexed, stored for 30 minutes in a -20°C freezer and then centrifuged for 20 minutes at 6500 xg (Eppendorf®, Mini Spin Plus). This step was to solubilise the lipid and precipitate the protein. The supernatant was discarded and the pellet left to air dry prior to addition of 500µl of 5mM HEPES buffer, pH 7.4. The sample was then vortexed and the pellet resuspended ultrasonically. Aliquots were taken and made up to a final volume of 200µl using the HEPES buffer and made up to 1ml with 800µls of 20% (v/v) Bradford dye (Biorad®) (Bradford 1976). Samples were incubated for 15 minutes at room temperature and then the absorbance at 595nm measured using a Jenway® 6305 spectrophotometer. In order to correlate the absorbance value for the SR membrane vesicles to protein concentration, a standard curve of protein versus absorbance at 595nm was constructed using lysosome.

2.5.1.2 Protein assay for solubilised protein

Aliquots (e.g. 10-30µl) were taken for analysis and made up to 200µl with HEPES buffer and, then, the assay was followed as described above. To evaluate the contribution of the buffer components (e.g. detergent) a blank with the same value of
buffer was used. It should be noted that due to the presence of the detergent the protein assay results only provide a rough approximation.

2.5.2 Gel electrophoresis

The protein composition of samples was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). For visualisation and gel loading purposes, a 5x sample buffer (60 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue) was added to each protein sample with incubation at room temperature for ~ 60 minutes (or boiling at 100°C for 10 minutes) for denaturation of the samples. Both the separating and the stacking gels contained 5% acrylamide in case of native samples. A low percentage separating gel was used because of the high molecular weight of RyR (4 x 565kDa homotetramer). However for recombinant protein samples the separating and the stacking gels contained 12% and 5% (v/v) acrylamide respectively. Bio-Rad® Kaleidoscope pre-stained markers with broad range of 206 – 7 kDa was employed.

Gels were stained with either Coomassie blue or silver stain. Following SDS-PAGE, the gel was carefully transferred to a container containing Coomassie stain (0.1% (w/v) Coomassie Blue R-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid). The gel was agitated for 10 minutes after which the stain was poured off and destain (10% (v/v) methanol, 10% (v/v) glacial acetic acid) was added. The container was agitated again for one hour after which visible strong bands appear on a light box. By changing the destain solution and agitating overnight the gel was completely destained. For preparations which failed to show any bands by Coomassie blue staining, silver staining was carried out. Gels were fixed with 50% (v/v) methanol, 12% (v/v) acetic acid for 1h - 45 minutes followed by three 20 minute washes in 50% (v/v) ethanol after which the gel was washed twice in solution A (1mM sodiumthiosulphate) for 30 seconds. Distilled water was then used to wash the gel for 2 minutes. The gel was then incubated in solution B (10mM silver nitrate) in the dark for 20 minutes. The gel was then washed in distilled water for 30 seconds after which solution C (500mM sodium carbonate, 2.5% (v/v) solution A and 0.05% (v/v) formaldehyde) was added. The reaction was stopped by the addition of 5% (v/v) acetic acid.
2.5.3 Western blotting

SDS-PAGE was followed by transfer of proteins to Whatman®-PROTRAN® nitrocellulose transfer membrane (0.2µM) for 75 minutes at 100V and 4°C. Kaleidoscope protein standards were employed as an indicator of protein transfer. The nitrocellulose membrane was washed in 10ml of tris-buffered saline (TBS) (150mM NaCl, 10mM Tris, pH 8.0) followed by a blocking step by incubation in 10ml TBS/tween/milk (0.5% (v/v) Tween 20 and 1% (w/v) non-fat dried Marvel milk) for 60 minutes at room temperature with gentle shaking. The nitrocellulose sheet was then incubated with gentle shaking for 60 minutes with the primary antibodies (details below, table 2.1) (prepared in TBS/tween/milk). The primary antibodies were removed and the membrane washed three times (5 minutes/wash) with 10ml of TBS/Tween/milk. Secondary antibodies with an alkaline phosphatase conjugate (details below, table 2.2) (prepared in TBS/tween/milk) were then added for a further 1 hour incubation step. The secondary antibodies solutions were discarded, the membrane washed 3x5 minutes in 10ml of TBS/Tween (without milk). For alkaline phosphatase staining, the membrane was incubated in 10ml of alkaline phosphatase buffer (100mM NaCl, 5mM MgCl₂, 100mM Tris, pH 9.5) for 10 minutes at room temperature with gentle rocking. A buffered solution of Sigma FAST 5-Bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium (BCIP-NBT) tablet dissolved in 10ml alkaline phosphatase buffer was added in order to visualise the reaction. To stop the reaction, the developing solution was poured off and the membrane was dried and kept in the dark.

2.5.4 Dot blotting

Dot blotting is another immunologically based technique used for detecting and identifying proteins. It differs from western blot in that protein samples applied directly onto the nitrocellulose membrane. It has the advantage of rapid screening of the sample. The possibility of non-specific interactions of the antibodies with protein components of the sample can be misleading and so other methods should be used to confirm data from dot blots. A grid was drawn on Whatman®-PROTRAN® nitrocellulose transfer membrane (0.2µM) by a pencil. 15µl of each sample fraction was spotted onto the labelled grid and the membrane was allowed to dry. The membrane was then treated as per western blotting from the blocking stage on.
Table 2.1: Primary antibodies

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<th>Antibody</th>
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<th>Antibody Supplier</th>
<th>Host Species</th>
<th>Dilution Factor</th>
<th>Blocking Agent</th>
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<td>Sigma</td>
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Table 2.2: Secondary antibodies

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<th>Host Species</th>
<th>Dilution Factor</th>
<th>Blocking Agent</th>
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</thead>
<tbody>
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<td>Sigma</td>
<td>Goat</td>
<td>1/500</td>
<td>1% (w/v) milk</td>
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<tr>
<td>Anti-Rabbit IgG (whole molecule)-Alkaline Phosphatase antibody</td>
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2.6 Investigation of the luminal Ca-sensor formation, RyR regulation; RyR-calsequestrin interactions

2.6.1 Surface plasmon resonance (SPR)

SPR experiments were carried out using both BIACore 3000 and Proteon XPR 36 instruments. Recombinant hCSQ2 was immobilised onto CM5 sensor chip (in case of BIACore 3000) and GLC sensor chip (in case of Proteon XPR 36). For a standard direct amine coupling, mixture of N-hydroxysuccinimide (NHS) (0.1M) / 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.4M) (1:1 volume ratio) was injected over both reference and test flow cells followed by injection of hCSQ2 (10µg/ml) in 10mM acetate buffer, pH 2.5, over test flow cell only; 10mM acetate buffer, pH 4.0, was also
employed in some experiments. As a deactivation step, an equal volume of 1M ethanolamine/HCl, pH 8.0 (to the volume of injected NHS/EDC mixture) was injected over both reference and test flow cells to block the unbound sites of the activated surface. Various immobilisation figures were achieved; 9500RU, 5500RU, 450RU and 150RU. The running buffer for the immobilisation step was 10mM HEPES, pH 7.4, 150mM NaCl and 0.005% (v/v) Tween 20. To remove the sucrose from the RyR1 samples, aliquots were dialysed in (Slide-A-Lyzer® (SLA) dialysis cassettes (7000MW) (Thermo Scientific)) against sucrose free buffer. For the open state of RyR1, the buffer employed for dialysis and as a running buffer was comprised of 10mM HEPES, pH 7.4, 150mM NaCl, 2mM DTT, 100µM CaCl₂, 5mM Na₂AMP and 0.5% (w/v) CHAPS. The buffer employed for the closed state of RyR1 had a composition of 10mM HEPES, pH 7.4, 150mM NaCl, 2mM DTT, 2mM EGTA and 0.5% (w/v) CHAPS. For the open and closed states of RyR2, similar buffers to that used for RyR1 were employed, with the inclusion of 0.25% (w/v) SBL. RyR1 samples (30µg/ml) were run as analytes over both reference and test flow cells. For experiments whereby RyR1 binding to hCSQ2 was indicated an anti-RyR antibody (Abcam, ab2827) (10µg/ml) was injected during the dissociation phase. Kinetic SPR experiments were carried out employing RyR2 at concentrations of 16, 4, 2, 1µg/ml which corresponds to 8, 2, 1, 0.5nM respectively. The data evaluation were carried out using the software program BIAevaluation version 4.0 determining $K_a$ (association constant; M⁻¹s⁻¹), $K_d$ (dissociation constant; s⁻¹), $K_A$ (association equilibrium constant; 1/M) and $K_D$ (M).

In another approach, an indirect coupling of hCSQ2 via its GST fusion tag using an anti-GST antibody was tested. The anti-GST antibody (Sigma, G7781) at a concentration of 50µg/ml in 10mM acetate buffer, pH 4.5, was immobilised on both reference and test flow cells to a figure of 5500RU. Then, hCSQ2-GST (200µg/ml) was run onto test flow cell. A figure of indirect immobilisation of 60RU was achieved. Dialysed RyR1 samples (40µg/ml) in both open and closed states were run onto both reference and test flow cells.

As another modality of the SPR experiment and using Proteon XPR 36, RyR1 (15µg/ml) in 10mM acetate buffer, pH 5.5, was immobilised by standard direct amine
coupling employing 10mM HEPES, pH 7.4, 150mM NaCl, 2mM DTT, 100µM CaCl₂, 5mM Na₂AMP and 0.5% (w/v) CHAPS as a running buffer. A lower concentration of NHS (0.05M)/EDC (0.2M) in 1:1 volume ratio was employed in the activation step. The immobilisation figure achieved was ~90RU. hCSQ2 (0.3mg/ml) was then run as analyte in 10mM HEPES, pH 7.4, 150mM NaCl and 0.005% (v/v) Tween 20.

### 2.6.2 Quartz crystal microbalance and dissipation (QCM-D) monitoring

To remove the sucrose and to decrease the CHAPS concentration, purified RyR1 samples were dialysed in (Slide-A-Lyzer® (SLA) dialysis cassettes (7000MW) (Thermo Scientific)) against buffer containing 10mM Hepes, pH 7.4, 150mM NaCl, 2mM EGTA and 0.17% (w/v) CHAPS. Examination of the samples by electron microscopy (negative staining as described in Section 2.7.1) found that reduction of the CHAPS concentration did not lead to aggregation of the RyR1.

The silicon dioxide sensor (SiO₂) chips were cleaned prior to use. The sensor chips were sonicated for 30 minutes in 2% (w/v) SDS followed by washing with Milli-Q water and then 100% ethanol. Using a stream of nitrogen gas, excess ethanol was dried off in fume hood. The chips were then plasma cleaned under UV/OZONE procleaner™ (Biofore, Nano sciences) for 30 minutes in fume hood. The SiO₂ sensor chip, immediately after the plasma cleaning, was mounted in the flow cell. Then, Hepes buffer (10mM Hepes, pH 7.4, 150mM NaCl, 1mM EDTA and 1mM DTT) was added at a flow rate of 150 µl/min using a peristaltic pump to fill the measurement chamber after which the QSoft programme was started to obtain and measure the SiO₂ sensor chip’s resonance frequency and dissipation values in buffer at several overtones. Hepes buffer was continuously applied to the flow cell at a flow rate of 20µl/min until a steady baseline was achieved. hCSQ2 (50µg/ml) in Hepes buffer (10mM Hepes, pH 7.4, 150mM NaCl, 1mM EDTA and 1mM DTT) was applied at the same flow rate. hCSQ2 was continuously applied until a stable and constant frequency and dissipation had been reached. Then, CHAPS buffer (10mM Hepes, pH 7.4, 150mM NaCl, 2mM EGTA and 0.17% CHAPS) was applied to the flow cell at the same rate. Steady frequency and dissipation was assured and then dialysed RyR1 sample (diluted to 50µg/ml) was applied to the flow cell at a flow rate of 20µl/min.
In another approach, RyR1 was first applied onto the chip. The CHAPS buffer (50mM MOPS, pH 7.4, 150mM NaCl, 2mM DTT, 0.1M EGTA, 0.17% (w/v) CHAPS and 6% (w/v) sucrose) was employed, as described above, initially until a steady baseline was achieved. Purified RyR1 sample was diluted to 50µg/ml using an identical CHAPS free and sucrose free buffer so that the final CHAPS and sucrose concentrations were 0.17% (w/v) and 6% (w/v) respectively. The diluted RyR1 sample (50 µg/ml) in CHAPS buffer (50mM MOPS, pH 7.4, 150mM NaCl, 2mM DTT, 0.1M EGTA, 0.17% (w/v) CHAPS and 6% (w/v) sucrose) was applied at a flow rate of 20µl/min until the rate of change of frequency and dissipation had considerably decreased. Then, Hepes buffer (10mM Hepes, pH 7.4, 150mM NaCl, 1mM EDTA and 1mM DTT) was applied at the same flow rate until a stable frequency and dissipation had been reached. Then, hCSQ2 (50µg/ml) in Hepes buffer (10mM Hepes, pH 7.4, 150mM NaCl, 1mM EDTA and 1mM DTT) was applied at the same flow rate.

2.6.3 Pull-down experiment investigating RyR1-CSQ2 binding

GST-hCSQ2 was applied to a Glutathione Sepharose™ 4B (GE healthcare) (50µl bed volume) as described above and then equilibrated in a buffer identical to the buffer used for the sucrose gradient fractions yielding pure RyR1 (50mM MOPS, pH 7.4, 185mM NaCl, 2mM DTT, 0.1M EGTA, 0.5% (w/v) CHAPS and 20% sucrose), as described in Section 2.1.6 above. Purified RyR1 was added in excess and incubated with the immobilised GST-hCSQ2 for 5 minutes at 4°C. Centrifugation in Eppendorf®, Mini Spin Plus at ~600 xg was then employed to separate the supernatant from the pellet which was then washed three times with 250µl of the above mentioned buffer. SDS-PAGE and western blotting using anti-RyR antibody (Abcam, ab2827) was then employed to determine whether RyR1 had bound to the hCSQ2. This experiment was preceded by a control experiment to investigate the non-specific binding of RyR1 to
Glutathione Sepharose™ 4B. Another control experiment was to wash the immobilised hCSQ2-GST in the RyR1 buffer (no RyR1) to ensure that it did not lead to elution of the protein.

2.7 Transmission electron microscopy (TEM)

Two approaches were taken to examine the protein samples negative staining and cryo-freezing. An overview of the two techniques is given in sections 2.7.1 & 2.7.2.

2.7.1 Negative staining of RyR

Using EMITech K100 X apparatus, 400 square mesh carbon-coated copper grids (Agar Scientific) were glow-discharged (rendered hydrophilic) and then applied (discharged surface down) onto an aliquots (~15µl) of purified RyR1 and incubated for 30 seconds. Excess protein was blotted with filter paper (Whatman® 50). Samples were then washed twice with distilled water and then negatively stained in 2% (w/v) uranyl acetate (Walsh, Davies et al. 2009).

For investigation of RyR1-hCSQ2 complex formation using EM, 800µl pure RyR1 (0.2µM) was mixed with purified hCSQ2 (3.3µM) for a molar excess of hCSQ2 in relation to RyR1. The mixture was then incubated at 4°C for 15 minutes and then ultracentrifuged through a sucrose cushion (~25%) at 4°C in Beckman® TLA-100.3 at 437,000 xg for 60 minutes. The upper 900µl containing unbound hCSQ2 (determined by SDS-PAGE and western blotting) was separated carefully and the bottom 100µl shown to contain both RyR1 and hCSQ2 was used for sample preparation as described above.

Grids were examined in a Phillips Tecnai 12 transmission electron microscope operated at an accelerating voltage of 100kV. Electron micrographs were recorded at a calibrated magnification of 47,800 x on Kodak Electron Image film 4489. Micrographs were scanned on an Imacon Flextight 949 scanner using Flex colour scanning software, for a resolution of 3.6 Å at the specimen level. The image quality was assessed by examination of the Thon ring of Fast Fourier transforms (FFTs) using CTFIT (EMAN software (Ludtke, Baldwin et al. 1999))
2.7.2 Cryo-EM

For cryo-EM protein samples > 1mg/ml are required. The RyR1 sample concentrated to ~1mg/ml prior to doing cryo-EM. This was accomplished by ultracentrifugation of 500µl pure RyR1 sucrose fraction at 4°C in Beckman® TLA-100.3 at 437,000 xg for 60 minutes followed by careful separation of the upper 400µl. This method yielded concentrated sample in the bottom of the tube (but not pelleted) used for cryo-EM. RyR1-hCSQ2 complex samples were prepared as described above. Quantifoil R2/2 400 mesh Cu holey carbon grids (SPI® supplies) were initially coated by a thin carbon layer (~2nm) using Edwards® Auto 306. The grids were then glow discharged using EMITech K100 X apparatus. Using the concentrated RyR1 samples (or RyR1-hCSQ2 samples), the freshly glow discharged carbon coated Quantifoil grids were prepared for cryo-EM by an FEI Vitrobot™ computer-controlled freeze-plunging instrument. Different blotting times were tested and 1.5 seconds was found to optimise the thickness of the ice layer.

The grids were transferred using Gatan® cryo-holder and cryo-transfer system to an FEI Polara field emission gun transmission electron microscope operated at an accelerating voltage of 300kV. The grids were examined at -170°C and digital micrographs were recorded, for a resolution of 3.13 Å at the specimen level, by the 4K x 4K Gatan® USC 4000 with low dose protocols between -1.5 and -4.5µm defocus range at a magnification of 50,000 x. The image quality was assessed by examination of the Thon ring of Fast Fourier transforms (FFTs) using CTFIT (EMAN software (Ludtke, Baldwin et al. 1999))

2.7.3 Image processing

EMAN was the image processing software employed in this research project. A comprehensive review of the EMAN software package is found in (Ludtke, Baldwin et al. 1999). Approximately 2600 and 2100 particles were manually selected for control samples (i.e. pure RyR1) from negatively-stained and cryo-images respectively. Comparable numbers were selected for RyR1-hCSQ2 complex samples. The particles were selected using the graphical interface boxer (EMAN image processing software) into a box of 128x128 pixels. Contrast transfer function (CTF) parameters for particles were determined in each micrograph using the CTFIT programme (part of EMAN
software). All particles were combined into “start.hed” file using “proc2d” command. Particles were band-pass filtered using hp and lp commands. The contrast was inverted for the cryo-EM dataset since RyR1 looks darker than the background and the software has been developed to recognise protein as white densities. The particles were centred using cenallgnint command and then reference-free classified with generation of class averages using “startnrclasses” programme (part of EMAN software). Presented below is a flow chart of EMAN image processing.

**EMAN Image processing Flow Chart**

- Particles Selection (using boxer)
- CTF Correction (using ctfit)
- Combining All Particles into start.hed (using proc2d command)
- Filtering and Centering The particles (using hp, lp and cenallgnint commands)
- Reference Free Classification of The Particles into Class Averages (using startnrclasses)
Chapter 3: Purification and Characterisation Ryanodine Receptors
Types 1 and 2

3.1 Introduction

To date the majority of studies use RyR purified from native tissues. The recombinant approach for isolating RyR poses several problems such as difficulties in producing a large sized plasmid cDNA encoding the full length open reading frame of RyR (~ 15,000 base pair), which is fragile and undergoes spontaneous rearrangement and deletions particularly in the case of RyR2. Specific strains of bacteria that are adapted for viable transformation with intact large plasmids as well as specific culture conditions are required. Furthermore, the production of intact functional recombinant RyR, with a $M_r > 2$MDa and with a complex 3D structure represents a major challenge (George, Yin et al. 2005)

The overall aim of this part of the project was the purification and characterisation of both cardiac and skeletal forms of ryanodine receptors. Over the past 25 years, the literature has reported numerous strategies for purification of ryanodine receptors from various animal species. Fleischer and co-workers (Inui, Saito et al. 1987) have described a 3-step chromatography method to purify ryanodine receptors from rabbit skeletal muscles. The three successive steps employed were heparin-agarose chromatography, hydroxylapatite column, and gel filtration HPLC. The ryanodine binding activity of the purified receptor determined yields of 393±65 pmol/mg protein. Later in the same year (Inui, Saito et al. 1987), a group also reported the purification of the cardiac ryanodine receptor (RyR2) from dog heart employing a similar methodology using heparin-agarose chromatography, $p$ aminobenzamidine-agarose column chromatography, and gel filtration HPLC. Yields were comparable to RyR1 from skeletal muscle (438±49 pmol/mg protein). Meissner and co-workers (Lai, Erickson et al. 1988) purified RyR1 from rabbit skeletal muscles using linear sucrose fractionation as a single purification step with a yield of 650 pmol/mg protein. The silver stained gel of fractions at the lower part of sucrose gradient revealed a single polypeptide band with an estimated $M_r$ of ~400kDa (see Fig. 3.1).
Figure 3.1: Purification of rabbit skeletal ryanodine receptor (RyR1) by (Lai, Erickson et al. 1988) employing a linear sucrose gradient fractionation as a single purification step after solubilisation of the isolated heavy SR using CHAPS / phosphatidylcholine. (A): Coomassie brilliant blue stain of SDS-PAGE of heavy skeletal SR membranes. (B): Silver stain of SDS-PAGE of sucrose gradient fraction of purified RyR1 showing a single high molecular weight band at ~ 400kDa.

Linear sucrose gradient fractionation was also employed for the purification of the cardiac ryanodine receptor (Anderson, Lai et al. 1989; Zhang, Kelley et al. 1997) (see Fig. 3.2). Anderson et al have purified cardiac ryanodine receptors from canine heart with a yield of 250pmol/mg protein (Anderson, Lai et al. 1989).
Figure 3.2: Purification of canine cardiac ryanodine receptor (RyR2) by (Zhang, Kelley et al. 1997) employing a similar protocol employed for isolation of the skeletal muscle RyR1 i.e. linear sucrose gradient fractionation after solubilisation of the isolated heavy SR using CHAPS / phosphatidylcholine. (A): Coomassie blue stained gel of cardiac junctional SR vesicles (JSR). (B): The purified RyR2 showing a single protein band that has a M, > 200KDa.

Size-exclusion chromatography as a single technique to purify cardiac ryanodine receptor has been reported once in the literature (West, Smith et al. 2002). A combination of both sucrose gradient fractionation and ion-exchange chromatography has been described in the literature as a purification strategy for RyRs; (Airey, Beck et al. 1990; Murayama and Ogawa 1992; Wagenknecht, Grassucci et al. 1996). They differ in the second ion-exchange chromatography step where Airey et al have used DEAE-sepharose column which was replaced by a MonoQ HR5/5 in the study by Murayama and Ogawa; however, Wagenknecht et al introduced a heparin agarose chromatography instead. Rardon et al (Rardon, Cefali et al. 1989) have used gel filtration chromatography and then linear sucrose gradient fractionation to purify RyR2 from canine heart. A previous study (Shoshan-Barmatz and Zarka 1992) has simply purified RyR from rabbit skeletal muscles using spermine-agarose column chromatography reporting that RyR was eluted with 2.0mM spermine (with a yield of 280±10pmol/mg
protein), however other proteins were eluted at higher concentrations of spermine. Immunoaffinity purification of RyRs has been described in some studies (Imagawa, Smith et al. 1987; Smith, Imagawa et al. 1988; Sharma, Jeyakumar et al. 2006). The first two studies have employed a monoclonal antibody produced against the ryanodine receptor to purify RyR1 from rabbit skeletal muscles. However, Sharma et al have used an isoform-specific RyR2 polyclonal antibody which was developed against a synthetic peptide whose sequence corresponded to 11 amino acids 4418-4229 of rabbit RyR2 with this method leading to a reported yield of ~450 pmol/mg protein.

The RyR, as a membrane protein, is extracted from a phospholipid bilayer with detergents and purified in detergent containing buffers. Detergents are amphipathic molecules which have a polar head group and a single hydrophobic tail in contrast to the two hydrophobic tails of the native phospholipids. Therefore, the detergents with the overall conical shape exhibit the unique property of self-assembling into spherical micellar structures; however there is a minimum concentration of detergent molecules to form micelles known as critical micelle concentration (CMC). Membrane proteins are usually soluble in the detergent micelles which mimic the natural lipid bilayer environment of the protein (Seddon, Curnow et al. 2004; Schmidt-Krey and Rubinstein 2011). It is important to choose a detergent that preserves the function of the membrane protein and that will not be prohibitively expensive for use during the purification process. The presence of detergents might cause the purified membrane protein complexes to assemble into a non-biological structure (e.g. an oligomer). In addition, the vitrification step required for cryo-electron microscopy (EM) can lead buffers containing a high concentration of detergents to form ice aggregates that can be mistaken as protein particles leading to the analysis of non-protein structures (Schmidt-Krey and Rubinstein 2011). Poor image contrast due to the presence of detergents is another difficulty when conducting structural EM studies on purified membrane proteins (Serysheva, Chiu et al. 2007).
3.1.1 Aims and Objectives

The purification of a protein is fundamental to address questions about structure, function and regulation. Purification of RyR was the initial and mandatory step before investigation of the assembly of the luminal Ca\(^{2+}\) sensor complex formed by RyR, CSQ and triadin; the main target of this research project.

The primary goal of this part of the thesis research was the purification and characterisation of the cardiac and skeletal forms of ryanodine receptor (RyR2 & RyR1). This was accomplished by first isolating heavy sarcoplasmic reticulum (SR) and SR junctional terminal cisternae (JTC) membrane vesicles from bovine cardiac and sheep skeletal muscles respectively. A series of purification protocols combining immunoaffinity chromatography, ion-exchange chromatography and sucrose gradient centrifugation were explored. A further goal was to develop/modify an assay to ascertain whether the isolated SR membranes contained ‘functional’ RyRs. These membrane vesicles were enriched with RyRs. A \(^{[3]}H\) ryanodine binding assay was employed. The \(^{[3]}H\) ryanodine binding assay was not only essential to assess the function of the protein, but also was exploited as a label to track the presence of RyRs throughout the purification process. Optimisation of this assay was also important for later sections of this thesis where it was employed to investigate the regulation of RyR by accessory proteins. The purified RyRs were assessed for purity using SDS-PAGE and western blotting. In addition, aliquots were examined by transmission electron microscopy to further assess the purity of the isolated protein and whether the classical ‘clover-leaf’ shape was discernible and if the protein was mono-disperse and thus suitable for subsequent structural studies. Yields of purified RyR were also an important consideration as this would be a limiting factor for the design of the structural studies.

3.2 Results and Discussion

3.2.1 Isolation of heavy sarcoplasmic reticulum (SR) membrane vesicles from bovine cardiac muscle

SR membranes were prepared following the method described by Sitsapesan (Sitsapesan and Williams 1990) with minor modifications. Ventricular tissue was initially homogenised using a kitchen blender. Mammalian cells are enclosed by a plasma membrane weakly supported by a cytoskeleton lacking any rigidity and hence
easy to disrupt by shear forces. Cardiac heavy SR membranes were, then, prepared by sequential low and high speed centrifugation followed by sucrose gradient fractionation of the mixed membrane population. The heavy cardiac SR membranes formed a light band at the 30%-40% interface (see Fig. 3.3). Typically from 200g ventricular tissue, 32.9±1.6mg heavy SR proteins (n=3) were isolated.

![Sucrose gradient fractionation of cardiac mixed sarcoplasmic reticulum (SR) membrane population for isolation of heavy cardiac SR.](image)

**Figure 3.3:** Sucrose gradient fractionation of cardiac mixed sarcoplasmic reticulum (SR) membrane population for isolation of heavy cardiac SR. The mixed membrane population, in 10% sucrose, was layered over three steps of sucrose (20%, 30%, and 40%) and then ultracentrifuged. Differential migration of the sarcoplasmic reticulum membranes resulted in formation of three light bands at three sucrose gradient interfaces (10%-20%, 20%-30%, and 30%-40%). The band at the 30%-40% interface corresponds to heavy cardiac SR which is widely reported to have the highest density of RyR2s.

### 3.2.2 Isolation of sarcoplasmic reticulum junctional terminal cisternae (JTC) from sheep skeletal muscle

A similar subcellular fractionation protocol was employed to isolate skeletal SR JTC. Sucrose gradient fractionation of the mixed membrane population was carried out overnight employing a discontinuous sucrose gradient. The skeletal SR JTC formed a light band at the 38%-45% interface. The protein yield of the isolated SK SR JTC was 51.0±2.0mg (n=3) from 200g of skeletal muscles, and hence much higher than that obtained in case of cardiac heavy SR consistent with the literature (Mahaney, Albers *et al.* 2005).
To examine the polypeptide profile of the isolated sarcoplasmic membranes, SDS-PAGE (Laemmli 1970) was carried out. Since the RyR has a high molecular weight (monomer unit is ~565kDa) low percentage gels were required in order for the receptor to be visualised. Analysis of cardiac SR membranes failed to show any bands after Coomassie blue staining. However, silver staining revealed a weak high molecular weight band at a possible position of ~565kDa with western blotting confirming the presence of RyR2 (Fig. 3.4).

![Image]

**Figure 3.4: SDS-PAGE analysis for the isolated bovine heavy cardiac SR.** (A): Silver stained gel illustrating the polypeptide profile of the isolated heavy cardiac SR with a high molecular weight band (indicated by arrow) expected for RyR2. (B): The corresponding western blot analysis using an anti RyR antibody (Cat. No. ab2827); there is a distinct band at ~565kDa (indicated by arrow) confirming the presence of RyR2.

Presented below (Fig. 3.5) is Coomassie blue stained gel and corresponding western blot of isolated sheep skeletal sarcoplasmic reticulum junctional terminal cisternae (JTC).
Figure 3.5: SDS-PAGE analysis for the isolated sheep skeletal sarcoplasmic reticulum junctional terminal cisternae (JTC). (A): Coomassie blue stained gel illustrating the polypeptide profile of the isolated skeletal JTC with a distinct high molecular weight band (indicated by arrow) expected for RyR1. (B): The corresponding western blot analysis using anti-RyR (Cat. No. ab2868) revealed an obvious band at ~565kDa (indicated by arrow) confirming the identity of RyR1.

The Coomassie blue stained gel shows a clear band at ~565kDa that would correspond to a monomer of RyR1. Western blotting confirmed the identity of the band as RyR1 (lane B). Therefore, in agreement with previous reports (Pessah, Waterhouse et al. 1985; Picher, Decrouy et al. 1997), it would appear that the density of RyR2 / mg SR membranes is considerably less than RyR1 as a band could not be visualised by Coomassie, despite a similar protein loading. In addition, several lower molecular weight bands are also present in the cardiac membranes. Although silver staining of skeletal muscle membranes may also reveal other protein bands, RyR1 is the principle component.
3.2.3 Functional characterisation of the isolated skeletal sarcoplasmic reticulum junctional terminal cisternae (JTC) & cardiac heavy sarcoplasmic reticulum (SR) membrane vesicles

The above experiments confirmed the presence of RyR1 and RyR2 in the isolated skeletal SR JTC and cardiac heavy SR membrane vesicles respectively. Therefore the next step was to ascertain whether the channels were functional.

Ryanodine is a plant alkaloid that has been suggested to have four binding sites on the ryanodine receptor, which are best described by negative cooperativity (Lai, Misra et al. 1989; Carroll, Skarmeta et al. 1991; Pessah and Zimanyi 1991). Binding of nanomolar ryanodine reflects a ligand-induced open state of the Ca$^{2+}$ channel and it is generally agreed that channel activation unmasks the high affinity sites for ryanodine. Ryanodine concentrations ≥ 10µM, in studies of reconstituted SR channels in planar bilayers, and in the absence of pre-incubation of the SR with the alkaloid, are shown to lock the channel in a persistent sub-conductance state as a consequence of ryanodine binding to “high affinity” sites. However, ryanodine binding to “low affinity sites”, at concentrations in excess of 1mM are required to completely block single channel activity (Hymel, Schindler et al. 1988; Smith, Imagawa et al. 1988; Anderson, Lai et al. 1989; Lai, Misra et al. 1989). The SR calcium release channel was named the “ryanodine receptor” due to the fact that ryanodine was found to bind with high affinity.

For these studies, it was first important to optimise radiolabelling of the isolated sarcoplasmic reticulum using $[^3]$H ryanodine. Upon reviewing the literature, it was noted that controversy exists regarding the use of the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) as a component of the binding buffer. Hamilton & Needleman, 1997, reported that the alteration in the ryanodine binding site which follows binding of ryanodine to RyR leading to a decreased affinity is prevented by the inclusion of CHAPS. In contrast, Maclennan and co-workers, 1998, found that presence of the detergent CHAPS decreased the number of high affinity binding sites of RyR to $[^3]$H ryanodine (Du, Imredy et al. 1998). The association of $[^3]$H ryanodine to the SR membrane preparations was examined in both the presence and absence of a low concentration of CHAPS (0.1% (w/v)). A filtration method described by Hamilton and co-workers (1997) to define $[9, 21(n)^3]$H ryanodine association
kinetics was employed with some modifications. In this approach, skeletal SR membranes were incubated with 10nM tritiated ryanodine. Aliquots were taken at increasing time points and filtered through glass fibre filters. The filters were then washed and their radioactivity was quantified by liquid scintillation counting. As shown below in Fig. 3.6, the binding of [³H] ryanodine to SR membranes at a [Ca²⁺] of 100µM and in the presence of 5mM AMP-PCP was examined at 25min intervals.

![Figure 3.6: [³H] ryanodine association kinetics of isolated skeletal SR junctional terminal cisternae (JTC) in presence and absence of CHAPS (n=3). The presence of 0.1% (w/v) CHAPS leads to increased [³H] ryanodine binding to the isolated JTC. The assay was conducted in the presence of 5mM AMP-PCP and 100µM CaCl₂. Significant increase in ryanodine binding due to presence of CHAPS (p<0.01 (*) & p<0.001 (**)) is observed after 75 minutes incubation and onwards.](image)

As can be seen from Fig. 3.6, in the presence of CHAPS more ryanodine bound to the SR membranes (kept constant) as judged by increased radioactivity measurements. Since the above curves illustrate the influence of two factors; time and the presence or absence of CHAPS, a two-way analysis of variance (ANOVA) test can be employed to investigate the statistical significance. ANOVA is a robust test which is reliable even with non-adherence to the assumptions of equal variance and normal distribution (Munro 2005). On applying a two-way ANOVA, the addition of CHAPS
was found to significantly increase $[^3$H] ryanodine binding to the isolated SR membranes (p<0.0001). Incubation time was also found to significantly influence the $[^3$H] ryanodine binding (p<0.0001). Moreover, an interaction between the presence of CHAPS and the length of the incubation of the assay was shown by the two-way ANOVA test (p<0.01). A Bonferroni post-test was carried out to test for false positives and showed that CHAPS significantly enhanced ryanodine binding after incubation times of 75 minutes (p<0.01), 100 minutes (p<0.001), 125 minutes (p<0.001), 150 minutes (p<0.001), 175 minutes (p<0.001), 200 minutes (p<0.01) and 225 minutes (p<0.001); however, no significant effects could be attributed to CHAPS after 0, 25, 50 minute incubation times (p>0.05). Hamilton and co-workers (Needleman and Hamilton 1997) also observed similar effects. It has been suggested that CHAPS permeabilises the SR membranes which enables the access of ryanodine to its binding site on RyR. A previous study has shown that the high and low affinity ryanodine binding sites are located close to, or in, the membrane spanning region of the ryanodine receptor (Callaway, Seryshev et al. 1994). On the basis of the experiments here, a low concentration of CHAPS (0.1% (w/v)) was included in the assay; and since the effect of CHAPS was significant after 75 minutes, an incubation period of 100 minutes was employed.

Following this optimization of the radiolabelling technique for skeletal SR membranes, $[^3$H] ryanodine binding at different [Ca$^{2+}$] ranging from 0.05-10000µM was investigated as shown below in Fig. 3.7.
Figure 3.7: Skeletal SR JTC $[^3\text{H}]$ ryanodine binding at different [Ca$^{2+}$]. Log ([Ca$^{2+}$]/µM) plotted against $[^3\text{H}]$ count per minute (cpm) (n=3). $[^3\text{H}]$ ryanodine binding at a concentration of 10nM was measured in skeletal SR membranes. The assay was conducted in the presence of 5mM AMP-PCP and 0.1% (w/v) CHAPS. This figure illustrates that there is maximum $[^3\text{H}]$ ryanodine binding when [Ca$^{2+}$] $= 100\mu\text{M}$.

The plot above shows that there is an increased association of $[^3\text{H}]$ ryanodine with increasing [Ca$^{2+}$] reaching a maximum around 100µM. This is in agreement with previously published reports (Pessah, Waterhouse et al. 1985; Pessah, Francini et al. 1986; Michalak, Dupraz et al. 1988; Bull, Marengo et al. 1989; Chu, Diaz-Munoz et al. 1990; Valdivia, Valdivia et al. 1990; Zimanyi and Pessah 1991; Zimanyi and Pessah 1991; Coronado, Morrissette et al. 1994).

The amount of $[^3\text{H}]$ ryanodine bound non-specifically to the SR membranes was determined by repeating the assay but in the presence of ~1500 fold excess of cold ryanodine (see Fig. 3.8).
Figure 3.8: Determination of non-specific binding of $[^3H]$ ryanodine at 50µM CaCl$_2$ to (A) cardiac heavy SR membranes and (B) skeletal SR JTC (n=3). The presence of 1500 fold molarity excess of cold ryanodine greatly reduced the measured radioactivity by 82%, in case of cardiac SR, and by 89%, in case of skeletal JTC.

The non-specific binding of $[^3H]$ ryanodine was determined to be ~ 11% and 18% for skeletal and cardiac SR membranes respectively. In agreement with the SDS-PAGE analysis and with previous reports (Pessah, Waterhouse et al. 1985; Picher, Decrouy et al. 1997), the density of RyR2 receptors is ~ 1/6th that calculated for RyR1, i.e. 0.1pmol RyR2/mg cardiac SR and 0.6pmol RyR1/mg skeletal SR.

3.2.4 Solubilisation of RyR1 & RyR2

In order to isolate the RyRs it was first necessary to solubilise the channels in order to separate them away from the other SR integral proteins. The literature shows that multiple detergents have been used for solubilisation of the ryanodine receptor. Campbell and co-workers used digitonin for solubilisation of the skeletal muscle ryanodine receptor prior to its purification using immunoaffinity chromatography (Imagawa, Smith et al. 1987). However, in later studies, they employed CHAPS reporting that ryanodine receptors solubilised with digitonin lacked some properties associated with functional channels. For example, digitonin-purified channels did not activate in the presence of millimolar ATP and micromolar Ca$^{2+}$ nor were they responsive to ryanodine (Smith, Imagawa et al. 1988). The zwitterionic detergent Zwittergent 3-14 has been demonstrated to trigger dissociation of the 30S rabbit skeletal
muscle ryanodine receptor complex into 9S components. Solubilisation with this detergent resulted in loss of $[^3H]_\text{ryanodine binding}$ (Lai, Misra et al. 1989). There are now many reports describing the successful isolation of ryanodine receptors solubilised from sarcoplasmic reticulum membranes in a buffer containing CHAPS and soyabean lecithin (SBL). The presence of exogenous phospholipid in the medium, during the isolation and purification of RyRs, has been reported as essential for the stability of $[^3H]_\text{ryanodine binding}$ to solubilised RyRs (Anderson, Lai et al. 1989). Therefore, CHAPS/SBL buffer was employed for solubilisation of RyRs from the isolated SR membrane vesicles in concentrations (w/v) of 2% CHAPS / 1% SBL for RyR1 and 0.5% CHAPS / 0.25% SBL for RyR2 as described in sections (2.1.5) & (2.1.6) of the Materials and Methods. Ultracentrifugation was applied to remove unsolubilised material. The supernatant, containing the solubilised protein, was kept and the pellet was discarded. $[^3H]_\text{ryanodine}$ was used for pre-labelling of an aliquot of the SR membranes to spike each membrane suspension before solubilisation in order to track the progress of RyRs throughout the purification process.

### 3.2.5 Purification of RyR2

#### 3.2.5.1 Method 1: Immunoaffinity purification of RyR2

Sharma et al. (Sharma, Jeyakumar et al. 2006) described immunoaffinity purification of RyR2 using an antibody raised against a region corresponding to amino acids EKFQEQKTKEEE in the rabbit RyR2 sequence. This region of the sequence is highly conserved between RyR2 species including bovine as shown below in the sequence alignment (Fig. 3.9).
Figure 3.9: CLUSTAL W (1.83) (Thompson, Higgins et al. 1994) multiple sequence alignment of the RyR2 domain in different species. The top sequence is the target sequence. There is a high identity between the peptides for example, the bovine sequence (indicated by arrow) shares 87% homology with the RyR2 peptide against which the polyclonal antibody was raised. Key: "*" (asterisk) indicates positions which have a single, fully conserved residue, ":" (colon) indicates conservation between groups of strongly similar properties, and "." (period) indicates conservation between groups of weakly similar properties.

A large scale preparation of a polyclonal antibody (Ab) raised against the peptide sequence shown above was undertaken commercially (AMS Biotechnology). An aliquot of the supplied Ab was examined by SDS-PAGE finding a polypeptide profile consistent with an IgG. An aliquot of the Ab was then incubated with protein A/G matrix followed by centrifugation. The supernatant and resin beads were analysed by SDS-PAGE finding that the antibody bound to the protein A/G matrix with no antibody detected in the supernatant (gel not shown). The next step involved scaling up to make an immunoaffinity matrix to isolate RyR2. The solubilised SR proteins were then applied to the protein A/G – Ab beads and incubated overnight with gentle agitation. The bound protein was eluted by incubation of the beads with elution buffer containing an excess of the epitope peptide. The beads were then washed using the elution buffer without the epitope peptide and the posteluate was collected. Lane 4, in the gel below (Fig. 3.10) shows the polypeptide profile of the unbound protein, which unfortunately is virtually identical to the sample prior to applying to the matrix with a distinct band at ~565kDa that we have determined corresponds to RyR2. The lack of bands in lanes 5 &
6, the eluted fractions, confirmed that little or no RyR2 had bound to the immunoaffinity matrix. These findings were confirmed by western blotting (not shown). The experiment was repeated several times increasing the amount of SR membrane protein loaded, incubation times etc.

![Figure 3.10: Silver-Stained SDS-PAGE of the different stages of the immunoaffinity purification of RyR2.](image)

A possible explanation for these findings may be that the epitopic domain was not accessible within the bovine RyR2 quaternary structure. Another reason might be that the bovine sequence, despite exhibiting a high sequence homology to the rabbit peptide sequence, is sufficiently different to result in a modified secondary structure. Secondary structure predictions for the rabbit and bovine peptide sequences were undertaken using “Jpred 3” (Cole, Barber et al. 2008). As shown in Fig. 3.11 the rabbit peptide is predicted to form two alpha-helical domains (7 & 8 residues in length)
whereas the corresponding bovine region is calculated to form a single helix 19 residues in length.

![Image with sequences and helical structures]

**Figure 3.11: Jpred 3 (Cole, Barber et al. 2008) prediction of the secondary structures of the RyR2 rabbit sequence and the corresponding bovine sequence.** The RyR2 rabbit sequence is predicted to form two α-helices corresponding to amino-acid segments: (5-11) and (16-23). However, the corresponding bovine sequence is predicted to form a single α-helical structure extending from the amino-acid (5) to amino-acid (23). The dashed boxes indicate the sequences which might dictate the differences in the predicted secondary structures.

Close examination of the amino acid sequence finds that there is a single amino acid difference in the region separating the two putative helices, i.e. Threonine-14 in the rabbit sequence is an Alanine in the bovine peptide. This would suggest that the K-A-K sequence of residues promotes a geometry and hydrogen bonding that is more conducive with a helical formation compared to K-T-K. Although it should be noted that this is a prediction and not based upon experimental data, it does, however, provide a possible reason as to why the polyclonal antibody raised against the rabbit sequence failed to efficiently bind the bovine RyR2.

**3.2.5.2 Method 2: Size-exclusion chromatography**

Williams and co-workers (West, Smith et al. 2002) have reported a method using size-exclusion chromatography (SEC) for purification of the cardiac isoform of ryanodine receptor. This is described as a rapid purification method with only one step and thus has the advantage that protein degradation (e.g., proteolysis, tetramer dissociation) which could result from prolonged exposure of the receptor to a non-native environment is minimised. A Sephacryl S-300 HR size exclusion column compatible
with a low-medium pressure system was used. It is suitable for high resolution macro-molecule separation if the sample volume is properly adjusted in relation to the total column. For high resolution fractionation, a sample volume from 0.5-4% of the total column volume is recommended; therefore, sample concentration is normally needed prior to its application onto the column. However, concentrations above 70mg/ml protein should be avoided as viscosity effects may interfere with the separation.

In order to detect the RyR2 a portion of SR membranes (e.g. 1mg) was first labelled with [³H] ryanodine as described in Materials & Methods section 2.1.5.2. Approximately 30mg of SR membrane proteins were then spiked with the [³H] ryanodine labelled membranes, corresponding to a total of ~95,000cpm. This was followed by protein solubilisation as described above. The solubilised protein was concentrated and then loaded onto Sephacryl S-300 HR gel filtration media packed column of ~20ml bed volume (all procedures were carried out at 4°C). Aliquots from each eluted fraction were taken and investigated for their radioactivity as well as protein concentration (Fig. 3.12).
Figure 3.12: Radioactivity & protein concentration profile of fractions eluted from the Sephacryl S-300 column. The volume of each fraction collected was 0.75ml. 100µl aliquots were taken for radioactivity counting, represented by the primary Y-axis. The secondary Y-axis displays the protein concentration (mg/ml). The protein concentration profile reveals that the solubilised cardiac SR proteins have eluted in one large peak between fractions 15-30 with several smaller peaks throughout the gradient. Given the amount of radioactivity introduced into the sample it is clear from the low-intensity radioactive peaks (red plot) that the majority of the RyR2s has been lost in the concentration step preceding the size-exclusion chromatography.

It appears from the plot above that the protein has eluted in one large peak between fractions 7 and 55 that then tapers off and that this peak includes 4-5 protein components. It was estimated that ~3000cpm was loaded on the column. This figure could account for the low-intensity radioactive peaks obtained in the elution profile. These results were in contrast to those reported by Williams et al who reported elution of a clear peak of [³H] ryanodine (corresponding to isolated RyR2) in the void volume, however in these experiments, a much larger peak (corresponding to unbound [³H]
ryanodine) appeared some time later. It was also reported that the majority of the other SR proteins (~93%) eluted after the RyR2 peak fraction. At this stage of the project we had the opportunity to talk to Professor Williams who reported that his group no longer uses size exclusion methods as they found the protein was too diluted and they achieved larger yields and more reproducible results from a sucrose gradient method.

3.2.5.3 Method 3: Sucrose gradient fractionation
Cardiac SR membranes protein, 1mg of which was pre-labelled with [³H] ryanodine, was solubilised as described above. The solubilised protein was layered onto 5-25% linear sucrose gradient which was then centrifuged overnight at 4°C. The 0.5ml fractions were collected and analysed for radioactivity and protein concentration (Fig. 3.13).

![Figure 3.13: Separation of solubilised heavy cardiac SR by linear sucrose gradient fractionation. The numbers of the 0.5ml fractions are displayed by the X-axis. The primary and secondary Y-axes represent the counted radioactivity from 100µl aliquots and the protein concentrations of the isolated fractions respectively. The solubilised cardiac SR proteins have separated into two large protein peaks. The radioactivity curve shows an initial large peak that presumably corresponds to free unbound [³H] ryanodine. A few minor peaks were observed between fraction 22-24 and after fraction 35.](image)
Fig. 3.13 shows that there are two large protein peaks, one towards the top of the gradient and thus likely corresponds to small low molecular components with a second peak between fractions 17–26. There is a large radioactive peak at the top of the gradient which likely corresponds to free $[^{3}H]$ ryanodine. Western blot analysis of fractions spacing the gradient failed to detect RyR2.

The sucrose gradient method was modified and a step-wise gradient was employed. After “spiking” an aliquot with $[^{3}H]$ ryanodine, the isolated heavy cardiac SR was solubilised and layered onto step-wise sucrose gradient (5-25%) on top of 35% sucrose cushion which was then ultra-centrifuged overnight at 4°C. The collected 0.5ml fractions were investigated for $[^{3}H]$ and protein concentration (Fig. 3.14).
Figure 3.14: Separation of solubilised heavy cardiac SR by step-wise sucrose gradient fractionation. 0.5ml fractions were collected and analysed for protein concentration and radioactivity. The solubilised cardiac SR proteins have separated into two large protein peaks with a further smaller peak found between fractions 31 and 36 as highlighted by the dashed box, corresponding to ~20% sucrose. The lower panel shows an enlargement of the region encompassed by the dashed box showing that there is also a radioactive peak of $[^3]$H] ryanodine detected in these fractions.
Fig. 3.14 shows that there is a large radioactive peak at the top of the gradient which likely corresponds to free $[^3]$H ryanodine; however, there is also a distinct, albeit small peak between fractions 32 and 38 (at ~ 20% sucrose) that coincides with a protein peak. Silver staining and western blot analysis of fractions 34-38 confirmed the presence of RyR2. However, SDS-PAGE gels (5% and 12%) showed that the RyR2 sample was far from pure with several high molecular weight bands (Fig. 3.15 A) as well as some low molecular weight components (Fig. 3.15 B). Compared to the protein profile of the crude SR membranes, the step gradient has only partially purified the RyR2 sample.

**Figure 3.15: SDS-PAGE analysis of step-wise sucrose gradient fractionation of solubilised heavy cardiac SR.** (A): Silver stained 5% SDS-PAGE analysis of the polypeptide compositions of fractions 34, 36 and 38 reveals that in addition to the high molecular weight RyR2 band (indicated by the arrow) there are also multiple lower molecular mass protein bands. The far-left lane shows the protein profile for SR membranes for comparative purposes. The presence of RyR2 in each of these samples was confirmed by western blotting (upper panel). (B): Silver stained 12% SDS-PAGE to examine whether any low molecular weight proteins were co-fractionating with the RyR2. As can be seen there are several low molecular weight components in these fractions.
It is not known whether the lower molecular weight components represent accessory proteins e.g. FKBP12.6, that are bound to the the receptor.

3.2.5.4 Heparin agarose chromatogaphy

In order to remove the impurities, a further purification step was introduced; heparin agarose chromatography, a type of ion-change chromatography separating polar molecules based on their charge. Sucrose gradient fractions corresponding to the radioactive peak of the previous purification step were pooled together and diluted to 0.2M NaCl using salt free equilibrating buffer (as described before in the materials and methods section 2.1.5.3). The pooled fractions were incubated with the heparin agarose resin. The resin was then washed and subjected to a step-wise NaCl gradient elution (0.25-1M NaCl). 0.5ml fractions were collected and characterised by dot blot as shown below in Fig. 3.16.

![Figure 3.16: Dot blot (anti-RyR, Cat. No. ab2827) of fractions eluted from a heparin agarose column. Fractions 2 and 4 eluted in 0.25M NaCl, fractions 6 and 8 eluted in 0.3M NaCl, fractions 10 and 12 eluted in 0.4M NaCl, fractions 14 and 16 eluted in 0.5M NaCl, fractions 18 and 20 eluted in 0.55M NaCl, fractions 22, 24 and 26 eluted in 0.6M NaCl, fractions 28 and 30 eluted in 0.65M NaCl, fractions 32 and 34 eluted in 0.7M NaCl and fractions 36, 38 and 40 eluted in 1M NaCl. There is no obvious hybridization of any of the fractions with the RyR2 antibody.](image-url)
Fig. 3.16 shows the dot blot analysis of fractions eluted off the heparin agarose chromatography using an anti-RyR2 antibody. When the blot was first developed it appeared that there had been hybridization of fractions 18 and 20 however, upon reproduction this was not readily discernible and so it was not possible to draw firm conclusions. However, protein yields were very low with RyR2 complexes barely detectable. The method was scaled up in an attempt to improve the yield, however the eluted sample still contained many impurities and analysis by electron microscopy found the density of RyR2 to be very low.

Since our aim was to employ cryo-EM (cryo-EM needs a higher protein yield), purification of RyR1 was investigated. Dulhunty & co-workers (Wei, Hanna et al. 2009) have suggested that CSQ2 binds to both RyR1 and RyR2 and the differential functional effects of CSQ upon RyR1 and RyR2 is a property of the CSQ isoform and not RyR type, i.e. CSQ2 exerts similar physiological effects upon both RyR1 and RyR2, and thus it seemed sensible at this point of the project to investigate purification of RyR1.

3.2.6 Purification of RyR1

3.2.6.1 Sucrose gradient fractionation of solubilised skeletal sarcoplasmic reticulum JTC

Isolated skeletal sarcoplasmic JTC, 1.5mg of which was pre-labelled with $[^3]H$ ryanodine, were solubilised using 2% (w/v) CHAPS/ 1% (w/v) SBL. Western blotting was employed to confirm that the RyR1s had been solubilised. The solubilised protein was then layered onto a step-wise sucrose gradient (5-25% w/v) on top of a 35% sucrose cushion which was then ultra-centrifuged overnight at 4°C and 0.5ml fractions were collected.
Figure 3.17: Separation of solubilised skeletal SR JTC by sucrose gradient fractionation. 0.5ml fractions were collected from the top of the gradient and counted for radioactivity and assayed for the protein concentration. The radioactivity profile shows there is little free $[^3]H$ ryanodine (i.e. no large peak at the top of the gradient suggesting unbound label) with a main peak between fractions 34-46 coinciding with a small distinct protein concentration peak.

Fig. 3.17 shows that there is a relatively small radioactive peak at the top of the gradient which likely corresponds to free $[^3]H$ ryanodine. However, it is clear that there is a large peak between fractions 34 and 46 with a corresponding protein peak. SDS-PAGE and western blotting confirmed the presence of RyR1 in fractions 34-46 as shown below in Fig. 3.18. Other fractions through the gradient were also analysed for RyR1 but none was detected. To test the purity, the samples were also analysed for low molecular weight components using a 12% SDS-PAGE. No polypeptide bands were observed (gel not shown). This result was in contrast to the same experiment carried out to purify RyR2 (compare Fig. 3.15 and Fig. 3.18)
**Figure 3.18: SDS-PAGE analysis of step-wise sucrose gradient fractionation of solubilised Skeletal SR JTC.** (A): Coomassie blue stained 5% SDS-PAGE gel of the sucrose gradient fractions corresponding to the radioactive peak each of which shows a single high molecular weight band. (B): Western blot analysis using anti RyR (Cat. No. ab2868) confirmed the identity of the protein bands as RyR1.

The protein yield in the pooled sucrose gradient peak fractions was 0.83±0.35mg (n=4) from 10mg skeletal JTC proteins

RyR1 samples were initially examined by negative staining as shown in Fig. 3.19. Negative staining results in visualisation of the protein as a white density. As can be seen from the figure the sample is extremely homogenous characterised by square shaped protein complexes ~30nm across, indicated by the black arrows. On close examination, the four-fold symmetry and “clover-leaf” appearance of the complexes can be seen which match previous reports of RyR1 structure from EM studies.
Figure 3.19: EM images of purified RyR1: field of negatively-stained (2% (w/v) uranyl acetate) RyR1 displaying the expected dimensions, four-fold symmetry and a characteristic “clover-leaf” appearance (indicated by arrows). Scale bar = 100nm.

Therefore, it was decided at this stage to progress EM studies using purified RyR1 rather than RyR2.

3.3 Conclusion

The purification procedures for RyRs involved sub-cellular fractionation to isolate the SR membrane populations enriched with RyRs (heavy cardiac SR and skeletal JTC). A radiolabelling technique using [3H] ryanodine identified the presence of functional RyR populations in each SR preparations and was found to be optimized by inclusion of a low percentage of CHAPS (0.1% (w/v)).

RyR2 purification was unsuccessful despite using a range of techniques: immunoaffinity purification, size-exclusion chromatography and linear sucrose gradient fractionation. RyR2 was partially purified using a step-wise sucrose gradient fractionation. Further purification of RyR2 using a heparin-agarose column, unfortunately, led to low protein yields which were not sufficient for extensive structural studies and so samples were reserved for the interaction studies described in the subsequent chapters. The density of RyR2 compared to RyR1 is known to be
significantly less as discussed in the introduction to this thesis and thus this might account for the very low RyR2 yields despite attempts to scale up the preparations. RyR2 has been reported by numerous groups (Inui, Saito et al. 1987; Anderson, Lai et al. 1989; Sharma, Penczek et al. 1998) to be less stable than RyR1. It has been found that the addition of exogenous phospholipid (phosphatidyle choline) to solubilise RyR2 from the isolated heavy cardiac SR helps preserve function and thus soyabean lecithin was employed in the studies described here. However, an alternative strategy for future work may include employing a combination of phospholipids (e.g. phosphatidyle choline (PC), phosphatidyle ethanolamine (PE), and phosphatidyle serine (PS)) as described by (Anderson, Lai et al. 1989). However, since the presence of phospholipids has been found to be detrimental to the contrast of cryo-electron microscopy (Sharma, Penczek et al. 1998), efforts would be needed to optimize the total and relative concentrations of these phospholipids in order to balance the stability and increased yields of RyR2 with preservation of suitable sample conditions for cryo-EM structural studies.

Optimization of the purification protocol may also be a factor for improving both purity and yields of RyR2. A fast protein liquid chromatography (FPLC) system is now available in Kitmitto laboratory which may allow better separation of RyR2 from the contaminating proteins through a more controlled elution from the various types of chromatography columns. A S-300 size-exclusion chromatography column was employed in these studies but future attempts may consider employing a Sephacryl S-400 size-exclusion chromatography column which is compatible with an FPLC system and is reported to be suitable for the high resolution separation of globular proteins of Mr ranging from 2x10^4 to 8x10^6Da and thus may be more appropriate for isolation of the 2MDa RyR2. Furthermore, GE Healthcare provides this matrix in pre-packed HiPrep 26/60 columns (320ml column volume) allowing the application of sample volumes up to 13ml, which would remove the need for a concentration step in which the membrane proteins including RyR2 might be lost.

There are no reports in the literature describing the purification of RyR2 from bovine heart. The hearts used in this study were purchased from a local abattoir where
animals are slaughtered by the “Cut Throat” method in which the duration of dying is ~15 minutes. This relatively long duration of dying, when compared to the 30 seconds sacrifice time in case of pentobarbitone injection, could expose the cardiac muscle to prolonged stress with energy decompensation, cellular disintegration and proteolysis. The heart is less resistant to hypoxia (resulting from hemorrhagic shock on slaughtering the animal) than skeletal muscle which might be able to rely on anaerobic glycolysis for energy compensation due to an up-regulation of the glucose transporter system in response to hypoxia (Xia, Warshaw et al. 1997). Moreover, it was difficult to acquire the heart and place it in isotonic fluid at the time of slaughter and thus it may be that the condition of the heart proteins in general was not suitable for this type of study due to e.g. proteolysis.

Fortunately, the purification of RyR1 was much more successful with negative staining of the RyR1 showing distinct, well-separated protein and thus these samples were subsequently employed to conduct detailed EM structural studies.
Chapter 4: Purification of RyR-Accessory Proteins Triadin and Calsequestrin

4.1 Introduction

The SR luminal proteins cardiac calsequestrin (CSQ2), triadin 1 (Trd1) and junctin are proposed to form, with the ryanodine receptor (RyR), a macromolecular complex that acts as luminal calcium sensor (Zhang, Kelley et al. 1997; Beard, Wei et al. 2009). It is suggested that this quaternary complex is responsible for termination of the SR Ca\(^{2+}\) release during the diastole (Terentyev, Viatchenko-Karpinski et al. 2002). Acquired and genetic defects of the protein components are associated with a phenomenon called “diastolic Ca\(^{2+}\) leak” (Gyorke, Gyorke et al. 2002; Gyorke and Terentyev 2008). This diastolic Ca\(^{2+}\) leakage with the associated delayed afterdepolarizations (DADs) is the arrhythmogenic mechanism in catecholaminergic polymorphic ventricular tachycardia (CPVT) (Liu, Ruan et al. 2008). Whereas RyR2 mutations are responsible for an autosomal dominant form of catecholaminergic polymorphic ventricular tachycardia (CPVT1), mutations of CSQ2 account for an autosomal recessive form (CPVT2) (Katz, Arad et al. 2009). Three percent of documented cases of CPVT have been attributed to homozygous CSQ2 mutations e.g. a nonsense R33X (Postma, Denjoy et al. 2002; Katz, Arad et al. 2009). CSQ2-D307H and CSQ2-R33Q are examples of missense mutations which, if existing in homozygosity, are associated with resting bradycardia and syncope respectively (Chopra and Knollmann 2009). The recombinant approach for expression and purification of cardiac calsequestrin provides a potential tool to study CSQ2 mutations at the molecular level. Terentyev et al have studied the CSQ2-R33Q mutation reporting that the ability of the mutant CSQ2 to inhibit RyR2 incorporated into lipid bilayers is impaired (Terentyev, Nori et al. 2006) and that CSQ2-R33Q overexpressing myocytes have a lower threshold for SR Ca\(^{2+}\) release termination (Terentyev, Kubalova et al. 2008).

Triadin 1 (35kDa) is the predominant triadin isoform expressed in the mammalian myocardium. A previous study had indicated that another isoform, triadin 2 Mr ~40kDa, was also present in the myocardium (Guo, Jorgensen et al. 1996). However, Kobayashi et al demonstrated that the band observed at 40kDa was in fact the glycosylated form of triadin 1. The glycosylation site of triadin 1 was localised to
asparagine residue 75 (Kobayashi and Jones 1999). The literature suggests two possibilities regarding triadin function in cardiac muscle. (1) A simple anchoring role concentrating CSQ2 near the junctional phase of the SR. In other words, triadin an integral SR membrane protein binds CSQ2 which buffers Ca\(^{2+}\) in the vicinity of the RyR, and thus indirectly facilitates SR Ca\(^{2+}\) release. (2) It is directly involved in the regulation of the RyR2 Ca\(^{2+}\) release channel (Gyorke and Terentyev 2008). One of the approaches to study triadin function employed transgenic mice with cardiac overexpression of triadin (Kirchhefer, Neumann et al. 2001). Kirchhefer and co-workers found that overexpression of triadin 1 leads to cardiac hypertrophy and selective down-regulation of other junctional SR proteins. The isolated cardiac myocytes, from the transgenic mice overexpressing triadin, showed a substantial prolongation of decay of the Ca\(^{2+}\) transient, rate of cell shortening and rate of whole heart relaxation. These physiological effects of triadin 1 overexpression occurred at low stimulation frequencies and were not evident on mimicking the normal heart rate. Another approach to investigate the function of triadin has been to overexpress triadin in rat ventricular myocytes by an adenoviral vector carrying the full coding sequence of canine cardiac triadin 1 (Terentyev, Cala et al. 2005). Terentyev and co-workers reported a reduced amplitude of Ca\(^{2+}\) transient in cardiac myocytes overexpressing triadin 1 and presented evidence to suggest that this reduction was due to a substantial reduction in the SR calcium content in these cells. A reduction of the Ca\(^{2+}\) spark amplitude and an increase in the spark frequency were also observed in myocytes overexpressing triadin 1. Single channel recordings employing SR microsomes prepared from cells overexpressing triadin 1 revealed a 5-fold higher open probability (P\(_{0}\)) of RyR2 compared with control cells. Moreover, an arrhythmogenic Ca\(^{2+}\) release and delayed afterdepolarizations (DADs) were observed in triadin 1 overexpressing cardiac myocytes and found to be similar to those observed in myocytes expressing CPVT-linked CSQ2 mutants.

This part of the project was aimed at expression, purification and characterisation of the RyR recombinant auxiliary proteins calsequestrin 2 (CSQ2) and triadin 1(Trd1). Once the individual components were purified, protein-protein interaction studies were undertaken to probe how the putative luminal Ca\(^{2+}\) sensor is formed as described in Chapters 5 and 6.
Several protein expression systems have been developed for recombinant protein expression over the past few decades. The strengths and weaknesses of each of these systems concern yield, protein folding, post-translational modification (PTM), cost, speed and ease of use. The employment of mammalian cells such as CHO or HEK293 cells as an expression system of physiological proteins has the merits of the proper PTMs, correct folding and processing, however some drawbacks such as the slow multiplication rates, the possibility of unsuccessful transfection, overall lower yield and the relatively high cost should be considered. *Saccharomyces cerevisiae*, one of yeast protein expression systems, has the advantages of being an inexpensive, fast growing unicellular organism as well as benefiting from the physiological properties of an eukaryotic cell. A disadvantage of this yeast system is the different glycosylation patterns from higher eukaryotes (Braun and LaBaer 2003).

The baculovirus expression system (BEVS) for protein production in insect cells combines the advantages of the robust and relatively inexpensive cell culture with the advantage that most eukaryotic PTMs are properly executed (Possee 1997). The need for an initial generation of recombinant baculovirus, a rate-limiting step, and the need to maintain a high virus titre are challenges of the BEVS (Zhao, Chapman et al. 2003).

The cell-free expression system is a relatively new technique removing the need for the harsh process steps associated with introducing DNA into cells because of the absence of a cell membrane. Expression systems from eukaryotic cell lysates have the additional superiority of executing appropriate PTMs. In addition to its high cost, the components of the commercially available cell-free expression systems are usually conventional standards, and this can be another disadvantage if salt or buffer concentrations need to be adjusted. Different requirements for the concentration of various ions, such as magnesium ions, is usually the case because the transcription and translation processes are very specific (Braun and LaBaer 2003).

Of particular interest to this research project is *Escherichia coli* (E.coli) which is the simplest and the most widely used organism for protein expression. A genetically compatible plasmid is normally used for expression induction in *E.coli*. The expression plasmid genetically includes the replication origin (replicon) *(ori)* a common example of
which is ColE1 replicon derived from pBR322. Furthermore an antibiotic resistance region is usually included within the plasmid sequence, and resistance to ampicillin, kanamycin, chloramphenicol or tetracycline are most common conferred by the recombinant expression plasmid. Another genetic element of the expression plasmid is the transcriptional promoters. The \emph{lac}, \emph{tac} and \emph{trc} promoter systems are examples of \emph{E.coli} promoter systems that are induced by isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) and are regulated by catabolite repression and the metabolic state which is represented by cyclic AMP level. An advantage shared by \emph{lac}, \emph{tac} and \emph{trc} promoter systems is the low basal expression levels which indicate a nature of tight control. The \emph{tac} and \emph{trc} promoters are stronger than the \emph{lac} promoter allowing the protein of interest to constitute up to 15-30\% of total cell proteins (Sorensen and Mortensen 2005; Terpe 2006). The requirements for ideal expression strains are to be deficient in the most harmful natural proteases, to maintain the stability of the expression plasmid and to confer the genetic elements relevant to the expression system (e.g. DE3). \emph{E.coli} BL21 and its derivatives, growing vigorously in minimal media and being deficient in \emph{ompT} and \emph{lon} proteases, are commonly employed in standard recombinant protein expression. \emph{E. coli} BL21(DE3) is a mutant which can be helpful for the soluble expression of membrane proteins (Sorensen and Mortensen 2005).

\textit{The \emph{E.coli} expression system has familiar genetics and is relatively simple and inexpensive. The fast doubling rates as well as the potential high protein yield are also among the outstanding advantages of \emph{E.coli} expression system (Sorensen and Mortensen 2005). Lack of eukaryotic PTMs and low solubility of some proteins can, however, be limitations in certain studies. Employing \emph{E.coli} for expression of recombinant integral membrane proteins (IMPs) might be a real challenge. Bacteria often have difficulty with the expression, folding, assembly and stabilisation of eukaryotic IMPs as a result of having a different bilayer composition and lacking the protein processing machinery of eukaryotic secretory pathways (Midgett and Madden 2007).}

\textbf{4.1.1 Aims and Objectives}

The main aim of this part of the thesis research was to express and purify (i) calsequestrin 2 (CSQ2) and (ii) Triadin 1 (Trd1). To achieve this goal human
calsequestrin 2 (hCSQ2) was expressed in *E.coli*. Attempts to express mouse cardiac triadin isoform 1 (Trd1), in *E.coli*, were also carried out. *E.coli* was employed as a high yield expression system since high yields were desirable for the structural protein-protein interaction elements of this project.

There have been numerous reports of successful expression of CSQ1 and CSQ2 in *E. coli*, therefore coupled with the availability of hCSQ2 in an *E. coli* compatible vector (pGEX-6P-3 vector), it seemed sensible to use this system to try to produce CSQ2 as a GST tagged protein. A protocol for expressing and purifying hCSQ2 with cleavage of the GST partner has already been developed by a member of the Kitmitto lab although some optimisation of the method was developed here.

A previous member of the Kitmitto lab has also produced both full length mouse cardiac Trd1 and the luminal domain in a pTrcHis2A vector suitable for expression in *E. coli*; however, no work was undertaken to express the protein. Glycerol stocks were provided. Trials to express the triadin constructs in *E.coli* were undertaken. Trd1 traverses the SR membrane with a putative single transmembrane helix, which might be challenging to overexpress as it is a membrane protein. Therefore, if difficulties were encountered with expression of the full-length Trd, studies would focus upon the luminal domain which should be soluble.

Characterisation of the expression and purification of the recombinant proteins was done by SDS-PAGE and western blotting. To determine whether the expressed proteins were folded circular dichroism (CD) was employed to investigate the secondary structure of the purified protein.

**4.2 Results and Discussion**

**4.2.1 Expression of cardiac triadin isoform 1 (Trd1); full length and luminal domain**

Previously, in the Kitmitto laboratory, total mouse ventricular RNA was isolated and subjected to RT-PCR, to create first strand cDNA. PCR, using primers designed against Trd1, was employed to obtain specific amplicons of interest; genes encoding full length mouse cardiac triadin isoform 1 (831-bp) and C-terminal mouse cardiac triadin
isoform 1 (630-bp). Formation of the recombinant vector was carried out by double digestion of triadin cDNA and pTrcHis2A (Fig. 4.2) by restriction enzymes SalI-HF™ and BamHI-HF™ followed by a ligation step using T4DNA ligase. A heat shock protocol was employed to transform E.coli BL21 (DE3) chemically competent cells with the recombinant vector. Presented below is a sequence alignment of mouse and human triadin 1 illustrating high sequence homology between the two triadin species.

| Mouse     | MTEITAEGNASTTTTVIDNKNGCIPKSPGKVLKKSVTETDIVTFSSPAAW |
| Human     | MTEITAEGNASTTTTVIDNKNGCIPKSPGKVLKRTVTEDIVTFSSPAAW |

| Mouse     | LLVIALITWSAVAVMFDDLVDYKNFSASSIAKIGSDPLKLVDNVEETT |
| Human     | LLVIALITWSAVAVMFDDLVDYKNFSASSIAKIGSDPLKLVRDAMEETT |

| Mouse     | DVIYGFSSLLSDIISSGDEDEDDEDDEDIDKGEIEEPPPLKREIHQKEAE |
| Human     | DVIYGFSSLLSDIISSEEDDEDDGDEDSKGEIDPEPLKKEIHDDKTE |

| Mouse     | KEEKPEEKIQTKASHREKGEKKEKKNKGEKPEKATHKEKKEKKEPTEK |
| Human     | KQEKPERKIQTKVTHKEKGEKKEKREKEKKEKKEKKEKKEKKEPTEK |

| Mouse     | MMAKEDKKIKTKKEEKEAKKEMKVKEKTKPAAKVKEVKEVKEPTEK |
| Human     | TVAKEQKAKTAKKEESKEETKKEKKEKKEKKEKKEKKEPTEK |

| Mouse     | KDDKEMPAS--HEQKGGHSRRBBQ---------------------QEVQRE |
| Human     | KDKKANKVSKHEQKGGQSPAIPPLPETRQARPTPSPALEGKYYFFS |

**Figure 4.1: CLUSTAL W (1.83) (Thompson, Higgins et al. 1994) multiple sequence alignment of mouse and human triadin 1.** There is high sequence homology (74%) between the two Trd species. Key: “*” (asterisk) indicates positions which have a single, fully conserved residue, “:” (colon) indicates conservation between groups of strongly similar properties, and “.” (period) indicates conservation between groups of weakly similar properties.
The Trd (full-length and luminal domain) was ligated into a pTrcHis2A vector for expression. This vector was selected due to the fact that the protein would be expressed with a C-terminal His-tag which will be useful for subsequent structural studies. There was no established protocol within Kitmitto laboratory for the expression of either full length or of the C-terminal mouse cardiac triadin isoform 1. Therefore the initial focus of this study was to try to express full length triadin 1 (Trd1) in E. coli; as a high yield was required for the proposed structural studies. Standard expression protocols were first tested, i.e. a post-induction temperature of 37°C and various concentrations of IPTG. Cells were lysed, and the pellets and supernatants were analysed by SDS-PAGE. However, as can be seen from the gel in Fig. 4.3 panel A, there was no obvious band at ~35kDa that would correspond to Trd1 in either the pellet or supernatant lanes. Comparison of lane 4 (control pellet, no IPTG) with the polypeptide

Figure 4.2: pTrcHis2A vector map (Invitrogen), illustrating origin of replication (pBR322), regions coding for ampicillin resistance, lac repressor (lacI'), trc promoter region (P_trc), lac operator (lac O), ribosome binding site (RBS), expression ATG, multiple cloning site (MCS) myc epitope and His\textsubscript{6} tag as well as the various restriction sites including BamHI (416) and SalI (511).
profiles in lanes 1-3 also finds no difference in the protein components indicating that triadin has not formed inclusion bodies. Furthermore, there were no additional protein bands in the supernatant fraction compared to the control sample. Therefore, under these conditions, Trd1 was not expressed. An explanation for this could be that at 37°C, the post-induction temperature, might have led to the production of non-functional protein with activation of unfolded protein response, leading to protein degradation. Modifying the temperature of the cell culture system can reduce the rate of protein expression (Midgett and Madden 2007). Therefore, further expression trials were undertaken at 28°C. Panel B in Fig. 4.3 shows again that there was no detectable Trd1 in either the pellet or supernatant of the cells. Western blotting (not shown) using an anti-His primary antibody also produced no evidence of Trd1 expression.

Figure 4.3: Comparison of IPTG concentrations and temperature on the production of recombinant full length mouse Trd1. (A): 4 h at 37°C, (B): 5 h at 28°C. Lanes 1, 2, 3, 4 are of resuspend pellets of lysed cells induced by 2, 1, 0.5, 0mM IPTG respectively. Lane 5, 6, 7, 8 correspond to supernatants of lysed cells (soluble protein fractions) induced by 2, 1, 0.5, 0mM IPTG respectively. Lanes 4 & 8 correspond to resuspend pellet and supernatant of lysed control cells, respectively. Lane 9 is of kaleidoscope marker. The recombinant proteins were analysed by SDS-PAGE and stained with Coomassie blue. Trd1 has an expected molecular weight of ~35kDa. It can be seen that the expression of the full length Trd1 was unsuccessful.

Kobayashi and Jones have purified recombinant cardiac triadin from Sf21 insect cells infected with baculovirus encoding canine cardiac triadin 1 (Kobayashi and Jones
Gyorke and co-workers have also expressed canine cardiac triadin in bacterial BJ5183-Ad electroporation competent cells. They proceeded to use the cell lysates, without any further purification (Terentyev, Cala et al. 2005). Furthermore, mammalian cells, rat myogenic L6 cells, have been used for expression of skeletal triadin (~95 kDa) in a previous study (Marty, Thevenon et al. 2000). The literature shows no evidence of successful bacterial expression and purification of full length skeletal or cardiac triadin. Accordingly, further efforts were directed towards expression of the C-terminal luminal domain of the mouse cardiac triadin isoform 1 (amino acids 69-277) since this should largely be soluble. Presented below to illustrate this point is a hydrophobicity plot and predicted topology for mouse cardiac triadin 1 generated using the TopPred (0.01) bioinformatic tool (Claros and von Heijne 1994) (Fig. 4.4 & Fig. 4.5). The luminal domain also houses the triadin KEKE motif (amino acids 200-224) which is thought to be essential for binding of RyR (Lee, Rho et al. 2004), CSQ (Kobayashi, Alseikhan et al. 2000) and histidine-rich Ca\textsuperscript{2+} binding protein (HRC) (Lee, Kang et al. 2001).

![Figure 4.4: Hydrophobicity plot of mouse cardiac triadin 1 as predicted by TopPred (0.01) (Claros and von Heijne 1994). The cloned C-terminal luminal domain of mouse cardiac triadin 1 (amino-acid residues 69-277) can be seen to be mainly hydrophilic with hydrophobicity values below the cutoff.](image-url)
**Figure 4.5:** TopPred (0.01) prediction (Claros and von Heijne 1994) for the topology of mouse cardiac triadin 1. Triadin is composed of an N-terminal cytoplasmic domain (47 amino acids), a single transmembrane domain (21 amino acids) and a C-terminal luminal domain (209 amino acids) including the triadin KEKE motif (the position of which is roughly indicated by the dashed ellipsoid) which is thought to bind RyR2 and/or CSQ2.

SDS-PAGE, Coomassie blue staining and western blotting were employed to characterise the expression trials of the luminal domain of mouse triadin 1 at different IPTG concentrations, temperatures and induction times. The Coomassie blue stained gels of these trials are presented below in Fig. 4.6 and Fig. 4.7.
Figure 4.6: Comparison of IPTG concentrations and length of induction on the production of the luminal domain of mouse Trd1 at 37°C. (A) 1 hr, (B) 2 hr (C) 3 hr and (D) 5 hr. Lanes 1, 2, 3, 4 are of resuspend pellets of lysed cells induced by 2, 1, 0.5, 0mM IPTG respectively. Lane 5, 6, 7, 8 correspond to supernatants of lysed cells (soluble protein fractions) induced by 2, 1, 0.5, 0mM IPTG respectively. Lanes 4 & 8 correspond to resuspend pellets and supernatants of lysed control cells, respectively. Lane 9 is of kaleidoscope marker. The recombinant proteins were analysed on SDS-PAGE and stained with Coomassie blue. The luminal domain of Trd1 has an expected molecular weight of ~26kDa. It can be seen that efforts to express the luminal domain of Trd using these conditions were not successful.
Figure 4.7: Comparison of IPTG concentrations and temperature on the production of the luminal domain of mouse Trd1 at 28°C; (A) 1h, (B) 2h and (C) 5h as well as at 20°C; (D) 3h. Lanes 1, 2, 3, 4 are of resuspend pellets of lysed cells induced by 2, 1, 0.5, 0 mM IPTG respectively. Lane 5, 6, 7, 8 correspond to supernatants of lysed cells (soluble protein fractions) induced by 2, 1, 0.5, 0 mM IPTG respectively. Lanes 4 & 8 correspond to resuspend pellets and supernatants of lysed control cells, respectively. Lane 9 is of kaleidoscope marker. The recombinant proteins were analysed on SDS-PAGE and stained with Coomassie blue. The luminal domain of Trd1 has an expected molecular weight of ~26kDa. It can be seen, again, that efforts to express the luminal domain of Trd using these conditions were not successful.
Although in some of the gels e.g. Fig. 4.6 C (lanes 5-7, soluble fractions) there does appear to be a band around 26kDa there is also a faint band in the control lane. Western blotting (not shown) did not detect the expression of Trd in any of the fractions. Therefore, as can be seen in the gels above expression of the luminal domain of mouse triadin 1 has not been achieved.

There are some reports using *E. coli* for the expression of various soluble domains of triadin. For example, Guo and Campbell, 1995, have reported expression and purification of both luminal (amino acids 100-706) and cytoplasmic (amino acids 1-47) domains of skeletal triadin as GST fusion proteins employing *E. coli* DH5α cells (Guo and Campbell 1995). However, this report did not show any gels or western blots of the purified triadin domains, but rather used GST-triadin domains in precipitation assays (i.e. the GST fusion proteins were immobilised on a glutathione matrix) to investigate the association of the luminal portion of triadin with RyR1 and CSQ1 in skeletal muscle homogenate. Similarly, Caswell *et al* (Caswell, Motoike *et al.* 1999) have also expressed various skeletal triadin domains as GST fusion proteins in *E. coli* BL21 cells, but employed the GST-tagged peptides to localise the ryanodine receptor binding site within the skeletal muscle triadin sequence. Furthermore, previous studies have expressed and purified the conserved region of the luminal domain of triadin (amino acids 69-264) as a GST fusion protein (H-triadin) from *E. coli* BL21 cells (Guo, Jorgensen *et al.* 1996; Zhang, Kelley *et al.* 1997). However, these reports, again, do not show any gels or western blots of the purified triadin domains, but rather employed the GST-tagged proteins in precipitation assays revealing interactions with junctin in CHAPS solubilised cardiac junctional SR vesicles and with purified RyR2 (Zhang, Kelley *et al.* 1997)

Inefficiency of transcription and/or translation can provide an explanation for unsuccessful expression trials. There might be a difference between the codon usage of the *E. coli* and the eukaryotic protein to be expressed. The mRNA transcript of a eukaryotic gene might contain rare codons that require one or more tRNAs which may be rare or lacking in the expression host. An insufficient tRNA pool can lead to translational errors such as translational stalling, premature translation termination,
translational frameshift and amino acid mis-incorporation (Terpe 2006). Another possible cause of inefficient translation is instability of the mRNA being readily degraded by RNases due to improper RNA folding and/or inefficient polyadenylation (Sorensen and Mortensen 2005). Proteolysis of the expressed proteins by proteases of the host E. coli strain can also be a problem. Time constraints prevented further attempts to try and address these problems, and an alternative approach was taken using a synthetic triadin KEKE motif. However, if time had allowed then another approach would have been employed to produce a glutathione S-transferase (GST) or maltose binding protein (MBP) fusion recombinant protein. These fusion tags adopt a stable globular fold and so enhance the solubility of their fusion partners. Furthermore, efficient initiation of the translation is usually ensured by GST and MBP at the N-terminus of the recombinant proteins (Waugh 2005).

4.2.2 Expression, purification & characterisation of cardiac recombinant calsequestrin

Full length human CSQ2 (hCSQ2) was inserted between restriction sites SalI and NotI of a pGEX-6P-3 vector (Fig. 4.8) and transformed into E. coli BL21 (DE3) competent cells.
Figure 4.8: pGEX-6P-3 vector map (GE Healthcare), illustrating origin of replication (pBR322), regions coding for ampicillin resistance, lac repressor (lacI<sup>q</sup>), tac promoter region (Ptac) and glutathione S-transferase (GST) tag, the various restriction sites including SalI (963) and NotI (974) as well as PreScission Protease cleavage site.

Using a protocol established in the Kitmitto lab, hCSQ2 was expressed as a GST (N-terminal GST-tag) fusion protein employing 0.5mM IPTG, 28°C post-induction temperature and 5 hours as induction period. hCSQ2 was purified from the expressing cells by following the protocol described in the Materials and Methods section 2.2 which involved an initial lysozyme cell lysis and Glutathione Sepharose™ 4B resin purification of GST-CSQ. The elution of GST-hCSQ2 was investigated at different concentrations of reduced glutathione (10mM, 20mM and 40mM). Each experiment employed one concentration of reduced glutathione; elution was performed in 3 successive steps the first of which was either done after 30 minutes incubation at 4°C or
after 10 minutes incubation at room temperature. The results of these optimisation experiments are shown below in Fig. 4.9.

**Figure 4.9: Optimisation of reduced glutathione elution of GST tagged hCSQ2.** Lane 1 corresponds to the Kaleidoscope marker. Lanes 2, 3, 4 correspond to 1st, 2nd and 3rd elution (respectively) after incubation with 10mM reduced glutathione for 10 minutes at RT. Lanes 5, 6, 7 correspond to the 1st, 2nd and 3rd elution (respectively) after incubation with 20mM reduced glutathione for 10 minutes at RT. Lanes 8, 9, 10 correspond to the 1st, 2nd and 3rd elution (respectively) after incubation with 40mM reduced glutathione for 10 minutes at RT. Lanes 11, 12, 13 correspond to the 1st, 2nd and 3rd elution (respectively) after incubation with 20mM reduced glutathione for 30 minutes at 4°C. Lanes 14, 15, 16 correspond to the 1st, 2nd and 3rd elution (respectively) after incubation with 40mM reduced glutathione for 30 minutes at 4°C. The purified recombinant proteins were analysed on SDS-PAGE, and stained with Coomassie blue. It is clear that a single incubation with 40mM reduced glutathione for 30 minutes at 4°C was sufficient to elute all bound hCSQ2.

Since the elution volume was essentially the same in all conditions, the lane showing the strongest band suggestive of hCSQ2-GST (~72kDa) was lane 14. Based on the results illustrated above in Fig. 4.9, elution of GST-hCSQ2 from Glutathione Sepharose™ 4B resin was always performed by incubation with 40mM reduced glutathione for 30 minutes at 4°C with a single elution step which was enough to elute all the bound recombinant protein as shown by the absence of protein in lanes 15 and 16 representing a 2nd and 3rd elution step respectively. The yield of the purified GST-hCSQ2 was 0.7±0.3mg (n=2) from ~200ml cell culture. In order to confirm the identity of the band with a molecular mass corresponding to GST-hCSQ2, western blotting using anti-CSQ was performed as shown below in Fig. 4.10. Western blot analysis using anti-GST confirmed the integrity of the GST tag within the eluted GST-hCSQ2.
Figure 4.10: Western blot analysis of purified GST-hCSQ2 using anti-CSQ (lane A) & anti-GST (lane B). Distinct bands confirmed the identity of GST-hCSQ2 and the integrity of the GST tag.

However, to purify hCSQ2 the GST fusion tag was cleaved in an overnight incubation with PreScission Protease as described in the Materials and Methods section 2.2. PreScission Proteases works accurately having stringent sequence specificity (LEVLFQ/GP), however the specificity determinants that are located on the C-terminal side of the scissile bond (/) (glycine and proline residues) remain on the purified hCSQ2 after the removal of the GST N-terminal tag. The yield of the purified hCSQ2 was 0.4±0.1mg (n=4) from ~200ml cell culture. The purified hCSQ2 was analysed by SDS-PAGE with Coomassie blue staining and western blotting as illustrated below in Fig. 4.11.

Figure 4.11: SDS-PAGE analysis for purified hCSQ2. (A): Coomassie blue stained gel showing a single band at ~46kDa. (B): The corresponding western blot using anti-CSQ confirmed the identity of the band.
The single band on the Coomassie blue stained gel, at the approximate position of 46kDa, indicates a high degree of purification. The identity of the band as CSQ2 was confirmed by western blotting.

Circular dichroism (CD) was employed to examine the secondary structure of the purified hCSQ2. The profile of the CD spectrum, shown below in Fig. 4.12, exhibits a double minimum at 208 and 220nm indicative of α-helical content. From the CD analysis and according to the Sholtz algorithm (Scholtz, Qian et al. 1991), the calculated percentage of the α-helical content was ~22%.

Figure 4.12: 190-260nm CD spectrum of purified hCSQ2 in Tris Buffer at 25°C. The spectrum confirms the folding of hCSQ2 with an α-helical secondary structure as evidenced by a double minimum at 208 and 220nm.

4.3 Conclusion

Expression of full length mouse cardiac triadin isoform 1 (Trd1), in a pTrcHis2A vector in E. coli BL21 (DE3), was unsuccessful as shown by SDS-PAGE, Coomassie blue stain and western blot characterization. Sequencing of the plasmid was done by a previous member in the Kitmitto laboratory therefore it was possible that the E.coli stock was devoid of the Trd1 recombinant plasmid. Triadin is an eukaryotic protein which requires eukaryotic translocon machinery in order to be transported from cytosol into the cisternal space of the endoplasmic reticulum for correct processing. E. coli BL21 (DE3), used in this PhD project, lacks this machinery which might result in misfolding of the expressed triadin. Misfolding of the expressed triadin could be also a
consequence of the different lipid composition of the SR membrane, where triadin is natively integrated, and that of the *E. coli* cytoplasmic membrane which lacks essential lipids such as cholesterol, cholesterol esters and sphingomyelin which are required for proper folding of membrane proteins. Misfolded triadin might be easily degradable by *E. coli* proteases accounting for its unsuccessful expression. Another hypothesis is the physical limitation of the membrane capacity of the *E. coli* for accommodating the overexpressed triadin which may jeopardize the optimal ratio between lipid and membrane proteins and hence induce stress responses in the host cell including protein degradation. However, the expression of the soluble C-terminal domain was also unsuccessful. This might suggest that triadin is toxic to the *E. coli* strain. In addition, the rate of triadin polypeptide chain elongation in *E. coli* might be too fast to allow the protein to develop its native conformation leading to exposure of hydrophobic parts with subsequent aggregation and formation of inclusion bodies. However, what might argue against this explanation is the absence of bands suggestive of either full-length or C-terminal triadin in the pellet lanes in Fig. 4.3, 4.6 and 4.7. The fast eukaryotic protein translation in prokaryotes could also contribute to misfolding of the expressed protein (due to overwhelming the folding machinery of the *E. coli*) making it again easily degradable by proteases. The “codon bias” problem and instability of the mRNA could also provide an explanation. Triadin isoform 1 is a relatively small protein (~35kDa) with a single transmembrane segment, and so further attempts to use the *E. coli* system for its expression in the future may still be worthwhile. An option would be trying to reduce the rate of expression of triadin by lowering the cultivation temperature (e.g. 16°C) and/or employing weak, yet tightly regulated promoters. Furthermore, co-expression of chaperones might help to accelerate the folding process in the *E. coli* expression system in order to cope with the increased protein load. Successful expression of Trd1 in *E. coli* might also consider parameters such as pH, osmolarity and/or aeration. Future attempts for Trd1 expression might also involve employing a GST or similar (e.g. MBP) fusion tag since these proteins adopt a stable globular conformation that may contribute to an enhancement of the solubility and stability of the expressed triadin.
A further strategy would be targeting a smaller luminal domain of Trd1 that does not include the relatively hydrophobic segment (e.g. amino acid residues 69-110) emerging from the SR membrane as shown in the hydrophobicity plot (Fig. 4.4). A more hydrophilic construct may enhance the solubility of the expressed triadin domain. Interestingly, secondary structure analysis, Jpred 3, (Cole, Barber et al. 2008) of mouse Trd1 revealed that the segment of amino acid residues between 69-88 is predicted to be mainly composed of random coil which might present a target for proteolytic cleavage. Removal of these putative hydrophobic or random coil segments from the Trd1 domain should not impair binding to RyR and/or CSQ, the focus of this research project, as the triadin KEKE motif, amino acid residues 210-224, which is believed to bind RyR and/or CSQ is preserved.

The *E.coli* BL21 (DE3), strain employed in this research project, has been shown to be promising for membrane protein expression; however there are other strains, e.g. Walker (C41(DE3) and C43(DE3)) and Lemo21 (DE3) that have also been shown to be successful for the expression of membrane proteins that were toxic to BL21(DE3) (Freigassner, Pichler et al. 2009), and thus might be more suited for Trd1 expression. Therefore future work may be directed at investigating other *E.coli* strains for Trd1 expression. However, another consideration that might hinder Trd1 stability when expressed in *E.coli* is PTMs such as glycosylation of Trd1. Triadin has been shown to be a glycosylated protein (the glycosylation site was identified at ASP 75) (Kobayashi and Jones 1999). Glycosylation might not be only essential for the function of the protein but also for a proper folding. As discussed in the Introduction to this Chapter, *E.coli* as a prokaryotic expression system is not capable of performing posttranslational modifications, including glycosylation, of the expressed eukaryotic proteins, and this might interfere with the successful expression of Trd1. Therefore, future work may also consider employing a “yeast” system to express Trd1. For example, *Schizosaccharomyces pombe* (*S.pombe*) is a yeast system capable of glycosylating the expressed mammalian proteins in a mammalian-like pattern (Takegawa, Tohda et al. 2009), and thus might be suitable for Trd1 expression in the future. Finally, higher eukaryotic systems, e.g. BEVS (which was employed before to express and purify
cardiac triadin (Kobayashi and Jones 1999)), mammalian cells and cell-free expression systems, should also be considered for future trials to express Trd1.

Fortunately hCSQ2 in a pGEX-6P-3 vector in *E.coli* BL21 (DE3) was successfully expressed and purified in yields suitable to continue this research work investigating the interactions with RyR1 and RyR2 as described in the subsequent chapters.
Chapter 5: Formation of a Luminal Ca-Sensing Complex: an Investigation into RyR Regulation by Triadin and CSQ2

5.1 Introduction

Activation of RyR2s by cytosolic Ca\(^{2+}\) is regulated by a process known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) as discussed in the introduction to this thesis. Ca\(^{2+}\) accumulated inside the SR (luminal [Ca\(^{2+}\)]) has also been shown by several groups to play a crucial role in controlling SR-mediated Ca\(^{2+}\) signalling in cardiac muscle (e.g. (Gyorke, Gyorke et al. 2002; Terentyev, Viatchenko-Karpinski et al. 2002)). Changes to the luminal [Ca\(^{2+}\)] are responsible for termination of CICR and cause a refractory state that continues until recovery of luminal [Ca\(^{2+}\)]. Disturbance of RyR2 sensing of luminal Ca\(^{2+}\) leads to diastolic Ca\(^{2+}\) leak, and hence arrhythmias and heart failure (Gyorke and Terentyev 2008). It is now generally accepted that the luminal Ca\(^{2+}\) sensor is a quaternary complex formed by the interaction of RyR with SR luminal proteins; calsequestrin, triadin and junctin. There are several reports demonstrating that both triadin and junctin couple calsequestrin to RyR at the junctional face membrane (Guo and Campbell 1995; Zhang, Kelley et al. 1997). Triadin, via a KEKE motif (amino acids 200-224) (Kobayashi, Alseikhan et al. 2000), is thought to bind to the asp-rich region at the carboxyl-terminus of calsequestrin (Shin, Ma et al. 2000). Triadin-CSQ binding has been shown to be Ca\(^{2+}\) dependent with the interaction enhanced at low [Ca\(^{2+}\)] (Kobayashi, Alseikhan et al. 2000; Shin, Ma et al. 2000). Shin et al have described the asp-rich region of CSQ as a major Ca\(^{2+}\) binding motif, and hence suggested that elevated [Ca\(^{2+}\)] could directly compete with triadin for the asp-rich motif or, alternatively, promote conformational changes to CSQ structure abolishing its interaction with triadin or junctin. A direct physical interaction between triadin and the ryanodine receptor has been reported (Guo and Campbell 1995; Lee, Rho et al. 2004). The triadin KEKE motif has also been shown to bind to RyR1 involving the negatively charged residues in the second intraluminal loop of RyR (Lee, Rho et al. 2004). Ryanodine receptor interactions with junctin and triadin are predicted to be Ca\(^{2+}\)-independent (Zhang, Kelley et al. 1997). An unexpected finding by Zhang et al, 1997, was Ca\(^{2+}\)-independent binding between triadin and junctin. Although it is generally believed that triadin and junctin mediate an interaction between CSQ2 and RyR2, there is some evidence to suggest that
there is also direct binding between RyR2 and CSQ2 (Wei, Hanna et al. 2009). For example, Varasanyi and co-workers have employed surface plasmon resonance (SPR) demonstrating an interaction between the ryanodine receptor and calsequestrin (Herzog, Szegedi et al. 2000).

Of particular interest to this research is the interaction of RyR with triadin and calsequestrin. The literature presents conflicting views regarding the regulatory function of triadin upon the RyR channel activity. Using single channel recording, Gyorke et al have reported an increase in purified native RyR activity at low luminal Ca\(^{2+}\) (20µM) upon the addition of recombinant triadin to the luminal side of the channel but found that increasing the luminal Ca\(^{2+}\) to 5mM had no effect on the channel activity. However, they did not investigate the regulatory effect of triadin on RyR activity at luminal Ca\(^{2+}\) concentrations between 20µM and 5mM (Gyorke, Hester et al. 2004). In another studying employing lipid bilayers, RyR activity was shown to be increased by triadin at a luminal Ca\(^{2+}\) concentration of 1mM (Wei, Gallant et al. 2009). As reviewed by Gyorke and Terentyev and consistent with these findings, altered Ca\(^{2+}\) handling including slowed relaxation and Ca\(^{2+}\) transient decay and depressed contractile strength is exhibited in cardiac myocytes from triadin overexpressing mice only at low stimulation frequencies (Gyorke and Terentyev 2008). In contrast, Ohizumi and co-workers have shown that \(^{3}H\) ryanodine binding activity (which reflects the channel open probability) was increased following depletion of triadin from solubilised heavy SR (HSR). They reported also that \(^{3}H\) ryanodine binding to solubilised HSR as well as activity of purified RyR incorporated into planar lipid bilayers was inhibited by purified triadin (Ohkura, Furukawa et al. 1998). Furthermore, Perez and co-workers have suggested that triadin-null cells have increased basal RyR1 activity finding that triadin-null myotubes had chronically elevated [Ca\(^{2+}\)] that were sensitive to inhibition with ryanodine. These results have been suggested to be due to impairment of FKBP12/RyR1 interactions in triadin-null muscles (Eltit, Feng et al. 2010). Controversy also exists in the literature regarding the regulatory role of CSQ upon RyR. Ohizumi and co-workers have reported enhanced \(^{3}H\) ryanodine binding to solubilised heavy SR (HSR) by calsequestrin (Ohkura, Furukawa et al. 1998). Another study, employing planar bilayer measurement to observe the current through the Ca\(^{2+}\) channel in HSR, has shown that
CSQ activates the Ca\(^{2+}\) release channel (Kawasaki and Kasai 1994). As reviewed by (Beard, Wei et al. 2009), single channel recording experiments with native RyR channels where triadin and junctin remain bound to RyR have shown that CSQ1 inhibits RyR1 while CSQ2 activates RyR2 at a physiological luminal [Ca\(^{2+}\)] of 1mM. At lower luminal Ca\(^{2+}\) concentrations, native cardiac RyR2 channels have also been shown to be activated by CSQ2 (Qin, Valle et al. 2008; Wei, Abdellatif et al. 2008). When luminal Ca\(^{2+}\) is ≤100µM, CSQ1 is reported to activate RyR1 (Wei, Varsanyi et al. 2006). Dulhunty and co-workers, have shown that the association of CSQ with RyR amplifies its response (relative \(P_0\)) to changes in luminal Ca\(^{2+}\) concentrations (Beard, Casarotto et al. 2005). In contrast, another study demonstrated that CSQ had a negative regulatory effect on RyR. Gyorke and co-workers, using an \textit{in vitro} reconstitution approach, i.e. lipid bilayers, have found that, at luminal [Ca\(^{2+}\)] of 20µM, CSQ2 inhibits RyR2 complexed with recombinant triadin and junctin; however it has no effect on RyR2 devoid of triadin and junctin. This inhibitory effect has not been observed at high luminal [Ca\(^{2+}\)] (2mM) (Gyorke, Hester et al. 2004). A CSQ2 knock-out model exhibited an increased fractional SR Ca\(^{2+}\) release and enhanced SR Ca\(^{2+}\) leak which would be consistent with the concept of CSQ2 having an inhibitory effect upon RyR2 (Knollmann, Chopra et al. 2006).

Screening of biomolecular interactions can be either label-dependent or label-free. Most label-dependent techniques are based on the measurement of fluorescence (e.g. fluorescence resonance energy transfer or fluorescence polarization) or radioactivity (e.g. filter binding assays and scintillation proximity assays). Examples of label-free technologies include optical biosensors (e.g. surface plasmon resonance), acoustic biosensors (e.g. Quartz crystal microbalance with dissipation monitoring) and calorimetry (e.g. isothermal titration calorimetry). The lack of labelling has the advantage of avoiding false negatives which might result from occluding a binding site by a reporter compound as well as false positives triggered by elevated background binding due to a hydrophobic reporter compound (Cooper 2003). Presented below is an overview of the label-free biosensor techniques employed in this research project.
5.1.1 Surface plasmon resonance (SPR)

Surface plasmon resonance, SPR, is an optical biosensor technique based on the transfer of a plane polarised light energy (photons) by means of a prism to a group of electrons on a thin layer (50nm) of gold (a component of the sensor chip). A deflection of incident photons (due for example to binding of a protein (analyte) to a ligand fixed onto the sensor chip) is monitored indirectly by determination of the angle (the SPR angle) of placement of the gold surface at which the electrons of this gold are made to resonate by interaction with the incident light beam. This surface plasmon resonance absorbs energy (evanescent wave) reducing the energy of the internally reflected light. The SPR response is detected by a fixed array of light sensitive diodes covering the wedge of reflected light. The angle at which resonance occurs depends on the refractive index into which the evanescent wave is propagated. The change of the index of refraction (and, hence, the resonance angle) is monitored in real time as a SPR sensogram (Fig. 5.1). A detailed review can be found in (Cooper 2003; Thillaivinayagalingam, Gommeaux et al. 2010).

The ligand is immobilised onto a layer of carboxymethylated dextran covering the gold surface of the SPR sensor chip. The analyte is injected by a microfluidic system over the sensor surface. Binding of the analyte to the immobilised ligand results in an increase in Resonance Units (RU). The SPR angle will cease to change as the binding of analyte to ligand reaches a steady state (at equilibrium). The SPR angle will change again as the analyte dissociates from the surface upon removal of the analyte from the flowing buffer. In biology, complexes often have long half-lives, therefore, regeneration solutions (e.g. high pH, high salt) are usually needed to disrupt interactions and regenerate free ligand (Fig. 5.2).
Figure 5.1: A cartoon illustrating the principles of surface plasmon resonance (SPR). (A): The change of the SPR resonance angle. (B): The corresponding sensogram. An increase in the mass adsorbed to the chip due to ligand immobilization and ligand-analyte complex formation results in changes in the refractory index in the immediate vicinity of the gold surface layer of the chip with subsequent changes in the resonance angles ($\theta_1$, $\theta_2$ and $\theta_3$). When the analyte binds to the ligand there is a further increase in mass resulting in a change to the resonance angle. The SPR sensogram, as shown in panel B, is a real time monitoring of the resonance signal which corresponds to the changes of the resonance angle and reflects binding events at the surface of the chip.
Figure 5.2: The SPR sensogram of a typical binding cycle. Initially the flow of buffer provides a baseline response. Injection of the sample results in the formation of an analyte-ligand complex (association) with subsequent mass changes at the chip surface which is translated into an increase in the SPR signal. Removal of the sample from the flowing solution is responsible for the dissociation phase of the sensogram which represents a partial separation of the analyte from the ligand fixed onto the chip. Regeneration of the surface with a free ligand is achieved by injection of regeneration solution.

In order to covalently link ligands to a biosensor surface, multiple immobilisation methods are available. Amine coupling (Fig. 5.3) is the most frequently employed technique to attach proteins to the biosensor surface; the protein is immobilised to the surface of a biosensor chip through free amino groups existing either in basic amino acid residues (e.g. lysine) or at the protein’s N-terminus. Other examples of coupling chemistries include thiol coupling based on an interaction involving thiol and disulphide groups and, to a lesser extent, maleimide and aldehyde coupling. A cross-linking step (by using chemical cross-linkers e.g. N-hydroxysuccinimide (NHS) / 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)) added to the immobilisation step aims to ensure the stability of the ligand onto the surface of biosensor chip, and helps to overcome the problem of removal of the surface ligand during regeneration.
Figure 5.3: Immobilisation of proteins by direct amine coupling. The carboxymethylated dextran coating the biosensor chip interacts with NHS/EDC producing a reactive succinimide ester. The protein (ligand) (R-NH₂) is then immobilised onto the chip via free amino groups of basic amino acid residues and/or that existing at the protein’s N-terminus.

A drawback of the direct coupling method is that it leads to the random orientation of the protein ligand immobilised onto the chip which may affect analyte binding if the binding domain is occluded or has been involved in coupling with the chip. An alternative approach to ensure a homogenous orientation of the protein of interest is to employ monoclonal antibodies for an indirect immobilisation of the protein (Fig. 5.4). However, the need for fresh protein to be re-applied prior to each binding cycle, as regeneration often removes the protein from the surface, is a limitation of this method.
Figure 5.4: Immobilisation of a ligand of interest to a biosensor surface. (A) Direct approach, (B) Indirect approach. In the direct approach, the ligand is immobilised directly onto the biosensor chip (e.g. by direct amine coupling). In the indirect approach, a capture molecule (e.g. a monoclonal antibody) is immobilised first onto the chip providing an indirect anchoring of the ligand.

There are several reports employing SPR to investigate binding of RyR to other proteins, peptides and ligands. For example, Groh and co-workers (Groh, Marty et al. 1999) have used the BIAcore biosensor system to test the binding of RyR1 to synthetic skeletal triadin peptides; peptide 1 (residues 2-17) and peptide 2 (residues 18-46). The triadin peptides were directly immobilized onto a biosensor chip and RyR1 was the analyte. Later, Mouton et al (Mouton, Ronjat et al. 2001) investigated the binding of RyR1 and RyR2 to structural elements of the C-terminal cytoplasmic domain of the α1c subunit of DHPR which had been biotinylated and immobilized onto a streptavidin coated biosensor chip. In another study (O'Reilly, Robert et al. 2002), RyR1 interactions were investigated with a His-tagged recombinant II-III loop from the skeletal DHPR α1s subunit (immobilized by anti-His antibodies covalently fixed onto the sensor chip) and with biotinylated synthetic peptides corresponding to sequences of the II-III loop of skeletal and cardiac DHPR α1-subunit (immobilized by streptavidin coated biosensor chip). Furthermore, Chen et al (Chen, Esteve et al. 2003) have studied the binding of synthetic maurocalcin (sMCa), a scorpion toxin which is a putative mimic of the II-III loop peptide of the DHPR α1s subunit, to RyR1. The biotinylated sMCa was immobilized onto the sensor chip surface coated with streptavidin (sensor chip SA).
More recently, Blayney and co-workers (Jones, Reynolds et al. 2005; Blayney, Jones et al. 2010) have employed SPR to investigate binding of RyR1 and RyR2 to recombinant GST tagged FKBP12/12.6 immobilized onto the biosensor chip by anti-GST coupling. A common characteristic, in all these studies, is the employment of RyR as the analyte in the SPR experiments. This is understandable because the immobilization step of the ligand, in the SPR experiments, involves dilution into acetate buffer (pH 3.5-5.5), and hence carries the risk of dissociation of the RyR homotetramer into monomers. However, a surprising feature of the SPR studies investigating RyR-ligand interactions is the minimal concentrations of detergents employed in the running buffers. For most studies involving purification of RyR1 or RyR2 CHAPS is employed and usually maintained at ~0.5% (w/v), just below the critical micelle concentration in order to maintain the solubilised RyRs in solution in the absence of the lipid bilayer. However, only very low concentrations of CHAPS (0.05% (w/v)) were employed in several of the SPR studies which is ten times lower than the CMC (Jones, Reynolds et al. 2005; Blayne, Jones et al. 2010), or other surfactants e.g. 0.005% (w/v) polysorbate were used (Mouton, Ronjat et al. 2001; O'Reilly, Robert et al. 2002; Chen, Esteve et al. 2003). This approach may increase the risk of RyR aggregation and artefacts.

5.1.2 Quartz crystal microbalance and dissipation (QCM-D)

Quartz crystal microbalance (QCM) is a piezoelectrically oscillating ultrasensitive weighing device. It consists of a thin circular piece of, so called AT-cut, quartz crystal sandwiched between two metal electrodes. Cycles of expansion and contraction of the crystal is induced by applying alternating electric fields to quartz (Dixon 2008). QCM can be considered as a nano-sensitive balance in that a frequency shift, Δf, is induced by and related to a mass change, Δm, in response to the adsorption of a molecule to the surface of the sensor. The high resolution with which very small frequency changes can be measured is associated to the high inherent sensitivity (<1ng/cm²) (Czanderna and Lu 1984). In air or a vacuum, Sauerbrey first showed a linear relationship between the mass adsorbed onto the surface of the sensor chip and the resulting frequency change (Sauerbrey 1959). The development of oscillator circuits convenient for liquids paved the route towards application of QCM to biological samples (Cooper 2003). Rigid mass adsorption is a mandatory requirement for the
Sauerbrey equation. Therefore, this relationship between $\Delta f$ and $\Delta m$ is not valid for liquid applications whereby the frequency change is affected by the viscoelastic properties of the liquid phase. QCM with dissipation monitoring (QCM-D) is a newer development of the technique considering the dissipation ($D$) parameter which addresses the issue of energy losses occurring with a viscoelastic mass layer. A characteristic dissipation ($D$) profile, dependent on the viscoelastic properties of the adsorbed material, is produced on periodic removal of the voltage exciting a resonant frequency of the crystal. In QCM-D, protein, vesicle, or cell adsorption results in a typical $f$ and $D$ response the size of which is tens to hundreds of Hz and single to tens ($\times 10^{-6}$) of dissipation units respectively. QCM-D detects $f$ and $D$ at a variety of overtones (harmonics) ($n = 3, 5, 7, 9\ldots$) of a resonant frequency on the timescale of milliseconds. Multiple harmonic data are needed to model the experimental data in order to extract meaningful parameters such as mass, thickness, density or viscosity (Dixon 2008).

Investigation of protein adsorption to biomaterial surfaces is one of the principal applications of QCM-D. In a previous study of biomaterial development, the extracellular matrix protein, fibronectin, was adsorbed as polymer films and then employed to investigate the adsorption of human umbilical vein endothelial cells (Wittmer, Phelps et al. 2007). For the selection of biomaterials for stents that do not induce clotting, QCM-D was employed to determine which material had the least amount of fibrinogen (a protein involved in blood clotting) adsorbed and was accordingly deemed as the best candidate to manufacture blood contacting medical devices (Weber, Pesnell et al. 2007). Su and co-workers have used QCM-D to study the conformational changes to the oestrogen receptor upon ligand binding (Peh, Reimhult et al. 2007). QCM-D applications relating to phospholipids usually involve formation of supported lipid bilayers (SLBs). For example, Trepout et al reconstituted a membrane protein in a selective orientation into an SLB which was then used to create a base to probe the binding of this protein with a component in an efflux complex (Trepout, Mornet et al. 2007).

### 5.1.3 Aims and Objectives

The main focus of this chapter was to investigate whether CSQ can directly interact with RyRs in the absence of triadin.
The previous Chapters have described the isolation of RyR1 and RyR2 from sheep skeletal and bovine cardiac muscle respectively and the purification of recombinant hCSQ2. Several approaches were taken to investigate RyR-hCSQ2 interactions including biochemical pull-down assays as well as label-free techniques such as surface plasmon resonance (SPR) and Quartz crystal microbalance and dissipation (QCM-D) monitoring.

Another objective of this thesis work was the use of the [³H] ryanodine binding assay to investigate changes to RyR function in response to the addition of exogenous CSQ and triadin. Since it was not possible to express the full-length or luminal domain of triadin a synthetic peptide encapsulating the ‘KEKE’ motif of triadin was employed instead. The [³H] ryanodine binding assay is a method for assessing the activity of RyR where ryanodine binds to the open state of the channel.

5.2 Results and Discussion

5.2.1 Calsequestrin binding to ryanodine receptor

In order to examine whether CSQ binds directly to RyR, several experimental approaches were employed in this thesis including a pull-down experiment as well as a number of label-free biophysical techniques

5.2.1.1 Pull down experiment

Purified hCSQ2 expressed as a GST fusion protein was applied to a 1ml Glutathione Sepharose™ 4B. After washing the column in a series of buffers (as described in Materials and Methods section 2.6.3), purified RyR1 was added. After a several washing steps, the presence of RyR1 binding to the hCSQ2 immobilised on the resin was probed by SDS-PAGE and western blotting using an anti-RyR antibody. A control experiment was carried out in the absence of hCSQ2 in order to eliminate the possibility of non-specific binding of RyR1 to Glutathione Sepharose™ 4B.
Figure 5.5: Pull down of RyR1 by hCSQ2. (A): Schematic drawing representing the assembly of the interacting bio-molecules onto Glutathione Sepharose™ 4B resin. (B lane 1): Western blot analysis using anti-RyR (Cat. No. ab2827) illustrating an interaction between hCSQ2 immobilised onto the resin via the N-terminal GST tag and RyR1. (B lane 2): The western blot analysis of the control experiment conducted in the absence of hCSQ2, no RyR1 has bound to the resin.

As shown above in Fig. 5.5 there is a clear band at ~565kDa which is consistent with the presence of RyR1. Therefore, this experiment suggests that RyR1 has bound to hCSQ2 and is immobilised onto the column. Control experiments (lane 2) show that RyR1 does not bind non-specifically to Glutathione Sepharose™ 4B in the absence of hCSQ2.

5.2.1.2 Surface plasmon resonance (SPR)

As detailed in the introductory part of this chapter, previous SPR studies studying RyR-ligand interactions did not employ detergents at concentrations typically employed to minimise RyR aggregation, which may produce false positive results. Therefore, we have only employed buffers containing 0.5% (w/v) CHAPS, as described in the Materials and Methods section 2.6.1.
5.2.1.2.1 Binding of hCSQ2 to the open state of RyR1

In order to investigate hCSQ2 binding to RyR1 employing SPR, the purified hCSQ2 was diluted to 10µg/ml in acetate buffer, pH 2.5, and then immobilised onto a CM5 sensor chip by direct amine coupling. An immobilisation figure of 9500RU was achieved. The RyR1 sample was diluted to 30µg/ml and dialysed in a sucrose free buffer promoting the open state of the channel (100µM CaCl₂, 5mM Na₂AMP) which was used as the analyte. The sensogram below shows that with the addition of RyR1, there was a deflection of the SPR signal (~1000ΔRU) (Fig. 5.6) reflecting a change of the refractory index.

![Sensogram showing binding of RyR1 to hCSQ2](image)

Figure 5.6: RyR1, in the open state, binds to hCSQ2. The association and dissociation phases of the interaction between RyR1 and hCSQ2 were monitored by following changes in the SPR signal over time. An SPR signal of ~1000ΔRU was detected following application of RyR1 to hCSQ2 immobilised onto a CM5 sensor chip. The arrow indicates the point at which RyR1 was injected.

To examine whether it is RyR1 binding to hCSQ2, an anti-RyR (10µg/ml) antibody was injected during the dissociation phase of the binding cycle (before regeneration). As can be seen below in Fig. 5.7 a further SPR signal (~300ΔRU) was detected indicating the binding of the antibody.
Figure 5.7: Antibody against RyR proves the interaction between RyR1 to hCSQ2. An SPR signal of ~300ΔRU was detected following the addition of an anti-RyR antibody during the dissociation phase of the RyR1-hCSQ2 binding cycle. This indicates the presence of RyR1 on the sensor chip. The arrow indicates the point at which anti-RyR antibody was injected.

The increase in signal upon injection of the RyR1 antibody indicates that RyR1 has bound to the chip and by inference immobilised hCSQ2.

5.2.1.2.2 Binding of hCSQ2 to the open and closed states of RyR2

RyR2 samples, from sucrose gradient fractionation, were partially pure when compared to RyR1 samples. However, as can be seen from gels in Fig. 3.15, bands corresponding to RyR2 were the most dominant. This was encouraging and SPR experiments to investigate RyR2 binding to hCSQ2 were undertaken. Following the same methodology as with RyR1, RyR2 was applied to immobilised hCSQ2. An immobilisation figure of 5500RU was achieved. Kinetic SPR experiments (Fig. 5.8) were carried out employing RyR2 (as an analyte) at concentrations of 16, 4, 2, 1µg/ml which corresponds to 8, 2, 1, 0.5nM respectively. RyR2 samples were dialysed in sucrose free buffers promoting either the channel open (100µM CaCl$_2$, 5mM Na$_2$AMP) or closed (2mM EGTA) states. The data evaluation was carried out using the software program BIAevaluation version 4.0 determining $K_a$ (association constant; M$^{-1}$s$^{-1}$), $K_d$ (dissociation constant; s$^{-1}$), $K_A$ (association equilibrium constant; 1/M) and $K_D$ (M). Table 5.1 summarizes values of $K_a$, $K_d$, $K_A$ and $K_D$ constants for the interaction of RyR2 in the open or closed states with hCSQ2.
Figure 5.8: A kinetic SPR experiment of the interaction between RyR2 in open (A) & closed (B) states and hCSQ2. hCSQ2 was immobilised (as a ligand) on a CM5 chip; and RyR2 was run (as an analyte) at concentrations of 8, 2, 1, 0.5nM from high to low concentration. The association and dissociation phases of the interaction were monitored by following changes in the SPR signal over time.
Table 5.1: Kinetic parameters determined by BIAevaluation version 4.0 using SPR measurements.

<table>
<thead>
<tr>
<th>Interaction of hCSQ2 with</th>
<th>$K_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$K_A$ (1/M)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RyR2 (open state)</td>
<td>$4.17 \times 10^5$</td>
<td>$1.49 \times 10^{-3}$</td>
<td>$2.8 \times 10^8$</td>
<td>3.57</td>
</tr>
<tr>
<td>RyR2 (closed state)</td>
<td>$4.68 \times 10^5$</td>
<td>$2.45 \times 10^{-3}$</td>
<td>$1.91 \times 10^8$</td>
<td>5.23</td>
</tr>
</tbody>
</table>

The BIAevaluation analysis indicates that the interaction between RyR2 and hCSQ2 fits a “1:1” binding model. This might suggest that 4 hCSQ2 monomers bind to a RyR2 tetramer. Kitmitto and co-workers (unpublished data) have employed negative-stain electron microscopy (EM) and single particle analysis (SPA) of purified hCSQ2 finding a large portion of the sample forms a stable dimer-dimer structure. As shown in the table above, the estimated $K_D$ for the interaction of hCSQ2 to RyR2 in the open and closed states were 3.57nM and 5.23nM respectively. Both values are indicative of high affinity binding of hCSQ2 to RyR2 in the two functional states and thus may suggest that CSQ2 is constitutively bound to RyR2. However, these kinetic experiments were carried out by injecting RyR2 samples from high to low concentrations and were not repeated in reverse order. Since RyR2 is a membrane protein containing a transmembrane hydrophobic segment, it should be considered that the protein might adsorb to the tubing of the injection micro-fluidics. This non-specific adsorption is usually followed by a slow release of the analyte over the course of the experiment creating artefacts in the data. This problem of adsorption carry over could be eliminated by injecting analyte samples from low to high concentration (Nguyen, Tanious et al. 2007). Furthermore, due to the low yield of RyR2 samples, a relatively narrow range of analyte concentration (from 0.5-8nM) was employed here. This adds a further limitation to the interpretation of the kinetic data since a robust analysis requires a wide (hundred fold) range of analyte concentration. Moreover, it would be more appropriate if each analyte concentration was run in duplicate or triplicate eliciting the same signal. This would ensure that the ligand level on the chip did not change with the injection of the regeneration buffer which would jeopardize robust kinetic analysis.
A further criticism of the above experiments would be the large amount of hCSQ2 immobilised onto the chip (~9500RU & 5500RU) which increases the possibility of non-specific binding to produce an SPR signal. Blayney and co-workers reported employing SPR with only ~100RU of FKBP12/12.6 as a ligand to investigate the binding to RyR (analyte) (Jones, Reynolds et al. 2005; Blayney, Jones et al. 2010). Other studies e.g., Ronjat and co-workers have tested the binding of RyR1 to synthesized peptides corresponding to sequences of DHPR, employing SPR with a signal of ~800RU upon peptide ligand immobilisation (O'Reilly, Robert et al. 2002). However, it should be noted, again, that these studies didn’t include detergents at an appropriate CMC in the analyte buffer which may lead to aggregation of RyRs. Therefore, the experiment was repeated several times with a much lower immobilisation figures (e.g. 450RU) of hCSQ2. An indirect immobilisation of hCSQ2 via its GST fusion tag using an anti-GST antibody was also tested. As another modality of the SPR experiment, RyR1 was immobilised onto the sensor chip to a figure of ~90RU and hCSQ2 was run as analyte. However, an SPR signal that would indicate RyR1-hCSQ2 and/or RyR2-hCSQ2 binding was not detected. A possible explanation for the absence of any interaction is that the highly acidic acetate buffer required for the ligand immobilisation steps might have denatured hCSQ2 and/or RyR1. Therefore, another biosensor technique, Quartz crystal microbalance and dissipation (QCM-D) monitoring, that does not require buffers at a pH extreme, was employed to probe RyR1-hCSQ2 binding.

5.2.1.3 Quartz crystal microbalance and dissipation (QCM-D)

Initially purified hCSQ2 (50µg/ml) was applied onto the biosensor chip in the absence of RyR1 (Fig. 5.9).
Figure 5.9: QCM-D analysis of hCSQ2 in 1mM EDTA buffer; (A): (∆f-t Plot), (B): (∆D-t Plot). hCSQ2 (50µg/ml) was applied directly onto the biosensor chip. Panel A shows the frequency change (∆f) corresponding to different overtones (f_{3,5,7,9,11,13}) over time. Panel B illustrates the dissipation change (∆D) corresponding to different overtones (D_{3,5,7,9,11,13}) over time. A fairly compact layer was formed onto the chip as indicated by the compact nature of the dissipation plots with ∆D values corresponding to the different overtones ranging from \( \sim 2 \times 10^{-6} \) to \( \sim 2.3 \times 10^{-6} \) units.
Fig. 5.9 represents a typical $\Delta f$-t and $\Delta D$-t plot of different overtones following the application of hCSQ2 onto the SiO$_2$ chip. The adsorption of hCSQ2 in 1mM EDTA buffer onto the chip results in a frequency change ($\Delta f$) (corresponding to the third overtone) of -55.7±2.1Hz (n=3). The formation of a layer of hCSQ2 onto the chip results in a dissipation change ($\Delta D$) (corresponding to the third overtone) of 1.9±0.2 x 10$^{-6}$ units (n=3). It can be seen that the dissipation plots are closely packed with very little separation. An elongated flexible molecule would be expected to exhibit a large $\Delta D$ with a spread of the individual dissipation plots.

After acquiring plots for hCSQ2 alone, experiments were developed to test binding of RyR1 to hCSQ2. The recombinant hCSQ2 (50µg/ml) was adsorbed onto the SiO$_2$ chip mounted into the flow cell, and the purified RyR1 sample (50µg/ml) was then applied to the flow cell. A frequency change ($\Delta f$) which would be indicative of RyR1-hCSQ2 binding was not observed (data not shown). A possible explanation (in addition to no interaction) is that CSQ2 (~50kDa) is very small when compared to RyR1 (2MDa) and thus the putative binding site may not be accessible once it has absorbed and packed onto the chip surface. Therefore, in an alternative approach, RyR1 sample was first applied to the chip surface (Fig. 5.10).
Figure 5.10: QCM-D analysis ($\Delta f$-$t$ Plot) of purified RyR1 (closed state) (n=3). (A): The RyR1 samples (50µg/ml) were directly applied to the sensor chip. (B): Formation of stable RyR1 plateau follows a subsequent washing step using CHAPS-free buffer. The data presented are that of the third overtone for each RyR1 sample. The $\Delta f$ values of the three RyR1 samples can be seen to vary which may be due to the amount of protein adsorbed onto the chip and the packing of the RyR tetramers resulting in different masses on the chip and thus a different $\Delta f$. Inset: Cartoon illustrating how RyR may associate with the sensor chip in a range of different orientations.
Fig. 5.10 shows the frequency changes (Δf) for three different experiments in which RyR1 (closed state) was applied directly onto the chip (panel A) followed by a washing step using CHAPS-free buffer which removed any loosely associated, or non-specifically bound, RyR1 resulting in formation of stable layer of RyR1 (panel B). The RyR1 plateaus of the three experiments are at different Δf values, which would suggest a different amount of RyR had associated with the chip surface. This may be due to the large size of RyR1 leading to different ‘packing’ combinations on the chip surface.

Once a stable layer of RyR1 had formed on the sensor surface hCSQ2 in 1mM EDTA buffer was applied. As can be seen in Fig. 5.11 the addition of hCSQ2 to the RyR1 results in an increase in Δf by ~60Hz, which would correspond to an increase in mass associated with the sensor chip. To remove non-specifically bound hCSQ2, EDTA buffer was applied to the cell. This resulted in only a minor fluctuation to the frequency measurements.

![Frequency changes](image)

**Figure 5.11:** A typical QCM-D analysis (Δf-t plot) illustrating the changes to the frequency profile of RyR1 (closed state) when hCSQ2 (50 µg/ml) in 1mM EDTA buffer is applied. The range of frequency overtones (f₃,5,7,9,11,13) is displayed. A Δf of ~60Hz corresponding to the third overtone was detected upon addition of hCSQ2. A washing step with 1mM EDTA buffer resulted in a minor change in the frequency measurements.

Fig. 5.11 illustrates a typical Δf-t plot of the addition of hCSQ2 in 1mM EDTA buffer to RyR1 (closed state) on the biosensor chip. Different Δf values were obtained
on repeating the experiment (e.g. a $\Delta f$ of $\sim$20Hz) which suggests less hCSQ2 had associated with the RyR1 layer. The change in frequency following the addition of hCSQ2 may indicate complex formation between hCSQ2 and RyR1. However, the possibility that hCSQ2 has bound non-specifically by filling the gaps between RyR1 complexes cannot be ruled out. However, examination of the dissipation plots (Fig. 5.12) reveals that upon addition of hCSQ2 the $\Delta D$ increased by $\sim 5 \times 10^{-6}$ units that would suggest that the layer associated with the sensor chip is now more flexible and elongated. If hCSQ2 had associated directly with the sensor surface, i.e. occupied the “holes” between the RyR molecules or had become sandwiched between the RyR complexes then although an increase in $\Delta f$ would be expected the layer would be more likely to be “compact” and thus an increase in $\Delta D$ would not be expected. The addition of EDTA buffer resulted in a minor change in dissipation measurements which would also suggest that hCSQ2 was not loosely associated with the chip.

**Figure 5.12: QCM-D analysis ($\Delta D$-t plot) of the addition of hCSQ2 (50µg/ml) in 1mM EDTA buffer to RyR1 (closed state).** The data presented are of different overtones ($D_{3,5,7,9,11,13}$) before and after the addition of hCSQ2. There is a substantial increase in the overall $\Delta D$ ($\sim 5 \times 10^{-6}$ units) following the addition of hCSQ2. There is also a slight increase in the separation of the overtones. A washing step with 1mM EDTA buffer resulted in a minor change in the dissipation measurements.
To confirm the presence of both RyR1 and hCSQ2 on the chip, the chip was carefully removed from the flow cell and washed with 2% SDS and analysed by SDS-PAGE and western blotting using anti-CSQ and anti-RyR. Two gels were run; 5% to detect the RyR1 and a 12% to examine the presence of hCSQ2. Western blotting confirmed the presence of both RyR and CSQ (see Fig. 5.13).

![Western blot characterisation of the QCM-D experiment investigating binding of RyR1 to hCSQ2 in 1mM EDTA buffer. The eluant of the SiO2 chip was characterised using anti-CSQ and anti-RyR.](image)

However, it should be noted that the association of both RyR1 and hCSQ2 with the sensor chip does not necessarily mean that they have formed a complex. In order to examine whether hCSQ2 binding to RyR1 is calcium dependent, the above experiments were repeated but using hCSQ2 in 1mM Ca^{2+} buffer; Fig. 5.14 & Fig. 5.15.
Figure 5.14: QCM-D analysis (Δf-t plot) of the addition of hCSQ2 (50µg/ml) in 1mM Ca\(^{2+}\) buffer to RyR1 (closed state) adsorbed to the chip. The data presented are of different overtones (f\(_3,5,7,9,11,13\)). A Δf of ~120Hz corresponding to the third overtone was detected upon the addition of hCSQ2. A washing step with 1mM Ca\(^{2+}\) buffer resulted in a minor change in the frequency measurements.

The example Δf-t plot shown in Fig. 5.14 illustrates that the addition of hCSQ2 (with Ca\(^{2+}\) bound) to RyR1 in the closed state results in an increase in Δf by ~120Hz. Although this value is substantially larger than that observed in the experiments with Ca\(^{2+}\) free hCSQ2 due to the large variability between datasets within each data set it is not possible to draw any further conclusions. Interestingly, examination of the ΔD-t profiles finds that there is also an overall increase in ΔD (corresponding to the third overtone) by ~13 x 10\(^{-6}\) units.
Figure 5.15: QCM-D analysis ($\Delta D$-t plot) of the addition of hCSQ2 ($50\mu g/ml$) in $1mM Ca^{2+}$ buffer to RyR1 (closed state) adsorbed to the chip. The data presented are of different overtones ($D_{3,5,7,9,11,13}$). The substantial increase in the overall $\Delta D$ ($\sim 13 \times 10^{-6}$ units) and the wider separation of the individual dissipation overtones following the addition of hCSQ2 support the possibility of forming a complex with RyR1. A washing step with $1mM Ca^{2+}$ buffer resulted in a minor change in the dissipation measurements.

Again, the increase in $\Delta D$ would suggest that the protein layer on the sensor chip is more flexible upon the addition of hCSQ2 (with $Ca^{2+}$ bound) to RyR1. As before, it can also be seen from Fig. 5.16 that both RyR1 and hCSQ2 were present on the chip surface although as discussed previously this does not conclusively show that RyR1 and hCSQ2 formed a complex.
**5.2.2 Investigation of RyR–protein interactions by tritium ryanodine binding experiments**

**5.2.2.1 Tritium ryanodine binding to SR vesicles in the presence of a triadin KEKE motif**

KEKE motifs are amino acid sequences (more than 12 residues in length) containing 60% (or more) alternating lysine and glutamic/aspartic acid residues, that lack five positively or negatively charged residues in a row and does not include tryptophan, tyrosine, phenylalanine and proline (Realini, Rogers *et al.* 1994). The luminal domain of triadin is rich in KEKE motifs and, hence, is highly charged raising the high possibility of an interaction site with other proteins. Triadin residues 200-224 satisfy all of the criteria for a KEKE motif (Realini, Rogers *et al.* 1994). The KEKE motif is the only one, among many within the luminal domain of triadin, which has been shown to be crucial for CSQ binding (Kobayashi, Alseikhan *et al.* 2000). Residues 210-224 of this motif form a β strand that have been shown to interact directly with charged residues of CSQ (Scott, Simmerman *et al.* 1988) and thus has been suggested to act as “a polar zipper” (Perutz 1994). This KEKE motif in triadin has also been shown to bind to RyR1 (Lee, Rho *et al.* 2004) as well as histidine-rich Ca$^{2+}$ binding protein (HRC) (Lee, Kang *et al.* 2001). Amino acid residues 1-264 (including the KEKE motif) are identical in all cardiac and skeletal muscle triadin isoforms (Fig. 5.17) (Guo, Jorgensen *et al.* 1996). Therefore, the KEKE peptide employed in these studies was appropriate to use for both RyR1 and RyR2. The KEKE peptide is highly conserved between species.
Figure 5.17: CLUSTAL W (1.83) (Thompson, Higgins et al. 1994) multiple sequence alignment between human skeletal triadin (Trdsk) and cardiac triadin 1 (Trd1). The two isoforms are identical in amino acid residues 1-264. Key: “∗” (asterisk) indicates positions which have a single, fully conserved residue, “:” (colon) indicates conservation between groups of strongly similar properties, and “.” (period) indicates conservation between groups of weakly similar properties. Highlighted is the Trd KEKE motif (residues 200-224).
In Chapter three, the binding of $[^3\text{H}]$ ryanodine to skeletal SR membranes was reported illustrating a maximum binding at 100µM [Ca$^{2+}$]. Using this assay work was developed to examine $[^3\text{H}]$ ryanodine binding to skeletal SR vesicles at different [Ca$^{2+}$] in the presence of a molar excess (11µM) of a peptide corresponding to the triadin KEKE motif, in relation to RyR1 (0.044 nM). The experiments were carried out as described in the Materials and Methods section 2.1.4 and the results were plotted in comparison to control experiments done in the absence of the triadin peptide as illustrated below in Fig. 5.18 & Fig. 5.19. The control data was obtained in a separate series of experiments; however the same batch of the SR vesicles (which was aliquoted on preparation) and the same bottle of $[^3\text{H}]$ ryanodine were employed in these experiments.

Figure 5.18: $[^3\text{H}]$ ryanodine association kinetics of skeletal SR membranes in the presence and absence of triadin KEKE motif (n=3). The assay was conducted in the presence of 0.1% (w/v) CHAPS, 5mM AMP-PCP and 100µM CaCl$_2$. A significant increase in ryanodine binding in the presence of the triadin peptide ($p<0.05$) is observed after incubation times of 100, 150 and 175 minutes.

Fig. 5.18 shows that the addition of the triadin KEKE motif increases the $[^3\text{H}]$ ryanodine binding to skeletal SR vesicles triggering a shift of the curve at 100µM CaCl$_2$. A two-way ANOVA test revealed a significant effect of the triadin KEKE motif on the
[³H] ryanodine binding (p<0.0001). The effect of incubation time was also shown to be significant (p<0.0001). However, an interaction between the two factors (the presence of triadin peptide & the incubation time) was not shown by the two-way ANOVA test (p>0.05). A Bonferroni post-test indicated that the triadin peptide produced a significant effect upon [³H] ryanodine at incubation times of 100, 150, 175 minutes (p<0.05); however a non-significant increase in [³H] ryanodine binding was detected at incubation times 0, 25, 50, 75, 125, 200, and 225 minutes (p>0.05). The lack of change to [³H] ryanodine binding after long incubation periods (e.g. 200 & 225 minutes) can be explained by saturation of [³H] ryanodine binding sites on RyRs within skeletal SR vesicles. Further experiments to explore the effects of the KEKE peptide upon [³H] ryanodine binding were undertaken employing [Ca²⁺] between 0.05µM and 10mM.

Figure 5.19: [³H] ryanodine binding to skeletal SR vesicles at different [Ca²⁺] (log scale) after 100 minutes incubation in the presence and absence of the triadin KEKE peptide. The assay was conducted in the presence of 0.1% (w/v) CHAPS and 5mM AMP-PCP. A significant increase in ryanodine binding (p<0.05) is observed at 50µM CaCl₂ in the presence of the triadin peptide (indicated by “*”).

As can be seen from Fig. 5.19 and in agreement with Fig. 3.7, maximal [³H] ryanodine binding occurs when [Ca²⁺] = 100µM irrespective of the presence of the KEKE peptide. Again, a two-way ANOVA test indicated that the effect of the triadin
peptide on ryanodine binding to the isolated SR vesicles was significant (p<0.001). The [Ca\(^{2+}\)] was also found by the test to have a significant effect on the \(^{3}\text{H}\) ryanodine binding (p<0.0001). However, a significant interaction between the presence of the triadin motif and Ca\(^{2+}\) concentration was not found (p>0.05). A Bonferroni post-test revealed that the significant effect of the triadin motif is at 50µM CaCl\(_2\) concentration (p<0.05). Surprisingly, a non-significant effect was detected for all other concentration points including 100µM (p>0.05). Since the previous experiments did indicate that at 100µM CaCl\(_2\) concentration there was a significant increase in \(^{3}\text{H}\) ryanodine to the SR membranes in the presence of triadin motif (Fig. 5.18) this was a little surprising and might indicate the need to increase the “n” number of these experiments in the future and that a longer incubation period of 150 or 175 minutes may be more appropriate since there was no difference found between \(^{3}\text{H}\) ryanodine binding at 125 minutes (Fig. 5.18). There was a lack of reproducibility of the assay for cardiac SR membranes when low and high CaCl\(_2\) concentrations were employed with large standard deviations. However, the data was more robust at Ca\(^{2+}\) concentrations of 50 and 100µM and so experiments to compare \(^{3}\text{H}\) ryanodine binding to cardiac SR membranes with and without the triadin peptide were conducted at only these two concentrations of Ca\(^{2+}\) with the results shown below in Fig. 5.20.

**Figure 5.20:** \(^{3}\text{H}\) ryanodine binding to cardiac SR vesicles in the presence and absence of triadin KEKE motif at (A) 50µM CaCl\(_2\) and (B) 100µM CaCl\(_2\) (n=3); (*) indicates significant difference (p<0.05). The assay was conducted in the presence of 0.1% (w/v) CHAPS and 5mM AMP-PCP.
Since in these experiments we are comparing two groups a t-test is most appropriate to statistically assess differences between the datasets. A t-test analysis revealed a significant increase in $[^3H]$ ryanodine binding upon the addition of the triadin KEKE motif to cardiac SR vesicles at 50µM CaCl$_2$ concentration (p<0.05) but not at 100µM (p>0.05). To investigate whether the addition of the KEKE peptide influenced the non-specific binding of $[^3H]$ ryanodine, control experiments were conducted. As can be seen from the data presented in Fig. 5.21, there was no significant difference (p>0.05) in levels of non-specific binding due to the addition of the KEKE motif.

![Figure 5.21: Assessment of non-specific binding of $[^3H]$ ryanodine in the presence and absence of triadin KEKE motif at 50µM CaCl$_2$ for (A) skeletal and (B) cardiac SR vesicles (n=3). The assay was conducted in the presence of 0.1% (w/v) CHAPS, 5mM AMP-PCP and 1500 fold molar excess of cold ryanodine.](image)

The data here indicates that when the [Ca$^{2+}$] is 50 µM the addition of the triadin KEKE motif increases tritium ryanodine binding to both skeletal and cardiac SR vesicles (and by inference binding to the RyRs) which is indicative of an increased open probability ($P_0$) of the channel. The experiments were carried out employing SR vesicles, which would also include triadin, an SR integral membrane protein. It therefore appeared that the triadin KEKE motif elicited an additive effect. Our results might also indicate that the peptide also shifted the sensitivity of the channel opening to 50µM [Ca$^{2+}$]. However, again, repeat experiments are needed to confirm these conclusion and different batches of SR membranes should be examined. Consistent with the results
here, Terentyev et al (Terentyev, Viatchenko-Karpinski et al. 2007) have shown that cardiac myocytes overexpressing a similar triadin peptide exhibited profound arrhythmogenic disturbances in Ca\(^{2+}\) handling and membrane potential (MP) manifested by after-Ca\(^{2+}\) transients and DADs. They have suggested that the triadin motif competed for CSQ2 and thus relieved its mediated inhibition. Furthermore, the literature suggests a state of dynamic association and dissociation (i.e. reversibility) between triadin (through its KEKE motif; amino acids 200-224) and the ryanodine receptor (Shin, Ma et al. 2000; Lee, Rho et al. 2004). Therefore, the addition of the triadin KEKE motif might change the equilibrium status of the triadin-RyR interaction occupying a population of RyRs without endogenous Trd bound within the SR vesicles. Even if an irreversible interaction is assumed between triadin and ryanodine receptor, the enhanced \(^{3}\)H ryanodine binding observed upon addition of the triadin KEKE motif may possibly be attributed to the stoichiometry of the RyR:triadin whereby not all RyRs have triadin bound. Since the triadin peptide corresponds to a luminal domain and it is unlikely that the peptide would be able to enter the SR lumen, another binding site of the triadin KEKE motif within the RyR complex might also be a possibility. It is interesting that the KEKE peptide elicits similar effects upon both RyR1 and RyR2.

5.2.2.2 Tritium ryanodine binding to CSQ-depleted SR vesicles (junctional face membrane (JFM))

5.2.2.2.1 Isolation of skeletal & cardiac junctional face membrane (JFM)

SDS-PAGE analysis of SR membrane preparations showed that there was some endogenous CSQ associated. To remove the CSQ, a series of washes containing detergent and high salt or EDTA were employed. SDS-PAGE & western blotting using anti-RyR and anti-CSQ (shown below in Fig. 5.22) were used to detect CSQ.
Figure 5.22: Western blot characterization of cardiac JFM preparation using anti-CSQ (panel A) & anti-RyR (panel B). Lane 1: Cardiac SR membranes contain both CSQ2 and RyR2. Lane 2: Treatment of SR membranes with 0.5% TX-100. The analysis of the supernatant found no CSQ2 (no bands in lane 2, panel A) indicating that TX-100 has not removed it. RyR2 was also retained following the treatment with TX-100 (no bands in lane 2, panel B). Lane 3 and 5: Treatment of the pellet washed with TX-100 with 2mM EDTA. The analysis of the supernatant (solubilised compartmental complex (CC)), lane 3, and of the pellet (cardiac JFM), lane 5, revealed minimal removal of CSQ2 (a faint band in lane 3 panel A and stronger band in lane 5 panel A). RyR2 was again retained following treatment with 2mM EDTA (no bands in lane 3 panel B and a distinct band in lane 5 panel B). Lane 4 and 6: Treatment of the TX-100 pellet with 0.5M NaCl. The analysis of the supernatant (solubilised compartmental complex (CC)), lane 4, and of the pellet (cardiac JFM), lane 6, revealed removal of CSQ2 (a distinct band in lane 4 panel A and no bands in lane 6 panel A) with preservation of RyR2 (no bands in lane 4 panel B with a band in lane 6 panel B).

It is clear from Fig. 5.22 that washing the cardiac SR membranes with 0.5M NaCl successfully removed CSQ2 from the membranes but had no effect upon the presence of RyR2 (lanes 6 Fig. 5.22 - panel A & B). The protocol was also employed for removing CSQ1 from skeletal SR vesicles as illustrated in Fig. 5.23.
Figure 5.23: Western blot characterisation of skeletal JFM preparation using anti-CSQ (Panel A) & anti-RyR (Panel B). Lane 1: SK SR membranes contain both CSQ1 and RyR1. Lane 2: Treatment of SR membranes with 0.5% TX-100 has not removed either CSQ1 or RyR1 as revealed by the analysis of the supernatant. Lane 3 and 4: Treatment of the pellet of the previous step with 0.5M NaCl resulted in a maximal removal of CSQ1 and preservation of RyR1 as indicated by the analysis of the supernatant (solubilised CC), lane 3, and of the pellet (SK JFM), lane 4.

Depicted in Fig. 5.23 is a distinct band at ~565kDa corresponding to RyR1 within SK JFM (lane 4 – panel B) as well as very faint band suggesting that the vast majority of CSQ1 has been removed (lane 4 – panel A). These preparations were subsequently employed to examine whether (i) removal of endogenous CSQ modified [³H] ryanodine binding characteristics and (ii) whether the addition of recombinant hCSQ2 would elicit a functional effect in terms of [³H] ryanodine binding.

5.2.2.2 Tritium ryanodine binding to skeletal and cardiac JFM in the presence of hCSQ2

It is now generally accepted that CSQ2 function is not restricted to just acting as a Ca^{2+} binding protein providing a storage reservoir. CSQ2 has a role as an active modulator of the Ca^{2+} release process potentially serving as luminal Ca^{2+} sensor for RyR2 (Gyorke and Terentyev 2008). As described in the introduction to this chapter, there are conflicting views in the literature regarding the regulatory role of CSQ upon RyR.

[³H] ryanodine binding to skeletal JFM was investigated at 100µM CaCl₂. Although the histogram below (Fig. 5.24) hints that there may be a drop in ryanodine
binding to JFM membranes upon removal of endogenous CSQ1, compared to control, this was determined not to be statistically significant as judged from a t-test analysis (p>0.05). Purified recombinant hCSQ2 was then added (in excess) to the JFM membranes and compared to control experiments employing skeletal JFM.

![Graph showing [3H] ryanodine binding to skeletal SR & skeletal junctional face membranes (JFM) in the presence and absence of hCSQ2 at 100µM CaCl2 (n=3). The assay was conducted in the presence of 0.1% (w/v) CHAPS and 5mM AMP-PCP.](image)

The addition of hCSQ2 seemingly increases [3H] ryanodine binding to skeletal JFM but was determined not to elicit a significantly different ligand binding profile compared to control JFM (p>0.05). Differential effects of CSQ1 and CSQ2 on RyR have been suggested in a previous study (Wei, Hanna et al. 2009). Wei et al showed that CSQ2 activated RyR1 following depolymerization of endogenous CSQ1. The authors proposed that CSQ2, which doesn’t polymerize, binds directly to RyR producing an activatory effect despite the occupation of the triadin/junctin binding sites by a CSQ monomer at low Ca^{2+} concentrations.

On repeating the above experiment employing cardiac membrane preparations (see Fig. 5.25), a different pattern of ligand binding was observed with a trend towards
an increase in radioactivity binding after removal of CSQ2; however, statistical analysis (t-test) found that there was actually no significant difference (p>0.05). The addition of excess hCSQ2 to the JFM membranes (endogenous CSQ2 removed) did not find a significant difference compared to either control SR and JFM membranes (p>0.05) in terms of $[^3]H$ ryanodine binding and thus agreed with the experiments with skeletal muscle membrane fractions.

![Bar graph showing $[^3]H$ ryanodine binding to cardiac SR, cardiac JFM, and cardiac JFM with hCSQ2 compared to control.](image)

Figure 5.25: $[^3]H$ ryanodine binding to cardiac SR & cardiac junctional face membrane (JFM) in the presence and absence of hCSQ2 at 100μM CaCl$_2$ (n=3). The assay was conducted in the presence of 0.1% (w/v) CHAPS and 5mM AMP-PCP.

Therefore, our results might indicate that the addition of hCSQ2 to RyR1 and RyR2 does not significantly inhibit or activate the receptor. A caveat to this conclusion is that the JFM may have re-sealed and thus hCSQ2 would be unable to pass through the lipid bilayer into the lumen; however, the data do clearly show that endogenous CSQ is removed and that the $[^3]H$ ryanodine binding is not significantly altered.
5.2.2.2.3 Tritium ryanodine binding to skeletal JFM in the presence of triadin KEKE motif

As previously reported the addition of the triadin KEKE motif to SR membranes (with a population of CSQ bound) resulted in increased [$^3$H] ryanodine binding. To examine the role of CSQ in triadin KEKE peptide binding, the same experiment was repeated on JFM preparations. Interestingly, no significant difference was observed between [$^3$H] ryanodine binding to SK JFM in the presence and absence of triadin KEKE motif at 50µM CaCl$_2$ concentration (Fig. 5.26).

![Figure 5.26](image)

**Figure 5.26: [$^3$H] ryanodine binding to (A) skeletal SR and (B) skeletal JFM in the presence and absence of Triadin KEKE motif at 50µM CaCl$_2$ (n=3). (*) indicates significant difference (p<0.05). The assay was conducted in the presence of 0.1% (w/v) CHAPS and 5mM AMP-PCP. It can be seen that the addition of the KEKE-peptide to SK SR membranes (that contain CSQ1) leads to increased [$^3$H] ryanodine binding whereas panel B illustrates that this additive effect is lost when the endogenous CSQ1 is removed.**

These data illustrate that the significant activatory effect of the triadin KEKE peptide upon SR membranes (p$_{t-test}$<0.05) is eliminated after removal of CSQ1 (p$_{t-test}$>0.05). This may suggest co-operativity in binding.

5.3 Conclusion

The results presented in this chapter suggest, using a range of protein-protein interaction techniques, that there is a direct interaction between RyR and CSQ. The pull-
down (co-precipitation) experiments of RyR1 (closed state) by GST-hCSQ2 suggest an association between RyR1 and hCSQ2.

The SPR experiments revealed binding of hCSQ2 to RyR1 and RyR2; however the large amount of the ligand (hCSQ2) immobilised onto the biosensor chip raises the possibility of non-specific binding events. Furthermore, the estimated K_D values of the SPR kinetic experiments investigating the interaction between hCSQ2 and RyR2 in open and closed states need to be verified by future experiments injecting the analyte (RyR2) from low to high concentrations. Future development of these SPR experiments would also include repeating the experiments multiple times as well as exploring the use other types of Biacore biosensor chips. For example, a CM3 sensor chip has a matrix with shorter dextran chains compared to the CM5 chip used in this thesis work. This permits an interaction to occur closer to the surface of the chip which can enhance the sensitivity of SPR detection system. The CM3 biosensor chip was employed in SPR experiments conducted in studies exploring the interactions of RyR1 and 2 with the FKB12/12.6 proteins (Blayney, Jones et al. 2010). The availability of L1 chips would also allow future experiments that could investigate the binding of RyR, incorporated into liposomes, to CSQ. The surface of the chip L1 is carboxymethylated dextran coated and modified with lipophilic groups which makes the surface suitable for direct attachment of lipid membrane vesicles and enables probing interactions involving transmembrane proteins. Successful reconstitution of RyR into unilamellar liposomes is reported in the literature (Lindsay and Williams 1991), although it should be noted that membrane protein reconstitution is a non-trivial task and much developmental work is required to determine the correct conditions for reconstitution with preservation of protein function. Proteoliposomes containing RyR could be, in theory, attached to the chip L1 surface without the need to incorporate anchor molecules within the lipid vesicles. The subsequent flow of CSQ would probe the potential interaction between RyR and CSQ. However, a control experiment, using RyR-free liposomes, would be needed to test for binding of CSQ to the phospholipids of the lipid vesicles. Other approaches may consider using a sensor chip with a plain gold surface (AU) that would provide the opportunity to design unique surface immobilisation chemistries. For example, FKBP12/12.6 adsorbed to the gold surface would immobilise RyRs by binding.
to the cytoplasmic assembly of the receptor, and thus the luminal domain of the protein will be exposed for an interaction with flowing CSQs. However, this would require the expression and purification of recombinant FKBP12/12.6.

Although the QCM-D results also suggested that there is a direct interaction between RyR1 and hCSQ2, we cannot completely rule out the possibility that the hCSQ2 has occupied the gaps between RyR1 complexes or has been trapped between the complexes adsorbed onto the chip. However, if this was the case then it might be predicted that if hCSQ2 was packed in between RyR1 complexes, and occupied any gaps on the sensor chip, then this would increase the packing density and thus lead to a more compact layer; however, the increase in the overall dissipation would hint that a more flexible, elongated, layer had formed. Interestingly, the data also suggests that the putative interaction between RyR1 and hCSQ2 is independent of the Ca\(^{2+}\)-bound state of hCSQ2. To develop these studies further several issues should be considered. For example, it has been shown that a more dense packing of the proteins can be achieved by coating the biosensor chip with a rough platinum layer (Dolatshahi-Pirouz, Rechendorff et al. 2008). Therefore, if the equipment were available then future studies may want to employ gold-coated AT-quartz crystals with added platinum films which can be deposited with an oblique angle (e.g. 10°) resulting in a rough surface on the biosensor chip. This may aid a more consistent, dense packing of RyRs onto the chip reducing the possibility of gaps in between the molecules that CSQ2 might occupy. However, conformational changes to RyR due to adsorption to this rough platinum layer might be an issue. A further limitation of the QCM-D results presented here is that we cannot exclude the possibility that non-specific binding between RyR1 and hCSQ2 has occurred. To address this possibility, future experiments should include control experiments employing other proteins not known to bind to RyR1 or CSQ2 e.g. bovine serum albumin which is of a similar mass to CSQ2. If an increase in mass was observed on application to the adsorbed RyR1 then that would provide evidence that the ‘dummy’ protein may be able occupy gaps in the surface layer. Another approach to assess whether a complex had truly formed might be to examine the surface topology of the chip using atomic force microscopy (AFM). AFM is a scanning probe microscope in which the surface morphology of a sample is sensed by a nanoprobe. The contact mode
of this technique in which the probe is in continuous contact with the surface can cause
deformation (flattening of proteins). However, the development of the tapping mode
advanced the study of protein deposits on solid surfaces (Casero, Vazquez et al. 2010). A
combination of QCM-D and AFM has been used to investigate the binding of
polyclonal antibody to laminin (Malmstrom, Agheli et al. 2007).

In order to be able to compare between the QCM-D data in the presence and in the
absence of Ca\(^{2+}\), CSQ2 and Ca\(^{2+}\)-CSQ2 should be ideally applied to the same RyR
plateau. Therefore, a development of the experimental design would be to try to
regenerate the RyR plateau following the application of hCSQ2 in the absence of Ca\(^{2+}\)
using e.g. NaCl to remove any bound hCSQ2 to then apply Ca\(^{2+}\)-hCSQ2.

A further novel finding from this research was that a luminal domain of triadin
(triadin KEKE motif) increased the P\(_0\) of the both RyR1 and RyR2 as shown by \([^3H]\)
ryanodine binding experiments. Interestingly, removal of CSQ (by JFM preparation)
eliminated the ‘additive’ effect of the the triadin peptide in agreement with an earlier
proposal (Terentyev, Viatchenko-Karpinski et al. 2007). It was proposed that the
increased RyR open probability was because the triadin peptide alleviates the inhibitory
effect of CSQ. However, what might argue against this proposal, and also suggests that
the interplay between RyR, triadin and CSQ is more complicated, is that we show here
that removal of endogenous CSQ from the membrane preparations does not result in an
increase in \([^3H]\) ryanodine binding.
Chapter 6: Electron Microscopy of RyR1 in Complex with CSQ2

6.1 Introduction

Membrane proteins have been described in the literature as the “Wild West” of structural biology (Torres, Stevens et al. 2003). In the Protein Data Bank (PDB), there is a striking shortage of membrane protein (MP) structures compared to water-soluble proteins (Granseth, Seppala et al. 2007). There are numerous challenges for structural studies of membrane proteins. For example, obtaining sufficient quantities of material for crystallization trials is a major constraint since eukaryotic MPs are difficult to overexpress. This is explained, as discussed before in Chapter 4, by the fact that Escherichia coli (the high yield expression system), as a prokaryote, has a different bilayer composition and lack the protein processing machinery of secretory pathways of eukaryotes (Midgett and Madden 2007). Another challenge is the preservation of the native tertiary and quaternary structure during MPs overexpression as well as their biological activity when extracted from the native membrane environment with detergents. Furthermore, obtaining well-diffracting three-dimensional (3D) crystals for X-ray crystallography is a major challenge for attaining high-resolution structures of membrane proteins of any size. Moreover, the application of solution NMR is limited for MPs due to the often large size of membrane protein / detergent complexes (Ubarretxena-Belandia and Stokes 2010). The technique of cryo-EM has firmly established itself as one of the major methods for studying viruses, filaments, ribosomes and membrane proteins. Cryo-EM has unique advantages, when compared to X-ray crystallography and NMR, including the relatively small amount of sample required to obtain a structure, removal of the rate-limiting crystallisation step and absence of an upper mass limit. Moreover, a sample can be imaged under near physiological or well characterized conditions as well as at different stages along a reaction pathway as the speed of vitrification allows trapping of samples in different conformations. The software employed for methods such as single particle analysis has the ability to sort out different biochemical or conformational species existing within the sample (Leschziner and Nogales 2007). Cryo-EM is applicable to three distinct modalities; electron crystallography, electron tomography and single particle analysis. Electron crystallography relies on the availability of two-dimensional crystals, either natural or
synthetic, of the molecules. It is particularly useful for membrane proteins which can be imaged in their lipid environment. The two-dimensional crystals can be grown by reconstitution of purified, detergent-solubilised membrane proteins into lipid bilayers under defined conditions, and this process involves removal of detergent, by e.g. dialysis or adsorption onto a hydrophobic resin, in the presence of specific lipid species with an optimal lipid-to-protein ratio (LPR) (Ubarretxena-Belandia and Stokes 2010). Electron crystallography has yielded near atomic structures of many membrane proteins, e.g. acetylcholine receptor (Miyazawa, Fujiyoshi et al. 1999; Unwin 2005), but is still technically difficult requiring substantive amounts of protein (low milligram) and optimization of the crystallisation process. Cryo-electron tomography has the potential to study macromolecular assemblies in situ, preserving the function of the structure and eliminating the risk of any artefacts accompanying the preparation procedures, however, it does not provide details of individual proteins. In this method, a series of transmission electron micrographs are taken from different viewing angles and can be used for reconstruction of an object (Robinson, Sali et al. 2007). Tomographic reconstructions of frozen-hydrated triad junctions were recently used to determine the structure of the macromolecular complex associated with calcium release from sarcoplasmic reticulum (Renken, Hsieh et al. 2009). Single particle analysis (SPA) is a cryo-EM technique whereby images of individual protein molecules, are used to determine the 3D structure with resolutions not usually greater than 10-15 Å. Single particle analysis involves two principle approaches: Random Conical Tilt (RCT) and Angular Reconstruction (AR). Single particle analysis techniques have proven to be highly successful for studying solubilised membrane proteins requiring only microgram amounts of material; for a review see (Mio, Maruyama et al. 2010). However, there are limitations of the method. SPA is generally not applicable to small proteins with low symmetry and therefore, in contrast to other techniques the larger the protein the better. Furthermore, the attainable resolution is limited. However, the new generation of field emission gun electron microscopes with electron beams of high coherence as well as the development of sophisticated software capable of retrieving fine structural details has advanced the field. SPA can be employed to resolve structures to medium resolution (~10 Å) and thus there is the potential to reveal the fold of the polypeptide chain (i.e. secondary structure
elements). This technique has been particularly successful in the case of icosahedral viruses due to the high symmetry and rigidity of the molecules (Orlova and Saibil 2004). For example, Crowther and co-workers have employed electron microscopy and SPA to solve the structure of hepatitis B virus core protein, which assembles into core shell particles very similar to the native virus core on expression in bacteria, to a resolution of 7.4 Å revealing the α-helical nature of the protein (Bottcher, Wynne et al. 1997).

Moreover, EM methods coupled with single particle analysis have been shown to be extremely useful for structural studies of RyR as discussed in the Introduction to this thesis. Wagenknecht and co-workers have determined the three-dimensional structure of the skeletal muscle calcium release channel from a random conical tilt series of images extracted from electron micrographs of purified channels prepared in a frozen-hydrated state (Radermacher, Rao et al. 1994). In addition, van Heel and co-workers have employed electron cryomicroscopy in conjunction with the angular reconstruction approach to visualise the skeletal muscle ryanodine receptor (Serysheva, Orlova et al. 1995). Both approaches yielded virtually identical structures. Discussed below is a brief outline of the electron microscopy and image analysis methods employed for the structural studies in this research project. A detailed review can be found in (Ruprecht and Nield 2001).

6.1.1 Transmission electron microscopy & Image analysis

Transmission electron microscopy (TEM) images of individual molecules are 2D projections of these molecules. A 3D reconstruction from 2D projections using single particle analysis can lead to volumes with resolutions ranging from 7-30 Å. Despite lagging behind in resolution compared to crystallography, these techniques can reveal the structural features and conformational changes that underlie the function of the protein (Torres, Stevens et al. 2003).

6.1.1.1 Specimen preparation

The incident electrons of TEM, breaking bonds and fragmenting molecules, are detrimental to biological specimens which are extremely sensitive to bombardment by electrons. Furthermore, the TEM images of proteins have low intrinsic contrast because the biological molecules contain mainly atoms (C, H, O, N, etc) which scatter electrons
weakly. Therefore, the aim is to minimise electron-beam damage to the specimen, and maximise image contrast, in order to attain a reasonable resolution and so specimen preparation plays a crucial role.

Two modalities of EM specimen preparation exist; negative staining and cryo-EM specimen preparation. Negative staining is a simple technique, and provides high contrast and a low sensitivity to the electron beam. However, negative staining has several potential drawbacks. It is the distribution of the heavy metal atoms rather than the density of the specimen that is imaged and thus provides details of the surface i.e. a cast of the protein. In addition, there is generally a resolution limit of ~20 Å imposed by the grain size of the negative stain. Another issue with negative staining is that flattening of the 3D structure by dehydration of the sample can occur, although that is not always the case. However, when dealing with a new structure it is sensible to first determine a negative stain structure due to the high signal to noise ratio. Cryo-EM involves specimen application to a “holey” carbon grid (see Fig. 6.1) which may have been glow-discharged to improve the hydrophilicity of the carbon film and therefore increase the amount of specimen adhering to the grid. The aim is however for the protein to occupy the holes rather than associate with the carbon film.

**Figure 6.1: Cryo-EM specimen.** A carbon film of ~ 500 Å thicknesses perforated with holes 1-2µm in diameter is applied to a standard 3mm electron microscope grid. The particles of interest (e.g. protein molecules) suspended in buffer are adsorbed onto the grid. The aim is for the protein to occupy the holes in the carbon film so that the absence of a carbon layer ensures random orientations of the particles are captured. Taken from (Wang and Sigworth 2006).
The grid is then blotted with filter paper to remove the excess buffer, and plunged into a bath of liquid ethane (see Fig. 6.2) leading to rapid freezing of the sample thereby embedding the specimen in vitreous ice. Therefore, damage of the specimen by ice crystal formation is avoided. The molecules, in vitreous ice, are well hydrated and are more like those in a native environment. The whole process of cryo-specimen preparation can be automated using a Vitrobot™, a computer-controlled freeze-plunging instrument. The frozen-hydrated specimens have to be kept at liquid nitrogen temperature throughout the process of cryo-EM (even at quite low temperatures, sublimation of ice may be appreciable causing dehydration of the specimen). Cryo-EM is technically much more demanding than negative staining and also requires significantly more protein.

Figure 6.2: Schematic diagram representing the steps of cryo-EM specimen preparation. Step 1: The process starts with sample application to a “holey” carbon grid. Excess buffer is then removed by blotting using filter papers as illustrated in Step 2. The blotting step is critical and requires optimisation regarding the number of blots and the timing of each blot to ensure an ideal thickness of the sample layer onto the grid. If the ice layer is too thick no protein will be visible whereas if it is too thin the sample will be unstable. Step 3: Finally, the sample is rapidly frozen by plunging the grid into a bath of liquid ethane.
Figure 6.2 illustrates the various steps of sample preparation for study by cryo-
EM.

6.1.1.2 Imaging

Images necessary for high-resolution work require minimising some commonly
encountered problems. For example, specimen drift is a consideration whilst an image is
recorded but this can be reduced by a high degree of mechanical stability of the
specimen stage. Secondly, a non-perfectly symmetrical objective lens can result in the
formation of line images of point objects, known as “astigmatism”. Astigmatism is
spotted if asymmetry of the granulity of the carbon film is detected at high
magnification (e.g. >105 000x), and can be corrected by adjusting the field applied by
electromagnetic lenses known as stigmators. Astigmatism is said to be corrected when
the carbon film granules remain rotationally symmetrical when cycling through in and
out of focus. Chromatic aberration results from variations in electron velocity and is
another common problem in TEMs with conventional thermoionic emission guns which
emit electrons with different energies. The development of the field emission gun (FEG)
has greatly decreased this problem, and its use should be the standard for a high
resolution work. Finally, spherical aberration is an inevitable problem, even in the most
advances types of electron lenses. It is a geometrical property of lenses leading to a
“zone of confusion” resulting from rays that travel to the lens margins being brought to
a slightly different focus point compared to those travelling very close to the lens axis.

In cryo-EM the electron dose is minimised (10-20 electrons/ Å²), and thus the
beam damage is reduced. Three different “modes”, search, focus and exposure, are used
in this “low-dose” technique. Search mode, set at low magnification (e.g. 2650x), is
employed to locate a good area which would be a one containing a hole in the carbon
film with a meniscus of ice spanning the hole with hopefully the sample embedded. The
selected area will be imaged at the required magnification (e.g. 40 000x) in the
exposure mode. Focus mode, set at high magnification (e.g. 175 000x), is used to focus
on an area near the ice hole.

EM data can be recorded on two available media: photographic film and charge-
coupled device (CCD) cameras. The machine at the University of Manchester is an FEI
Polara field emission gun transmission electron microscope operated at an accelerating voltage of 300kV equipped with a 4K x 4K CCD camera.

6.1.1.3 Image processing and single particle analysis

EM images still require substantial processing to minimise contribution from artefacts e.g. spherical aberration. This is particularly true in the case of low signal noise ratio (SNR) of images collected employing low-dose techniques. Therefore, image processing methods provide a means of converting raw data into meaningful information. Software packages for carrying out single-particle image reconstruction of three-dimensional (3D) macromolecular assemblies from cryo-electron microscopy (Cryo-EM) image data include SPIDER (System for Processing Image Data from Electron microscopy and Related fields) (Frank, Radermacher et al. 1996), Imagic-5 (van Heel, Harauz et al. 1996) and EMAN (Electron Micrograph ANalysis). EMAN, the software package employed in this research project, is free for the academic community and has the advantages of an efficient processing capacity that has been employed for reconstructions beyond 10 Å. Furthermore, the relative simplicity especially for the new users and the robust method for contrast transfer function (CTF) correction make it popular for EM studies (Ludtke, Baldwin et al. 1999).

6.1.1.3.1 Particle selection

Locating individual particles, in the raw micrographs or CCD frames, is the first mandatory step for the single particle analysis. EMAN includes a graphical program, known as “boxer”, for manual or semiautomatic particle picking. The single particles are selected into pixel boxes the size of which should be 25-50% larger than the particle. This program provides the option of contrast inversion for cryo-images as the protein appears darker than the background whereas the software has been developed to recognise protein as white densities (Ludtke, Baldwin et al. 1999).

6.1.1.3.2 Contrast transfer function (CTF) correction

As discussed, the images generated by electron microscopes suffer from a set of artefacts including the contrast transfer function (CTF). The CTF of a microscope is a function of many variables including drift, lens errors, astigmatism, electron wavelength, and the temporal and spatial coherence of the electron beam. Without CTF
correction, serious artefacts can skew the analysis leading to a 3D reconstruction containing significant local mass displacements, and, hence, bearing only a vague resemblance to the true structure. CTF correction aims at retrieving the true 2D projections of the specimen and is therefore essentially required prior to further image analysis in particular for cryo-EM images.

Contrast, in an image, has two components; amplitude and phase. The phase component is due to the interference between the elastically scattered waves, by the object and still passing through the objective aperture, and the unscattered, i.e. transmitted, waves. Amplitude contrast, however, is produced by high angle elastic scattering or inelastic scattering, in both of which electrons are lost falling outside the objective aperture. In cryo-EM work, as discussed above, the biological specimen scatters electrons weakly; therefore, the amplitude contrast is low. Furthermore, the defocusing, employed to enhance the contrast, contributes to the phase component of the CTF (PCTF).

The CTF oscillates rapidly at high spatial frequencies providing detailed information. However, the coarse information given by CTF at low spatial frequencies is important for localisation of the particles in the micrograph (van Heel, Gowen et al. 2000). In practice, an envelope function is applied to the CTF (see Fig. 6.3). In other words, the illumination has finite divergence and a finite energy spread. The CTF is dampened by the resulting partial coherence as we go towards higher spatial frequencies, and the resolution is ultimately limited (Frank 1996).
Figure 6.3: The contrast transfer function, and the resolution limits. The continuous line illustrates the phase contrast function (PCTF) variations against spatial frequency \((\alpha/\lambda)\), where \(\lambda\) is the electron wavelength and \(\alpha\) is the angle of scattering in the microscope. The dashed line represents the damping effect of an envelope function which limits the attainable resolution to a point known as “information limit”. Without CTF correction, the resolution limit corresponds to the first zero crossing of the CTF. Taken from (Ruprecht and Nield 2001).

As can be seen from Fig. 6.3, the CTF shows multiple zero crossings with alternating peaks in both positive and negative directions. Such maxima and minima of CTF can be represented by an optical diffraction pattern, the fast Fourier transform (FFT), or the power spectrum of an EM image revealing the Thon rings. Thon rings appear as bright bands where the CTF is positive or negative, and as black rings at zero crossings. The zero crossings correspond to the missed spatial frequencies, an inevitable artefact of electron microscopy which can be compensated for by using several sets of particles from images taken at a different defocus. Accordingly, without CTF correction, the first zero crossing of the CTF indicates the limit of resolution of an image. However, even with CTF correction, extending the resolution, beyond a point known as “the information limit” (see Fig. 6.3), is impossible. This is due to the damping effect of applying an envelope function to CTF. Within the EMAN software package there are
well-defined algorithms for correcting both the phase and amplitude of the CTF. After correction for the CTF the images can then be employed for SPA.

6.1.1.3.3 Alignment & Classification

Prior to further analysis, it is important to first center and then align the selected images with respect to each other in order to eliminate the “in-plane” translational and rotational degrees of freedom among the images. It is recommended that this initial alignment is not performed using a reference image so as to avoid biasing the data set towards the properties of the reference image used. Therefore, “reference-free” alignment, using cross correlation functions (CCFs), is preferred to produce a first version of the various views present in the data. These views represent the different arbitrary rotational orientations of all the protein molecules (van Heel, Gowen et al. 2000). An iterative statistical procedure, know as multivariate statistical analysis (MSA), is then employed to search the whole data set so as to find similar images, in similar rotational orientations creating classes of images. MSA involves determination of eigenvalues and eigenvectors (also called eigenimages or factors) which show the variation in the data set, and thus can be used to reduce the total amount of data by considering only the significant eigenvectors. This speeds up the image processing. After many iterations, class averages are generated which are then used to start the multi-reference alignment procedures. This multi-reference alignment uses the first class averages as references to search through and, now, align the entire data set using CCFs. Translational and rotational alignment is performed in this multi-reference alignment. New class averages are produced which after a few iterations exhibit a greatly improved SNR.

6.1.1.3.4 Angular reconstruction (Euler angle determination)

The generated class averages, in the previous step, represent 2D projections of a 3D structure, and thus can be used to construct a 3D model. Euler angles are directional angles used to indicate a position and orientation in 3D space relative to a common centre, and their determination for each of the class averages is the first step towards the construction of a 3D model. This can be achieved by the angular reconstruction technique which is based on the common line projection theorem stating that two different 2D projections of the same 3D object have at least one 1D line projection in
common. The relative Euler-angle orientations of set projections can be determined from the angles between such common lines. Random Conical Tilt (RCT) is an alternative approach to AR. In RCT, each field of particles is imaged at a relatively high tilt (typically around 50°) followed by an image of the same area but at 0° i.e. untilted. For 2D alignment and classification, the untilted images are used; however, reconstruction is performed on the tilted dataset. In RCT, there may be unavailable views because of the physical limitations as to how far the sample can be tilted in the microscope, and accordingly the 3D reconstruction is missing information. This is known as “missing cone problem”; describing the shape of the missing data in Fourier space (Leschziner and Nogales 2007). Leschziner & Nogales described a novel approach to overcome this by applying the Orthogonal Tilt Reconstruction (OTR) in which there are two orthogonal tilts (-45° and +45°); one for 2D alignment and classification and the other for reconstruction (Leschziner and Nogales 2006). Utilising exclusively untilted data, the AR technique builds upon analytical rather than geometrical principles and thus is technically easier and faster. AR is a zero-tilt single particle approach and thus data collection can be less technically demanding than the RCT method. However, the AR method requires multiple orientations of the protein to be captured, and if the protein adopts a preferred orientation, then this method is not suitable. The macroscopic tilt, required for the RCT approach, creates huge differences in defocus existing within the tilted image which are very difficult to tackle computationally limiting the achievable resolution level. Presented below is a flow chart representing the reconstruction process in EMAN.
6.1.2 Aims and Objectives

Since the earlier chapters provided some evidence of an interaction between RyR and CSQ, the next goal of this thesis work was to employ negative stain and cryo-electron microscopy to examine a putative complex formed by RyR1 and hCSQ2.

For the EM studies RyR1 samples were used since the level of purity and yields were much higher than for the cardiac channel. Moreover, it has been shown in the previous chapters that hCSQ2 also interacts with RyR1.

Control images of RyR1 were first acquired followed by those of a complex of RyR1-hCSQ2 isolated on a sucrose gradient. In order to examine the quality of the protein samples negative staining methods were first employed followed by cryo-EM. Single particle analysis (SPA) was then used to probe the complex formation between
RyR1 and hCSQ2. For a robust analysis and to extract potential high resolution data the cryo-EM data sets were CTF corrected. The aim was to identify the domain within the RyR1 volume to which hCSQ2 binds.

6.2 Results and Discussion

As described in Chapter 3, the skeletal ryanodine receptor (RyR1) was purified to homogeneity as judged by biochemical analysis and negative staining showing a field of well separated particles. These data encouraged further studies of RyR1 employing two modalities of electron microscopy (EM); negative stain and cryo-EM.

6.2.1 Negative stain and cryo-EM of RyR1

The purified skeletal RyRs from sucrose gradient fractionation were examined by transmission electron microscopy (Tecnai 12 transmission electron microscope operated at an accelerating voltage of 100kV) after negative staining of the sample using uranyl acetate (panel A, Fig. 6.4). Higher concentrations are required for cryo-EM. Therefore, prior to cryo-EM, the RyR1 was concentrated by ultracentrifugation through a sucrose cushion as detailed before in Materials and Methods section 2.7.2. Initial attempts using cryo-EM revealed a preference of RyRs to adhere to the carbon film rather than to occupy the holes of the Quantifoil holey grids. This problem was solved by coating the grids by a thin carbon layer as described before (Radermacher, Rao et al. 1994) and as described in Materials and Methods section 2.7.2. For cryo-EM the grids were examined in a Polara TEM with a field emission gun operated at an accelerating voltage of 300kV under low dose conditions (see panel B Fig. 6.4).
Figure 6.4: Electron microscopy images of purified RyR1. Panel (A) shows field of negatively stained RyR1. Panel (B): unstained images of RyR1; proteins in ice show low contrast. The protein appears white when negatively stained; however, it appears black/grey in the absence of stain. The black arrows highlight the densities corresponding to the RyR1 homotetramers. The characteristic four-fold symmetry and “clover-leaf” appearance of RyRs is very clear in these images. Scale bar = 100 nm.

As shown above in Figure 6.4 above, the negative stain as well as the cryo-EM images showed a homogenous population of protein particles. These protein particles are characteristic of previous reports of RyR1 with the four-fold symmetry and “clover-leaf” appearance. Examination of the images found that the RyR1 had a preferred orientation on the grid with the top (cytoplasmic) and bottom (luminal) views predominating although some side views were identified as shown below.
Figure 6.5: Examples of negatively stained (2% (w/v) uranyl acetate) RyR1 raw particles (prior to filtering and alignment). Box size 461x461 Å. (A): Top and bottom views of RyR1. Since these are raw particles, it is not possible to distinguish between top and bottom orientations at this stage. However, the “clover-leaf” structure is clear. (B): Side views of RyR1 with white arrows highlighting the putative transmembrane (TM) stalk domain.
Therefore, the angular reconstruction method may not be optimal for a 3D reconstruction. This problem of preferred orientations of RyRs has been reported before in the literature and dealt with by the alternative approach of random conical tilt (RCT) (Radermacher, Rao et al. 1994; Benacquista, Sharma et al. 2000; Sharma, Jeyakumar et al. 2000; Meng, Xiao et al. 2007; Meng, Wang et al. 2009). However, since several 3D structures have been determined for RyR1, all of which agree extremely well, this was not an objective of this study. The earlier chapters in this thesis describe a direct interaction between RyR1 and hCSQ2 and thus the aim of this portion of the research project was to determine whether a RyR1-hCSQ2 could be imaged using TEM/single particle analysis methods. Initially, images of purified RyR1 in negative stain and cryo-EM were acquired to gather a control dataset. Approximately 2600 and 2100 particles were selected, using the graphical interface “boxer” (EMAN image processing software) from the negative stain micrographs and digital cryo-EM images respectively (see Fig. 6.5 and Fig. 6.6). Following CTF correction, the selected particles were filtered, centred, aligned (rotational and translational). Distinct views of the RyR1 were determined using
reference-free classification methods using the “startnrclasses” command. As can be seen below none of the classes were of side views of RyR1 (see Fig. 6.7).

![Figure 6.7: Class averages of negative stain RyR1 dataset. These class averages were created from approximately 2600 particles using “startnrclasses” programme, part of EMAN software. None of the class averages were suggestive of the side views of RyR1.](image)

This might be due to the fact that although some raw particles that appeared to be side views were identified as shown in Fig. 6.5 B & Fig. 6.6 B these were too few for the software to form a class average.

6.2.2 EM imaging of RyR1-hCSQ2 complex

The purified RyR1 sample (closed state; 0.1M EGTA) was incubated with purified hCSQ2, and the mixture was ultracentrifuged through a sucrose cushion. SDS-PAGE and western blot characterisation have confirmed the presence of RyR1 and hCSQ2 in the bottom part of the sucrose cushion with free unbound CSQ2 identified at the top of sucrose which was well-separated from the complex. Examination of negatively stained grids prepared from the fraction containing both RyR1 and hCSQ2 revealed extra-densities extending out from the RyR1 complexes (see Fig. 6.8) indicative of hCSQ2 binding to the closed state of RyR1.
**Figure 6.8: hCSQ2 binding to RyR1 (closed state).** Examples of negatively stained RyR1-hCSQ2 complexes; top & bottom (A) and side (B) views. Box = 533x533 Å. The hCSQ2 appear as an extra density extending out from the characteristic RyR1 complex; indicated by white arrows.

As can be seen from Fig. 6.8, hCSQ2 binds to RyR1 (closed state) and can be visualised by EM as densities varying in size (e.g. 30-50nm) extending out of RyR1 complex. Given the size of a monomeric unit of CSQ2 < 5nm (Park, Park et al. 2004) these densities would suggest that hCSQ2 has polymerized which is surprising since the CSQ polymerization is reported to be calcium dependent (Park, Wu et al. 2003; Park, Park et al. 2004), and in these samples 0.1M EGTA was present. This may suggest that other factors may influence the formation of linear chains of CSQ2. It was difficult to identify single RyR1-hCSQ2 complexes that were separated from adjacent molecules and had a similar size CSQ2 density associated. In fact many areas were found with “chains” of RyR1s as shown below (Fig. 6.9)
Figure 6.9: Selected fields of negative stain electron microscopy of RyR1-hCSQ2 complex micrographs. The presence of hCSQ2 appears to lead to a linear arrangement of RyR1s as indicated by the white arrows.

This may indicate that CSQ2 can link individual RyR1 molecules and thus may have a role in “coupled gating” of RyRs. Marks and co-workers suggested a phenomenon called “coupled gating” between individual RyRs whereby a physical association mediates co-ordinated regulation of RyR channels during EC coupling (Marx, Ondrias et al. 1998; Marx, Gaburjakova et al. 2001). Careful examination of the EM micrographs of RyR1-hCSQ2 complex has also revealed polymers of hCSQ2 linking RyR1s complexes as illustrated below in Fig. 6.10.
The association between RyR1s through polymers of hCSQ2 provides new insights into how RyRs are arranged, and a possible role of CSQ2 polymers in this arrangement. CSQ2 polymers might provide the required physical link for the spread of the $\text{Ca}^{2+}$ signal from a single RyR1 origin.

A cryo-EM dataset of RyR1-hCSQ2 complexes (approximately 2100 particles) was also collected and subjected to CTF correction and then filtered, centred, aligned and subjected to reference-free classification. The resultant class averages were examined in comparison with the class averages of the control dataset as seen below in Fig. 6.11.
Figure 6.11: Class averages of control (RyR1) (A) and complex (RyR1-hCSQ2) (B) cryo-EM datasets. The contrast was reversed. These class averages were created from approximately 2100 particles using “startnrclasses” programme, part of EMAN software. No clear differences can be described on examination of the two groups of class averages i.e. there are no obvious additional densities associated with the RyR1 homotetramers.

As can be seen from Fig. 6.11, no clear differences can be determined between the control (RyR1) and complex (RyR1-hCSQ2) class averages in agreement with the analysis of the negatively stained data. A possible explanation for this may be that the single RyR particles selected from the complex (i.e. RyR1-hCSQ2) dataset were of RyR1s devoid of hCSQ2 as when hCSQ2 is bound, it promotes association of the RyR1 complexes to form the clusters shown in Fig. 6.9.

6.3 Conclusion

Adding to our findings of a direct interaction between RyR1 and hCSQ2, as described in the earlier chapters of this thesis, the negative stain EM images show what appears to be polymerization of hCSQ2 upon binding to RyR1 in the absence of Ca\textsuperscript{2+}. This novel finding may suggest that an interaction with RyR, rather than calcium, dictates the polymerization of CSQ2. Further examination of the raw EM images has revealed chains of RyR1 form in the presence of hCSQ2 raising the intriguing possibility that CSQ2 contributes to the “coupled gating” mechanism between individual
RyRs by providing a link, or bridge, between adjacent RyR1 homotetramers. The “coupled gating” theory postulates a coordinated activation and inactivation of the Ca\textsuperscript{2+} release channels during EC coupling. Marx et al (Marx, Gaburjakova et al. 2001) proposed a model of coupled gating between RyR2s allowing synchronous opening (during systole) and closing (during diastole) of all RyR2s in a T-tubule/SR junction. This model raised the possibility of a role shared by the drop in SR [Ca\textsuperscript{2+}] and the coupled gating mechanism in decreasing RyR2 open probability and termination of Ca\textsuperscript{2+} release.

Examination of the class averages of control (RyR1) and complex (RyR1-hCSQ2) datasets failed to find any clear differences which might be due to the fact that these particles were well-separated and not part of a ‘chain’ which may suggest that they are free RyR1 homotetramers (i.e. hCSQ2 is not bound) and thus explain why there is no difference when examined in comparison with the control dataset. This might also be due to the fact that the presence of RyR1 considerably enhances the process of hCSQ2 polymerization leaving only a minor population of RyR1 to bind to a monomeric hCSQ2. This minor population would then be “averaged out” during the image processing.
Chapter 7 Conclusions

This research project has investigated the somewhat controversial issue regarding the structural assembly of the quaternary SR luminal Ca\(^{2+}\)-sensor formed by RyR, CSQ, triadin and junctin; namely whether CSQ can bind directly to RyRs in the absence of triadin and junctin. This thesis work has provided evidence of a direct interaction between RyR and CSQ using a variety of biochemical and biophysical techniques. RyR1 (closed state) was co-precipitated with hCSQ2 as shown by western blot analysis of pull down experiments. Surface plasmon resonance (SPR) data shows binding of hCSQ2 to RyR1 (open state) and to RyR2 (open and closed states). However, as discussed in Chapter 5 there are limitations of the SPR data. In a third approach, quartz crystal microbalance and dissipation (QCM-D) monitoring has provided additional evidence of binding between RyR1 (closed state) and hCSQ2 with data to suggest that this interaction might be Ca\(^{2+}\) independent, although further experiments are required to develop this concept. Finally, transmission electron microscopy (TEM) was employed showing complexes of RyR1 and hCSQ2. Intriguingly, it appeared that the addition of hCSQ2 to RyR1 lead to a linkage of the RyR complexes. These data therefore suggest a novel role for hCSQ2 for forming a bridge between RyR1 homotetramers that would fit with the idea of “coupled gating” between RyRs. Therefore, the experiments described in this thesis provide new data that would suggest that RyR and CSQ can interact in the absence of triadin and junctin. Furthermore, the manner in which hCSQ2 binds to RyR1 shown here leads to a new concept of hCSQ2 having an additional role as a ‘linker’ molecule facilitating the clustering of RyR1 complexes. Furthermore, the fact that CSQ is a Ca\(^{2+}\) binding protein then a direct interaction between CSQ and RyR might support the idea that CSQ serves directly as transducer of luminal Ca\(^{2+}\) sensing for RyR and thus contributes to the control of the RyR open probability throughout the cardiac cycle. Accordingly, these results might not only have implications for clarifying mechanism underlying normal physiology but also for understanding pathological conditions such as CPVT where a disruption of a direct interaction between RyR and CSQ might underlie the failure of luminal Ca\(^{2+}\) sensing with a subsequent increased RyR open probability.
This thesis work has also involved the optimisation and employment of \[^{3}\text{H}]\) ryanodine binding experiments to investigate the regulation of RyR1 and RyR2 within isolated SR membrane vesicles by CSQ and triadin. An intriguing finding was that a synthetic peptide corresponding to the triadin KEKE motif led to increased ryanodine binding which reflects the open probability of the RyR channel, this effect was abolished when CSQ was removed suggesting a co-operativity between the peptide and CSQ binding to RyR. This interesting finding, that a small peptide can bind to RyR1 and RyR2 and elicit changes to the channel function, might be of interest to pharmacologists for the design of a compound similar to this triadin motif, although ideally it would be antagonistic to RyR2 and thus suppress the increased open probability of the channel in patients with arrhythmia and heart failure.

**Future work**

This thesis work has employed numerous techniques to explore the interaction of purified hCSQ2 with RyR1 and RyR2. The luminal Ca\(^{2+}\)-sensor quaternary complex formed by RyR, CSQ, triadin and junctin is believed to be in a dynamic state. It is possible that a population of a binary complex formed by RyR-CSQ interactions is present in native tissue. One approach to investigate RyR-protein complexes would be to employ blue native PAGE (BN-PAGE); for detailed reviews about BN-PAGE see (Miernyk and Thelen 2008; Dresler, Klimentova et al. 2011). In order to preserve the protein (or protein complex) in its near native state both sample preparation and electrophoresis are conducted employing non-denaturing conditions e.g. neutral pH, low salt concentration, with no reducing and denaturing agents and at 4\(^\circ\)C. To apply this technique to examine RyR complexes would first require solubilisation of the SR membrane proteins using mild non-ionic detergents such as Triton X-100 and CHAPS. These detergents should provide a balance between effective solubilisation of membrane protein complexes and non-disruption of the protein-protein interactions. The sample would then be incubated with the anionic dye Coomassie Brilliant Blue G-250 (CBB G-250) which binds to the protein surface adding negative charges to the protein complexes and thus promoting electrophoretic migration. CBB should be included in the electrophoresis buffer in order to replace the dye which dissociates from the proteins during the electrophoresis. The BN-gels, after complete separation, can be destained
with 10% (v/v) acetic acid, followed by silver staining and immunoblotting as required to detect protein components. Since the molecular mass of RyR2 homotetramer is \( \sim 2.3 \text{MDa} \) and the MW of CSQ2 is \( \sim 50 \text{kDa} \), bands suggestive of an RyR2-CSQ2 binary complex would be at the approximate positions of 2.35, 2.4, 2.45 and 2.5MDa for 1:1, 1:2, 1:3 and 1:4 stoichiometries of RyR2:CSQ2, respectively. Bands of complexes of MW higher than 2.5MDa may indicate that polymers of CSQ2 were bound to RyR2. Given the high molecular mass of RyR, separation by gel electrophoresis would be technically demanding since a 5% gel is required to separate out (under denaturing conditions) a monomer of RyR at \( \sim 565 \text{kDa} \). However, since BN-gels are usually run in the cold room extended electrophoresis times e.g. overnight may allow the non-denatured protein (2.3MDa) to enter the gel. Moreover, the resolution of the gel may be limited so that it would be difficult to distinguish between e.g. a 2.3 and 2.35MDa band. The bands observed on a one-dimensional BN-PAGE could be further analysed by a second dimension SDS-PAGE (denaturing electrophoresis), and an RyR2-CSQ2 binary complex would be confirmed by separating bands of an RyR2 monomer (at \( \sim 565 \text{kDa} \)) and CSQ2 (at \( \sim 50 \text{kDa} \)).

BN-PAGE has the advantages of being inexpensive; however, the drawbacks include limited resolution and the possibility of complex dissociation during the electrophoretic separation. Some protein-protein interactions are also affected by the anionic CBB dye. Therefore, an alternative approach would be clear native (CN) PAGE. In CN-PAGE, there is no employment of CBB dye, and thus the migration of protein complexes is driven only by their intrinsic charge. Therefore, CN-PAGE is only suitable for acidic proteins (PI<7) (Krause 2006). The theoretical PI values for bovine RyR2 and CSQ2 (ExPASy-ProtParam tool (Gasteiger, Hoogland et al. 2005)) are 5.73 and 4.26 respectively, and so would suggest that CN-PAGE may be appropriate. However, a lower resolution due to diminished mobility when compared to BN-PAGE is a disadvantage of CN-PAGE.

Other approaches that could be taken to investigate RyR-CSQ interactions might include employing a \(^{125}\text{I-CSQ}\) overlay assay. This assay has been described previously to examine the interaction between junctin and CSQ (Jones, Zhang et al. 1995). In brief
this method would involve SDS-PAGE of the purified RyR followed by transfer to nitrocellulose. The nitrocellulose sheet would then be stained with Amido Black to localise the RyR band. A series of blocking and washing steps would be followed by the addition of purified CSQ that has been iodinated by 3-(trifluoromethyl)-3-(m-[125]I) iodophenyl) diazirine ([125]I TID) label. Autoradiography would indicate whether the 125I-CSQ has bound to the RyR. However, a limitation of this technique would be that the RyR is denatured i.e. the protein band corresponds to a RyR monomer and thus the CSQ2 binding site may be disrupted either by the denaturing SDS-PAGE conditions or by the loss of the quaternary structure of the homotetramer.

Another approach for probing RyR-CSQ interactions might include the development of a Yeast Two-Hybrid (Y2H) system. For example, Lai and co-workers have successfully expressed fragments of human cardiac RyR2 in a pGBK7 vector using Y190 strain with employment of β-galactosidase assay in order to demonstrate that oligomerization of RyR2 occurs through the C-terminal tail (Stewart, Zissimopoulo et al. 2003). They have employed a Y2H system to show an interaction between SNARE-associated protein snapin and the RyR2 C-terminus (Zissimopoulos, West et al. 2006). A similar approach could be used in future experiments to probe the proposed interaction between RyR2 and CSQ2. Fragments corresponding to the C-terminal one fifth of human RyR2 (which involve the luminal portion of the protein) can be expressed as fusions to GAL4 DNA-binding domain (BD) (bait protein) whereas hCSQ2 is expressed as fusion to GAL4 activation domain (AD) (prey protein). Co-expression of the bait and prey proteins is essential for the β-galactosidase assay in Y2H analysis. An interaction between an RyR2 fragment and CSQ2 would be indicated by a blue phenotype (in β-galactosidase assay) of the strain Y190 transfected with the two constructs. The interaction should be investigated by swapping around the GAL4 DNA-BD and AD fusion partners. This analysis would not be only useful to investigate an interaction between RyR2 and CSQ2 but also to probe the binding domain of RyR2 to CSQ2.

Given the problems in isolating pure RyR2 from cardiac tissue described in this thesis future work may want to concentrate on producing RyR2 recombinantly. There
are some reports showing successful cell-free synthesis of human RyR2 fragments employing TNT Quick-T7 coupled rabbit reticulocyte lysate system (Promega) (Stewart, Zissimopulos et al. 2003; Zissimopulos, West et al. 2006). Furthermore, the cell free expression system could be employed to probe an interaction between RyR2 and CSQ2 where individual Myc-tagged human RyR2 fragments are co-expressed with hCSQ2 followed by investigation of co-precipitation of hCSQ2 with an RyR2 fragment immunoprecipitated with Ab\textsuperscript{Myc} which would indicate that binding occurs between the two proteins. The presence of pancreatic microsomal membranes in the cell-free lysate might be required to elicit the proposed interaction between RyR2 and CSQ2. These pancreatic microsomal membranes were found, in a previous study, essential to illustrate an interaction between snapin and RyR2 C-terminus using this cell-free TNT system, suggesting that a membrane environment might be crucial for the association of RyR2 to accessory proteins (Zissimopulos, West et al. 2006). Moreover, Wagenknecht and co-workers have reported the successful construction of the 15 kb cDNA encoding full length RyR2 within a mammalian cell expression vector (pCDNA3) followed by successful expression and purification of the recombinant RyR2 from HEK293 cells (Zhao, Li et al. 1999; Liu, Zhang et al. 2004). However, Wagenknecht and co-workers ensured the high yield required for their structural studies by employing a purification protocol using Glutathione-Sepharose beads containing bound GST-FKBP12.6 with subsequent elution using reduced glutathione. The purified RyR2 was eluted as a GST-FKBP12.6-RyR2 complex. Therefore, a future study may focus upon developing the HEK293 mammalian cell expression system to express recombinant RyR2 to isolate RyR2 devoid of any accessory proteins and produce yields required for structural studies. The other advantage of generating a recombinant RyR2 is that mutations found in for example CPVT can be introduced and the mutants examined in terms of both structure and protein interactions. HL-1 cells are a cardiomyocyte cell line that combines the advantages of continuous division, spontaneous contraction and keeping a differentiated cardiac phenotype (White, Constantin et al. 2004). Lai and co-workers (George, Higgs et al. 2003) have transfected this cell line with plasmids encoding mutations of hRyR2 in order to investigate the effects of these mutations on phenotypic properties of HL-1 cardiomyocytes. The literature shows no evidence of isolating RyR2
from HL-1 cells overexpressing the protein; however, it might be interesting in the future to try to employ this cell line for generating high yields of purified hRyR2 for structural and functional studies.

The EM studies revealed that the addition of hCSQ2 to RyR1 leads to some form of polymerisation and thus makes analysis of the irregular polymer chains difficult. The hCSQ2 structure is formed by three almost identical thioredoxin-like domains. Each of these domains contains about 100 amino-acid residues; domain I (22-142 amino acid residues), domain II (143-246 amino acid residues), and domain III (247-370 amino acid residues) (Kim, Youn et al. 2007). There is no evidence in the literature as to which of these domains is the region involved in binding to the ryanodine receptor. To attempt to remove the polymerisation problem and to ‘dissect’ the region of hCSQ2 that houses the RyR binding domain one approach might be to express and purify these three structural domains separately, and then investigate their binding to ryanodine receptor. It may then be possible to select well-separated particles of RyR-hCSQ2 domain complexes, to determine a three-dimensional reconstruction of the complex, and to conduct a difference mapping study with control RyR particles. However, a limitation of this approach would be if the RyR binding domain is formed by multiple regions of the hCSQ2 and not restricted to one domain.

As described in this thesis difficulties were encountered trying to express both full-length triadin and the luminal domain. Several reasons for these difficulties are discussed in Chapter 4 along with ideas for future development. Once triadin (full-length and/or luminal domain) is available, investigations into its binding to RyR and/or CSQ for the formation of binary and tertiary complex can be carried out. However, an alternative approach might be to purify triadin from native tissue. For example, Dulhunty and co-workers (Wei, Gallant et al. 2009) isolated skeletal triadin employing preparative gel electrophoresis. Therefore, application of this approach or development of chromatographic methods to isolate triadin from cardiac tissue may provide an alternative depending upon the attainable yields.

A direct interaction between hCSQ2 and RyR1 and RyR2 has been successfully demonstrated here. To advance this work further it would be interesting to next
introduce CPVT mutations (e.g. R33X, D307H and/or R33Q) to hCSQ2 and to investigate whether binding to RyR was altered or even eliminated. This would help to elucidate the molecular mechanisms of CPVT2, the autosomal recessive form of catecholaminergic polymorphic ventricular tachycardia which is triggered by homozygous mutations of CSQ2.

The [³H] ryanodine binding assay is a very useful method to probe the open probability (P₀) of RyR. Future work would encompass exploring complementary assays such as a Ca²⁺ uptake and leak spectrophotometric assay which is described in the literature as an in vitro functional assay for SR membranes (Yano, Ono et al. 2000). In this assay, Ca²⁺ uptake is triggered by ATP which stimulates SERCA and the time course of the Ca²⁺ uptake is monitored spectrophotometrically using Flou-3 as a Ca²⁺ indicator. Following a plateau of Ca²⁺ uptake, the accessory proteins relevant to this research project (CSQ and triadin) would be added and the Ca²⁺ leak monitored in the presence of thapsigargin to inhibit SERCA. Although this approach may work for the triadin peptide employed in this thesis research work it would be technically difficult to add CSQ2 and/or triadin as the SR vesicles (or JFM) would be sealed and thus CSQ2 and/or triadin would not be able to enter the vesicles and bind to the luminal domain of RyR. Therefore a more appropriate approach might be single channel recordings which have the advantage of segregating the luminal (trans) and cytoplasmic (cis) sides of the channel which allows control of the luminal and cytoplasmic [Ca²⁺]. This arrangement would allow the addition of CSQ2 or luminal domain of triadin separately and/or sequentially to the trans chamber, and investigating the effects upon the channelling activity of RyR. This would provide a robust understanding of the physiological and pathological functional assembly of the luminal Ca-sensor.
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