The structural / functional relationships of peptides derived from the II – III loop of the skeletal muscle dihydropyridine receptor

by

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Statement

This thesis presents the results of work undertaken in the Muscle Research Group at the John Curtin School of Medical Research, Australian National University, Canberra, Australia. This research was supported by an Australian National University PhD scholarship.

All experiments and data analyses presented in this thesis are my own original work, accomplished under the supervision of Dr Marco G Casarotto and Prof. Angela F Dulhunty, except where otherwise acknowledged.

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Abstract

The skeletal muscle dihydropyridine receptor (DHPR) II – III loop plays a crucial role in skeletal type excitation-contraction coupling. Up to this point in time there have been very few studies into the atomic structure of the skeletal DHPR II – III loop. This study describes investigations into the structure of several peptides derived from the skeletal muscle DHPR II – III loop using nuclear magnetic resonance spectroscopy (NMR) and circular dichroism spectroscopy (CD). The structures have been correlated with functional results obtained from SR vesicle release and single channel bilayer studies. The skeletal DHPR II-III loop has been broken down into 4 segments, denoted as A, B, C and D. Peptides investigated included several variants of the A peptide (T^{671}-L^{690}), variants of the AB peptide (T^{671}-K^{710}) and the C peptide (E^{695}-P^{760}). Some preliminary structural studies were also performed on the full recombinant II – III loop protein (SDCL) (E^{666} – L^{791}).

Eight peptides derived from the sequence of the A region and comprising 7 – 20 amino acids were investigated. It was found that these peptides contained either α-helical, nascent helical-like structures or were random coil (unstructured) in nature. There was a strong correlation between the degree of α-helical secondary structure and the peptides ability to activate Ca^{2+} release through the RyR1. The presence of positively charged amino acids from the region R^{681} – K^{685} increased the peptides ability to activate Ca^{2+} release through the RyR1. The alignment of positively charged residues along one surface of the A peptides as well for the toxin molecules imperatoxin A and maurocalcine was deemed one of the defining features responsible for activation.

The AB peptide contained two regions of α-helical secondary structure, separated by a flexible linker region. Unlike the A1 peptide, the AB peptide (a 40 amino acid fragment of DHPR) did not activate the RyR1, even though the structure of the A region was virtually identical to that found in the A1 peptide. Two mutant peptides derived from the AB region were investigated. The results suggested that several negatively charged residues located in the C terminal end of the AB peptide were preventing the A region of the AB peptide from activating the RyR1 thus providing
further evidence for the importance of positively charged residues in the A region in activating the RyR1. The inactivity of the native AB peptide underlines potential problems with the interpretation of functional data obtained with peptide fragments of larger proteins.

Peptide C contained no fixed secondary structure. Functional studies performed with peptide C and RyR1 have shown that it is capable of both high affinity activation and lower affinity inhibition. As the net charge of peptide C is negative and it has no defined secondary structure, it is highly likely that the C peptide interacts with the RyR1 at a different site to the A1 peptide.

Preliminary studies into the secondary structure of the SDCL indicated a high proportion of α-helical structure in the A, B and D regions and an undefined structure in the C region. The SDCL has been shown to activate the RyR1, but the regions responsible for activation have not yet been defined. The preliminary results for the SDCL have supported the structural profile obtained for the individual peptides studied in this thesis and imply that some of the mechanisms of activation found with the individual peptides may be the same as those in the SDCL.

Overall, the results obtained in this study have helped solve the structure for several portions of the skeletal DHPR II-III loop and will make a contribution to understanding the molecular nature of excitation-contraction coupling.
Publications

Refereed Publications


Commonly used Abbreviations

\(\alpha H_i\) alpha hydrogen of residue i
AID alpha interacting domain
BID beta interacting domain
CaM calmodulin
CaMKII calmodulin kinase II
CD circular dichroism spectroscopy
CDCL cardiac recombinant protein spanning RyR2 residues 788 - 922
CICR calcium induced calcium release
COSY correlated spectroscopy
CSI chemical shift index
CSQ calsequestrin
DHPR dihydropyridine receptor
ECC excitation – contraction coupling
IpTx\(_a\) imperatoxin A
Mca maurocalcine
NH\(_i\) amide hydrogen of residue i
NMR nuclear magnetic resonance spectroscopy
NOE nuclear overhauser effect
NOESY nuclear overhauser effect spectroscopy
PKA protein kinase A
RyR ryanodine receptor
SDCL skeletal recombinant protein spanning RyR1 residues 666 - 791
SR sarcoplasmic reticulum
TOCSY total correlation spectroscopy
T-Tubule transverse tubule
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Chapter 1 - Introduction and Background

1.1 Muscle

1.1.1 Function and Structure

Muscle contraction is an essential part of life for a wide variety of life forms. Muscles are used for numerous functions including: respiration; transporting of blood; locomotion; eyesight; postural positioning; facial expression; and digestion. In humans there are two main forms of muscle present, smooth and striated.

Striated muscle is formed of two major types, skeletal and cardiac. Striated muscle gained its name from the pattern of cross banding of myofibrils, which are about 1 – 3 µm in diameter and are located within the muscle cell (Laki, 1971). Myosin molecules (thick filaments) are arranged in the form of bipolar filaments approximately 1.6 µm long and 10 nm in diameter (Huxley, 1963). The globular subunit of the myosin molecule, the myosin head, projects sideways from the filament shaft and reaches across to a neighboring thin filament, which consists mainly of polymerized actin and tropomyosin (Ruegg, 1986). In a muscle contraction an allosteric change occurs (see section 1.1.2) which draws the thin filament a short distance (approximately 10 nm) along the thick filament. ATP is then required to remove the bound myosin head and it rebinds further along the muscle fiber and the process is repeated. This theory of muscle contraction, proposed by Huxley (1969) has been termed the sliding filament hypothesis (Huxley, 1969).
Figure 1.1 Organization of skeletal muscle. Muscles (a) are made up of a collection of muscle fibers (b), each of which is composed of several myofibrils (c). Within the myofibrils (d) is the contractile machinery, that is, the actin and myosin filaments (g). The striation that can be seen under a light microscope are a result of the highly organized positioning of the actin and myosin filament and these contribute to the formation of A bands, Z lines and H zones (d and e). The filaments (f) are actin, myosin and troponin. The myosin filaments slide along the actin filaments via interactions with the myosin globular head (g). Reprinted with permission (Eckert, 1988).

Skeletal muscle is generally under voluntary control. Skeletal muscles are attached to various points on the skeleton and are generally used for movement. Based on their functional and metabolic abilities skeletal muscle fibers have been termed either, slow twitch (type I) and fast twitch (type II) fibers. Slow twitch fibers are loaded with
mitochondria and therefore depend on aerobic metabolism for ATP production. They are activated by slow conducting motor neurons, are resistant to fatigue and they are often dominant in muscles responsible for movements relating to body posture. On the other hand, fast twitch fibers, have few mitochondria and are dependent on glycolysis for ATP production (anaerobic metabolism). They are activated by fast conducting motor neurons and they fatigue easily for several reasons, including the build up of lactic acid during glycolysis.

It has been recognized recently that the build up of lactic acid is not responsible for muscle fatigue (Dutka & Lamb, 2000), but merely something that often occurs in tandem with fatigue. In fact, lactic acid build up seems to counter some types of muscle fatigue (Nielsen et al, 2001).

on and off via the regular rise and fall of intracellular free calcium and is never maximally stimulated and rarely exceeds half activation of the contractile proteins (Ruegg, 1986). This is a protective mechanism so the heart always has reserves to cope with varied demands of the body.

Smooth muscle is found mainly in the walls of the hollow organs of the human body, with the exception of the heart. In contrast to skeletal muscle, contraction of smooth muscle is generally not under voluntary control and may occur in response to nervous or hormonal stimulation (Ruegg, 1986). The contractile apparatus of smooth muscle is arranged in a less orderly manner and no striation pattern is visible under the light microscope (Ruegg, 1986). Contraction of smooth muscle usually reduces the volume of the bodily organ. It is involved in functions such as digestion, blood flow, movement of urine from the urinary bladder and respiration (by regulating the flow of air through the lungs).
mitochondria and therefore depend on aerobic metabolism for ATP production. They are activated by slow conducting motor neurons, are resistant to fatigue and they are often dominant in muscles responsible for movements relating to body posture. On the other form of striated muscle, cardiac muscle, is an essential component in vertebrate life. The cardiac muscle is able to rhythmically contract and relax without fatigue for a whole lifetime. The contractile machinery of cardiac muscle is switched on and off via the regular rise and fall of intracellular free calcium and is never maximally stimulated and rarely exceeds half activation of the contractile proteins (Ruegg, 1986). This is a protective mechanism so the heart always has reserves to cope with varied demands of the body.

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1.1.2 Excitation-Contraction Coupling

Excitation contraction coupling (ECC) in skeletal muscle fibres, is the process linking electrical activation of the sarcolemma to the release of calcium from the sarcoplasmic reticulum (SR)(the intracellular calcium store). Calcium release during this process is essential for contraction in skeletal muscle. The release of calcium from internal stores in general occurs through two main calcium channels, the inositol-1,4,5-trisphosphate receptor (InsP3) and the ryanodine receptor (RyR). Both of these calcium channels are located in endoplasmic reticulum, while the RyR is also present in the SR (Meissner, 1994). RyRs, with their presence in the SR and large calcium conductivity, are essential for ECC, where a large release of internal calcium from the SR is required (Franzini-Armstrong and Protasi, 1997). During ECC, a wave of depolarisation travels down the integral foldings of the sarcolemma, known as the transverse tubule or t-system. The t-system penetrates deep into the muscle fibre and contains areas that are in close physical contact with the terminal cisternae of the SR (see Figure 1.2). The areas of close physical contact, known as triadic junctions, contain dihydropyridine receptors (DHPRs) and RyRs (see Figure 1.2). The DHPR is an L-type calcium channel embedded in the t-system membrane. It functions as a voltage sensor detecting the depolarization of the t-tubule membrane (evidence for the role of the DHPR as a voltage sensor in presented in section 1.2.3). Upon detecting a depolarization the DHPR activates the RyR and causes a release of Ca$^{2+}$ from the SR. The released calcium interacts with troponin C, a peptide chain consisting of a molecular mass of 19 kDa and 159 amino acids (Ruegg, 1986). Troponin I is a globular protein with a molecular weight of 23 kDa which binds to actin and inhibits
the interaction between actin and myosin filaments (Ruegg, 1986). The inhibitory actions of troponin I are relieved when troponin C binds Ca\textsuperscript{2+}. Troponin I and troponin C are bound to tropomyosin via a 259 amino acid residue protein, troponin T. A schematic representation of the components involved in ECC is shown in Figure 1.3.

*Figure 1.2 An example of an isolated frozen-hydrated triad junction. The sarcoplasmic reticulum vesicles (labeled TC) and the transverse tubule (T-Tub) are indicated. Reprinted with permission (Wagenknecht et al., 2002).*
Figure 1.3 Schematic representation of the main components involved in excitation contraction coupling (ECC). The two proteins that have been found to be essential for ECC are the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR) respectively located in the t-tubule and sarcoplasmic reticulum membranes. The triadin/junction complex has also been shown to be important in ECC (see section 1.5).
1.1.3 Two types of ECC

The process of ECC is different in cardiac and skeletal muscle, where there are different isoforms of the RyR and the DHPR. Each of these isoforms performs essentially the same function, but structural and functional differences distinguish the different isoforms.

In skeletal muscle it has been proposed that the DHPR activates the skeletal RyR (RyR1) via a mechanical interaction (Schneider and Chandler, 1973). Support for this comes from the finding that ECC occurs in the absence of extracellular calcium (Melzer et al., 1995). While the precise mechanism by which the DHPR interacts with the RyR1 is still unknown, it has been shown that the putative cytoplasmic regions between repeats II and III of the alpha 1 subunit of the DHPR (shown in Figure 1.3) are crucial components for skeletal ECC coupling (Tarabe et al., 1990b). Also, the S4 segment of each of the four DHPR membrane spanning segments has been shown to act as the voltage sensor, detecting the depolarization of the t-tubule membrane (Adams et al., 1990). Evidence for the involvement of the S4 region and the II – III loop of the alpha-1 subunit is outlined in sections 1.2.4 and 1.2.6 respectively.

In contrast to skeletal muscle, the release of calcium in cardiac muscle is dependent on the presence of extracellular calcium, as contraction of cardiac myocytes does not occur in its absence (Nabauer et al., 1989). The hypothesis of calcium induced calcium release (CICR) was first proposed in 1975 and is now widely accepted as the mechanism by which the cardiac RyR is activated (Fabiato and Fabiato, 1975). The
process of CICR occurs when a depolarisation of the t-tubule membrane activates the DHPR, which results in a flow of calcium ions from the extracellular to the intracellular domain (Fabiato and Fabiato, 1975). This calcium current activates the cardiac RyR (RyR2) causing a much larger release of calcium from the SR (Fabiato and Fabiato, 1975). In cardiac muscle a functional coupling exists between the DHPR and the RyR, but this coupling is not essential for CICR, but is essential for normal Ca\textsuperscript{2+} release during ECC (Sham et al., 1995). Sham and collaborators, showed that calcium entering cardiac myocytes through the DHPR (in response to a t-tubule depolarisation) elicited a calcium release from the SR that was 1-2 orders of magnitude greater than a similar SR calcium release caused by Ca\textsuperscript{2+} entry through the Na\textsuperscript{2+}/Ca\textsuperscript{2+} exchanger (Sham et al., 1995).
1.2 The Skeletal Muscle Dihydropyridine Receptor (DHPR)

1.2.1 The primary structure of the skeletal and cardiac DHPR.

The skeletal and cardiac muscle DHPRs consist of four main subunits including, the alpha-1s (or Ca\textsubscript{v1.1}), the beta-1a, the alpha-2delta-1 and the gamma-1 with a molecular mass of 465 kDa. In 1987, the primary sequence of the skeletal alpha-1s subunit was published (Tanabe et al., 1987). A 6083 nucleotide sequence of cDNA encoded 1873 amino acids, with a molecular mass (M\textsubscript{r}) of 214 kDa and containing four internal repeats which exhibited sequence homology: I, residues 38-337; II, residues 418-664; III, residues 786-1068; and IV, residues 1105-1384 (Tanabe et al., 1987). The DHPR was found to be homologous in amino acid sequence with the sodium channel, particularly in the region of the four internal repeats (Tanabe et al., 1987). Each internal repeat has five hydrophobic regions (S1, S2, S3, S5, and S6) and one positive charged segment (S4) (Figure 1.4) (Tanabe et al., 1987).

These repeats all exhibit predicted secondary structure that are consistent with alpha helical membrane spanning segments and it was thought that the amino and carboxyl termini were both located on the cytoplasmic side of the membrane (Tanabe et al., 1987). The S4 segment contained five or six Arginine or Lysine residues at every third position (Tanabe et al., 1987). These positive charges, many of which form dipoles, were thought to represent what has been termed the voltage sensor of the DHPR and further evidence for this will be presented in section 1.2.3.
Figure 1.4 Schematic representation of the structural characteristics common to both the DHPR and the voltage-dependent sodium channel. The four units of homology spanning the membrane are displayed linearly. The putative transmembrane alpha-helical segments S1-S6 (from left to right) in are shown in the repeats I-IV. Residues that are conserved within the DHPR and three known sodium channels are shown in one letter code. Reprinted with permission from Tanabe et al., 1987.

It was thought that one or two helical segments from each repeat would form the inner wall of the ion channel and at its narrowest point would have a diameter of approximately 6 Å (Tanabe et al., 1987). The high selectivity of the DHPR arises from the high affinity calcium binding sites located in the pore region of the channel (Tanabe et al., 1987). In light of these results and a great many results by many previous researchers, Tanabe et al (1987) proposed that the skeletal DHPR alpha-1s subunit would primarily function as the voltage sensor for ECC, but would also act as a calcium channel to ensure long term calcium replenishment.

The alpha-2-delta subunit is a transmembrane disulfide-linked glycoprotein dimer, consisting of the 143 kDa alpha-2 and 24 kDa delta subunits (Takahashi et al., 1987). It has been found that the alpha-2 subunit is located entirely extracellularly (Brickley et al., 1995). The delta subunit possesses one transmembrane domain, which anchors the alpha-2-delta subunit to the membrane adjacent to the alpha-1s subunit (Catterall, 1995). The beta and gamma subunits are the two smallest subunits of the skeletal
DHPR each comprising 54 kD and 30 kDa respectively (Takahashi et al., 1987). The beta subunit is thought to reside completely within the cytoplasm, while the gamma subunit is located within the t-tubule membrane (Catterall, 1995). The functions of the alpha-2-delta, the beta and the gamma subunits are discussed further in section 1.2.4.

In cardiac muscle, where Ca$^{2+}$ influx across the sarcolemna is essential for contraction, the DHPR alpha-1 subunit (alpha-1c or Ca$_{v1.2}$) represents the major entry pathway of extracellular Ca$^{2+}$ (Mikami et al., 1989). The rabbit cardiac alpha-1c is composed of 2171 amino acids, having a relative Mr calculated as 243 kDa (Mikami et al., 1989). The alpha-1c, like its skeletal muscle counterpart, contains four repeated units of homology (amino acid residues 141-438, 540-786, 917-1,199 and 1,236-1,509) with each repeat containing five hydrophobic segments (S1, S2, S3, S5, and S6) and one positively charged segment (S4) (Mikami, et al., 1989). The degree of amino-acid sequence homology between the cardiac and the skeletal muscle DHPR alpha-1 subunit is 66% (Mikami, et al., 1989) and the regions corresponding to the four internal repeats are highly conserved (Mikami et al., 1989). Both the amino-terminal and the carboxy-terminal region of the cardiac DHPR alpha 1 are larger than those of the skeletal muscle DHPR alpha 1 (Mikami, et al., 1989). Messenger RNA specific for the cardiac DHPR alpha 1 subunit was synthesized by transcription in vitro of the cloned cDNA and was injected into Xenopus oocytes (Mikami et al., 1989). In a series of experiments in which various ionic concentrations were used, a sustained inward Ba$^{2+}$ current was evoked by depolarizing pulses (Mikami et al., 1989). The experimenters replaced external Ca$^{2+}$ with Ba$^{2+}$, as Ca$^{2+}$ had previously been shown to activate an endogenous chloride channel. It was concluded that the
inward Ba\(^{2+}\) current was through the cardiac DHPR alpha 1 subunit, identifying the DHPR as a functional Ca\(^{2+}\) channel (Mikami et al., 1989).

1.2.2 The secondary structure of the skeletal DHPR

The secondary structure of the skeletal DHPR has been determined using cryo electron microscopy (Serysheva et al., 2002). The DHPR consisted of an asymmetric structure with heart shaped and handle shaped regions (Serysheva et al., 2002). The heart shaped region was seen to be about 120 \(\text{Å}\) in height and 115 \(\text{Å}\) in width, while the handle shaped region is 60 \(\text{Å}\) high with a thickness of 30 – 50 \(\text{Å}\) (Serysheva et al., 2002). These two regions were connected at an oblique angle on the upper lobes of the heart shaped region and the whole shape has dimensions of approximately 115 \(\text{Å}\) x 130 \(\text{Å}\) 120 \(\text{Å}\) (see Figure 1.5 (a)) (Serysheva et al., 2002). A cavity of 30 \(\text{Å}\) in diameter appears between the two regions (Serysheva et al., 2002).

Hypotheses regarding the arrangement of subunits within the DHPR complex were tentative due to the resolution of 30 \(\text{Å}\) (Serysheva et al., 2002). It was proposed the 170 kDa alpha-1 subunit would be similar in shape to that previously determined for the Na\(^{+}\) channel (Sato et al., 2001), that is, orientated in a pseudo-fourfold symmetry fashion and located within the membrane (Serysheva et al., 2002). The heart shaped region of the DHPR was estimated to have a molecular mass of approximately 280 kDa and is thought to contain the alpha-1, gamma and beta subunits as well as a portion of the delta subunit (Serysheva et al., 2002). The smaller handle-shaped region, consisting of a estimated mass of 140 kDa, most likely contains the alpha-2 subunit (Serysheva et al., 2002) (see Figure 1.5 (b)).
Figure 1.5 (A) Surface representation of the 3D reconstruction of the skeletal DHPR shown in the front view (center). All other views were obtained by rotation of the front view along the horizontal axis (top and bottom) or around the vertical axis (left and right). (B) A membrane topology model of the locations of the subunits of the skeletal DHPR (left-hand image). The 3D reconstruction is partitioned into two regions based on the proposed membrane topology (right-hand image). Reprinted with permission (Serysheva et al. 2002).
1.2.3 The role of the skeletal DHPR alpha-1 subunit in ECC.

Many definitive studies investigating the role of the DHPR in ECC have used the single, recessive, lethal, autosomal mutation in mice known as muscular dysgenesis. In dysgenic muscle the sarcolemma is still able to generate action potentials (Powell and Fambrough, 1973) and the SR retains the ability to sequester calcium and release it in response to caffeine (Bowden-Essien, 1972), however sarcolemmal depolarization fails to elicit SR calcium release (Klaus et al., 1983). Beam and co-workers (1986) reported that dysgenic skeletal muscle cultures were incapable of ECC because they lacked the alpha 1 subunit of the DHPR. This was indeed the case and results from restriction endonuclease analysis of genomic DNA (Tanabe et al., 1988) found that there were differences between normal and dysgenic mice in two of the nine probes which represent the entire protein-coding cDNA sequence of the DHPR alpha 1 subunit. Blot hybridization analysis of poly(A)^+ RNA further revealed that dysgenic mice contain greatly reduced amounts of the DHPR alpha 1 subunit mRNA or an RNA related to it (Tanabe et al., 1988).

Other techniques used to determine the role of the DHPR in ECC have included measuring calcium currents from skeletal muscle cells. In 1983, using patch-clamp techniques, it was discovered that two kinetically different calcium currents existed in skeletal muscle (Donaldson and Beam, 1983) and these currents have been termed I_{fast} and I_{slow}. The I_{fast} current is approximately 1 pA / pF in amplitude, a fast onset (approximately 10 ms) and a duration of approximately 100 ms. In contrast the I_{slow} current has an amplitude of 5 pA / pF, a much slower onset (approximately 100 ms) and a duration of approximately 250 ms.
In addition to the kinetic differences between the calcium currents, numerous methods have been used to distinguish these two currents. In 1986, Bean et al., showed that single muscle cells from rat mesenteric arteries elicited a fast transient inward current, termed $I_{\text{fast}}$, when test potentials were in the range of −40 mV to −10 mV. Test potentials of greater than 0 mV activated an additional slower, maintained inward current, termed $I_{\text{slow}}$. It was also shown that calcium channel antagonists could distinguish between the two currents. A high concentration of cadmium (> 1 mM) was able to block both $I_{\text{fast}}$ and $I_{\text{slow}}$ (Bean et al., 1986). However, lower concentrations (< 100 µM) produced a significantly larger block of $I_{\text{slow}}$ compared to $I_{\text{fast}}$. Similarly, nitrendipine (5 µM) (a dihydropyridine Ca$^{2+}$ channel antagonist) caused an approximate 75% reduction in $I_{\text{slow}}$ with little effect on $I_{\text{fast}}$ (Bean et al., 1986).

Another difference between the two components of calcium currents was found in dysgenic mice. In dysgenic mice the $I_{\text{slow}}$ was no longer present. $I_{\text{fast}}$ was present with values similar to that determined in normal myotubes (Bean et al., 1986). Interestingly, both components of the calcium current were present in cardiac and neuronal cells of dysgenic animals, indicating that the dysgenic mutation is specific to the slow calcium current of skeletal muscle.

In 1985, PN-200/110 was used to investigate the number of DHPR binding sites present in skeletal muscle. It was observed that there are 35-50 times more dihydropyridine (DHP) binding sites, than there are voltage-dependent Ca$^{2+}$ channels that can be activated to pass current (Schwartz et al., 1985). At this time it was speculated that the DHPR could represent a large reserve of dormant Ca$^{2+}$ channels kept for an unknown physiological emergency (Schwartz et al., 1985). However,
considering the DHPR has a low opening probability (Lamb and Walsh, 1987) it is now generally accepted that most, if not all, of the DHPRs also act as calcium channels.

The critical role played by the DHPR in ECC was proven beyond doubt by Tanabe and co-workers (1988) who injected an expression plasmid (pCAC6), carrying the entire protein coding sequence of the rabbit skeletal muscle DHPR alpha 1 subunit cDNA, into dysgenic myotubes. The injection of this plasmid restored both spontaneous and electrically evoked contractions to dysgenic myotubes (Tanabe et al., 1988). Further experiments revealed that even after ten minutes in a calcium free solution electrical stimulation caused contraction of both normal myotubes and pCAC6 injected myotubes, indicating skeletal type ECC (Tanabe et al., 1988). Also, the L-type Ca$^{2+}$ current detected in pCAC6 injected myotubes (Tanabe et al., 1990b), had a rate of activation that was slow and similar to that observed in normal skeletal muscle (Sanchez and Stefani, 1978; Donaldson and Beam, 1983).

Another expression plasmid (pCARD1), carrying the entire protein coding sequence of the rabbit cardiac DHPR alpha 1 subunit cDNA, was also able to restore spontaneous and electrically evoked contractions in dysgenic myotubes (Tanabe et al., 1990b). However, pCARD1 injected myotubes only displayed electrically-evoked contractions in normal rodent ringer, but not in Ca$^{2+}$-free ringer, or in 0.5mM Cd$^{2+}$ containing ringer, therefore displaying cardiac-type ECC (Tanabe et al., 1990b). The L-type Ca$^{2+}$ currents that were detected with dysgenic myotubes that had been injected with pCARD1 had kinetics and voltage dependence different from those of the pCAC6-injected myotubes (Tanabe et al., 1990b). The L-type calcium current in
pCARD1 injected myotubes was activated more rapidly (Tanabe et al., 1990b), like the L-type Ca\(^{2+}\) current in normal cardiac muscle (Lee and Tsien, 1982).

The expression of skeletal muscle DHPR alpha 1 subunit cDNA restored both skeletal type ECC and skeletal like L-type Ca\(^{2+}\) currents in dysgenic myotubes and the expression of cardiac muscle DHPR alpha 1 subunit cDNA restored both cardiac type ECC and cardiac like L-type Ca\(^{2+}\) currents to dysgenic myotubes. It therefore appears the alpha-1 subunit is a critical requirement for both forms of ECC and L-type Ca\(^{2+}\) currents and that the tissue specific differences found between skeletal and cardiac muscle could be encoded within the type of alpha-1 subunit cDNA.

In 1973, Schneider and Chandler provided the first insight that the DHPR may function as a voltage sensor in ECC by successfully detecting an ionic current or movement of charge across the T-system membrane. As mentioned above, their results suggested that there was a fixed amount of free charge to move between different locations across the T-system membrane and this has been termed immobilization resistant charge movement. The charged group was found to have a valence of magnitude between two and three. The number of charge movement sites was estimated to be 500-600 sites \(\mu\text{m}^{-2}\), which roughly correlated with the estimated density of DHPRs located in the T-system membrane. Therefore it was suggested that both the charge movement and the DHPR were involved in linking the depolarization of the T-system with the release of calcium from the SR.

To provide further evidence for the role of the skeletal muscle DHPR as the voltage sensor in ECC, Adams et al. (1990) used dysgenic mice to examine the properties of
immobilization resistance charge movement. They found that the maximum quantity of charge in dysgenic myotubes was smaller than in normal myotubes (Adams et al., 1990). In these types of experiments, the amount of immobilization resistant charge movement was taken as an indication of the level of expression of DHPR alpha 1 subunit encoded by the injected cDNA. They found that the immobilization resistant charge movement, deficient in dysgenic myotubes, could be restored by injecting them with the expression plasmid pCAC6 or pCARD1 (Adams et al., 1990). All these expression plasmids restored immobilization resistant charge movements with very similar voltage dependent behaviour, independent of the nature of the L type current (slowly or rapidly activating) or ECC (skeletal or cardiac type) appearing after their injection (Adams et al., 1990). This similarity in voltage dependence of charge movement seemed reasonable to Adams and co-workers given that the S4 segments, which are assumed to sense voltage, are highly conserved between the skeletal and cardiac DHPRs. Thus both the decreased charge movement and absence of ECC evident in dysgenic myotubes is restored with the injection of DHPR cDNA. This provides conclusive evidence that the DHPR functions as the voltage sensor in ECC.

More evidence for the role of the DHPR as the voltage-sensor in ECC, came from a study which independently investigated the importance of charge movement in initiating a calcium release from the SR. Rios and Brum (1987) looked at the effects of dihydropyridines (DHPs), such as nifedipine, which act specifically on the DHPR (Rios and Brum, 1987). Nifedipine has the ability to reduce charge movement in the DHPR in a concentration dependent manner. It was also found that nifedipine inhibited calcium release from the SR (Rios and Brum, 1987). As the concentration of
nifedipine was increased, the peak amount of calcium released decreased, directly relating charge movement to the ability of the DHPR to cause a release of Ca\(^{2+}\) from the SR.

Further to the studies above, the importance of the DHPR in eliciting skeletal type ECC was demonstrated using skinned muscle fiber studies performed by Lamb and Stephenson (1990). It was discovered that depolarization induced contractions were possible by the replacement of potassium with sodium in the bathing solution (Lamb and Stephenson, 1990). The contraction noted with depolarization could be terminated by repolarizing the fiber in the original potassium bathing solution (Lamb and Stephenson, 1990). Further to this, depolarization was seen to initiate a contraction in the presence of high and low cytoplasmic Ca\(^{2+}\) concentrations (Lamb and Stephenson, 1990). This indicates that the DHPR, shown to be sensitive to changes in membrane potential, is able to tightly control skeletal type ECC i.e. the release of Ca\(^{2+}\) from the SR irrespective of localized Ca\(^{2+}\) concentrations.

1.2.4 *The role of the beta-1a, alpha-2-gamma-1 and delta subunits*

In skeletal muscle the beta subunit has been found to be crucial: in targeting the DHPR to the endoplasmic reticulum (Bichet et al., 2000); for increasing the coupling between charge movements of the S4 region and pore openings (Neely et al., 1993); for regulating the kinetics of the DHPR as a calcium channel (Beurg et al., 1997); and for regulation of ECC (Bichet et al., 2000).
The beta-la isoform from skeletal muscle has been found to be tightly bound to the cytosolic loop spanning repeats I and II of the alpha subunit (De Waard et al., 1994; Pragnell et al., 1994). Two conserved regions have been found within the family of DHPR beta subunits and these have been labeled domains D2 and D4 (Perez-Reyes and Schneider, 1995). The non-conserved regions of the beta-la subunit have been labelled D1 (N-terminus region), D3 (linker region) and D5 (C-terminus region) (Perez-Reyes and Schneider, 1995). The D4 region is approximately 30 amino acids in length and contains what has been termed the beta interaction domain (BID). The interaction between the I-II loop of the alpha-I subunit (contained within the alpha interacting domain (AID)) and the beta subunit is located within this BID of the D4 region (De Waard et al., 1994; Pragnell et al., 1994).

In 1993, a study by Neely and co-workers investigated the properties of charge movements within the alpha-la subunit expressed in Xenopus oocytes. The half-activation potential for charge movement was found to be 35 mV more negative than that for pore opening (Neely et al., 1993). When the beta subunit was co-expressed with the alpha-ls subunit the difference between half-activation potentials was reduced without any effect on charge movement (Neely et al., 1993). It was thus concluded that some form of intramolecular coupling existed between the voltage and pore opening and that this coupling could be modulated by the beta subunit (Neely et al., 1993).

The functional role of the beta-la subunit has also been investigated through the use of beta-1 null mice (Beurg et al., 1997). It was found that 10 – 15 day old beta-la null myotubes exhibited: an L-type calcium current that was approximately 7-fold lower
than in normal cells of the same age; a significantly faster activation of L-type calcium currents; calcium currents that were not elicited by depolarization; and no spontaneous or evoked contractions (Beurg et al., 1997). Transfection of beta-1 null myotubes with beta-1a cDNA reestablished spontaneous or evoked contractions in approximately 30% of cells after 13 days (Beurg et al., 1997). The contracting cells exhibited skeletal type ECC and there was a complete recovery of the L-type current density, the magnitude of charge movements and the amplitude and dependence of calcium transients evoked by depolarisations (Beurg et al., 1997).

In the absence of the beta-1a subunit, the alpha-1s subunit contains what has been termed a ‘brake’ in the AID (Bichet et al., 2000). This brake located within the alpha-1s subunit I-II loop decreases the amount of protein that is expressed in the surface membrane (Bichet et al., 2000). It is hypothesised that the reduced amount of plasma membrane expression trigged by the I-II loop is caused by an increased retention of the proteins in the endoplasmic reticulum (Bichet et al., 2000). The presence of the beta-1a subunit inhibits the ‘brake’ found within the AID and in this way assists in targeting the DHPR to the surface membrane (Bichet et al., 2000).

The alpha-2-delta subunit is a 170 kDa complex thought to be located within the lumen of the t-tubule (Serysheva et al. 2002). It has been shown that the subunit plays a role in gating of Ca$^{2+}$ release of the alpha-1 subunit (Alden and Garcia, 2002). It was also proposed, by the authors, that the alpha-2-delta subunit may play a role in coordinating channel openings. In cardiac muscle the alpha-2-delta subunit has been found to have a role in modulating charge movement (Shirokov et al., 1998).
The role of the gamma subunit of the skeletal muscle calcium channel is still not clearly defined. The fact that ECC occurs in skeletal muscle of a gamma knock out mouse suggests that the gamma subunit is not required for either targeting or expression of the DHPR (Freise et al., 2000). In a study using a co-sedimentation technique the gamma subunit was found to bind to the alpha-1s subunit (Arikkath et al., 2003). Further to this, using chimeric constructs, Arikkath et al. (2003), discovered that the first half of the gamma-1 subunit was important for the interaction with the alpha-1s subunit and for restoring calcium conductance in gamma null myotubes.

1.2.5 Localisation of the sequence of the DHPR critical for skeletal ECC

In order to localize the DHPR sequence critical for supporting skeletal type ECC, three major experimental techniques have been used, dysgenic (see section 1.2.3), chimeric and deletion studies. Chimera studies have mostly used the cardiac DHPR as a template whilst changing portions of the protein to skeletal sequence. These chimeras have been expressed in dysgenic myotubes and ability of the DHPR to support skeletal type ECC has been examined. Deletion studies have looked at the functional properties of the skeletal DHPR with portions of sequence removed. The relevant findings for the role of the skeletal DHPR in ECC determined from chimera and deletion studies are presented within this section.

The major technique used to investigate the mechanisms of skeletal ECC involves peptides derived from the skeletal or cardiac DHPR alpha-1 subunit. The ability of the peptides to either activate or inhibit the RyR1, are assessed using either single RyR channels incorporated in lipid bilayers, Ca²⁺ release from SR vesicles, ryanodine
binding to SR vesicles or skinned fiber studies. Results from studies utilizing these techniques are presented in section 1.2.6.

The first study to use dysgenic mice as a model to investigate the proteins critical for skeletal type ECC was conducted by Tanabe and co-workers in 1988. By injecting cDNA encoding the skeletal DHPR into cultured cells from mice with muscular dysgenesis the experimenters were able to restore skeletal type ECC (Tanabe et al., 1988).

In 1990, work conducted by the same research group narrowed down the portion of the DHPR alpha-1 subunit that interacted with the RyR to mediate skeletal ECC. Tanabe et al. (1990a) constructed five different expression plasmids carrying chimeric DHPR alpha-1 subunit cDNAs. These constructs encoded the cardiac DHPR as the backbone structure. All of the four large putative cytoplasmic regions were replaced by the corresponding sequence of the skeletal muscle DHPR (Tanabe et al., 1990a). Dysgenic myotubes injected with chimeric plasmids did indeed display electrically evoked contraction, and the number of responsive myotubes relative to the number of tested myotubes was comparable to that reported previously for pCAC6 (cDNA encoding the skeletal DHPR) (Tanabe et al., 1988) or pCARD1 injected myotubes (cDNA encoding the cardiac DHPR) (Tanabe et al., 1990b). Each of the four large, putative cytoplasmic regions was then individually replaced in separate chimeras (Figure 1.6) (Tanabe et al., 1990a).

The results with the chimera pCSk2 (cardiac DHPR sequence with a skeletal sequence spanning the residues on the loop between trans-membrane sections II and III)
indicated that the II-III loop of the skeletal muscle DHPR is a major determinant for skeletal type ECC (Tanabe et al., 1990a). This was because when the chimera pCSk2 was injected into dysgenic mice skeletal type ECC was restored, i.e. in a Ca\(^{2+}\) free solution (Tanabe et al., 1990a). A weaker skeletal type ECC was also observed with the chimera pCSk3 (cardiac DHPR with skeletal I-II loop), indicating that the I-II loop may also be involved in determining skeletal type ECC, but to a lesser extent than the II-III loop (Tanabe et al., 1990a). While the expression plasmids pCSk1 (cardiac DHPR with skeletal NH terminus) and pCSk4 (cardiac DHPR with a skeletal COOH terminus) were unable to produce ECC in the absence of Ca\(^{2+}\), showing that the amino-terminal and carboxy-terminal regions are insufficient to elicit skeletal type ECC by themselves (Tanabe et al., 1990a).

The determinant site for skeletal ECC was later found to be confined to approximately 55 amino acids in the central portion of the II-III loop (Nakai et al., 1998). This was again shown using chimeras of cardiac/skeletal sequence while monitoring the type of ECC (Nakai et al., 1998). By systematically changing portions within the II-III loop it was found that skeletal DHPR residues 711-765 were the minimum essential residues for skeletal type ECC (chimera CSk33) (Nakai et al., 1998). When the area was further reduced to 18 amino acids between residues 725 and 742 (chimera CSk58), skeletal type ECC was still present, however slightly weaker than with the larger 55 amino acid segment (Nakai et al., 1998). Thus these studies identify the region between residues 711-765 of the skeletal DHPR to be responsible for controlling the ability of the skeletal DHPR to support skeletal ECC,
presumably through a protein-protein interaction with RyR1, which occurs in the absence of external calcium.

Figure 1.6 (A) Schematic representation of the DHPR chimera used by Tanabe et al (1990a). Skeletal sequence is depicted by bold lines, cardiac sequence in standard lines. (B) Comparison of electrically evoked contractions in dysgenic myotubes expressing the corresponding chimera. Contractions were initially recorded in normal rodent ringers (1) and subsequently in a test solution that was either Ca\(^{2+}\) free (2) or 0.5 mM Ca\(^{2+}\) containing ringers (3) in an attempt to identify skeletal type ECC. Reprinted with permission (Tanabe et al. 1990a).
Figure 1.7 Schematic representation of the II-III loop from a series of chimeras of the cardiac and skeletal DHPR. Thin lines indicate the cardiac sequence, bold lines indicate the skeletal sequence. Calcium transients elicited in the presence (left-hand trace) and absence (right-hand trace) of Ca\(^{2+}\) are shown for each chimera investigated. Reprinted with permission from Nakai et al. 1998.

The studies mentioned above show that the cardiac DHPR is a useful template to investigate the regions of the alpha-1 subunit that are required for the interaction of the DHPR with the RyR to elicit skeletal type ECC. However because of the similarity between the skeletal and cardiac DHPR II-III loops it is possible that in the chimeras investigated the portion of cardiac sequence has played a role similar to that present with in the skeletal sequence. Therefore a study was conducted by Wilkens and co-workers (2001) using a DHPR chimera of skeletal sequence except for the II-
III loop which contained sequence derived from the house fly, *Musca domestica* (Wilkens et al., 2001). This chimera was used because the house fly II-III loop has only a 25% sequence similarity compared to the skeletal II-III loop and it is especially divergent in the regions of the Arg\textsuperscript{681} - Leu\textsuperscript{690} and Leu\textsuperscript{720} - Gln\textsuperscript{765} (Wilkens et al., 2001). The chimera was expressed into dysgenic myotubes and displayed normal surface expression levels and correct triad targeting (Wilkens et al., 2001). However, the chimera was not able to restore skeletal ECC in dysgenic myotubes and displayed strongly reduced Ca\textsuperscript{2+} current densities (Wilkens et al., 2001). Once the residues spanning Glu\textsuperscript{724} - Thr\textsuperscript{755} were replaced with the sequence of skeletal residues previously found to be critical for skeletal type ECC (residues Leu\textsuperscript{720} - Leu\textsuperscript{764}) the expression of the chimera in dysgenic myotubes was able to completely restore bidirectional skeletal type ECC (Wilkens et al., 2001).

The issue surrounding the location of sequences critical for skeletal type ECC was complicated by the findings of Ahern et al. (2001). They conducted a deletion analysis of two regions of the skeletal DHPR including residues 671 – 690 (spanning the A region) (see section 3.1.1) and the region previously shown to be critical for skeletal ECC (Nakai et al., 1988; Wilkens et al., 2001), residues 720 – 765. The deletion alpha-1s mutants were expressed in dygenic myotubes and analysed by voltage-clamp and confocal fluoro-4 fluorescence (Ahern et al., 2001). Based on past results it was no surprise that the deletion of residues 720-765 abolished voltage evoked Ca\textsuperscript{2+} transients entirely, i.e. transients reflecting Ca\textsuperscript{2+} release from the SR in the absence of external Ca\textsuperscript{2+} (skeletal type ECC) (Ahern et al., 2001). The absence of residues 671 – 690 had no effect on eliciting skeletal type ECC (Ahern et al., 2001). However, when both portions of sequence were removed (that is, regions 671 – 690
and 720 – 765) moderate voltage evoked Ca\(^{2+}\) transients were recovered (Ahern et al., 2001). This implies that there are other regions of the alpha-1s or beta subunit, which play some part in controlling skeletal type ECC. In light of these findings it is possible that the region 720 – 765 of the skeletal muscle DHPR is responsible for the successful coupling of the DHPR and the RyR into tetrads (see Section 1.4), whilst another region may play the critical determinant in releasing Ca\(^{2+}\) through the RyR1.

From the evidence provided in the studies described above, it seems highly likely the major region critical for skeletal type ECC is located within residues 720 – 765, while other portions of the DHPR seem to be less important. However the results obtained by Ahern et al. (2001) demonstrate that other regions within the DHPR also play a role in eliciting skeletal type ECC and that the mechanical interaction between the DHPR and the RyR1 is not exclusively isolated to residues 720 – 765. In order to further resolve the mechanical interactions which occur between the DHPR and the RyR in skeletal type ECC several regions has been investigated in peptide form.

1.2.6 Peptides probes derived from the skeletal DHPR

Peptides corresponding to regions in proteins can provide useful information about the potential role of that region of the protein. In the case of skeletal type ECC, the functional effects of peptide segments of the DHPR on the RyR can provide insights into the mechanisms of the interactions between these two proteins. The results of these studies must be interpreted with caution as the peptides' structural and functional characteristics may not resemble those found for that region in the complete protein and can be greatly altered by the addition or removal of even a single amino acid.
Also, when peptides are investigated they may be able to interact with regions of the target protein that are not accessible to the sequence corresponding to the peptides in the whole protein. Taking these limitations into account, studies of the interactions between peptides derived from the DHPR and the skeletal RyR have provided important data regarding mechanisms of activation and inhibition of RyR1. Indeed some of the peptides have provided useful experimental probes for RyR activity (Gallant et al., 2001; Chen et al., 2003; Haarmann et al., 2003) and the possibility remains that the peptide studies will provide relevant information for the interaction between the particular sequence of the DHPR and the RyR in skeletal muscle at rest and during skeletal type ECC.

In the first study assessing the ability of peptides derived from the DHPR II-III loop to alter RyR function, it was found that the whole recombinant II-III loop fusion protein activated RyR1 (Lu et al., 1994). Sequences taken from the DHPR II-III loop, of both the skeletal and the cardiac receptor, were expressed in E.coli. These fusion proteins were termed the SDCL (spanning RyR1 residues 666 – 791) and CDCL (spanning RyR2 residues 788 – 922) for the skeletal and cardiac DHPR II-III loop respectively (Lu et al., 1994). It was found that both the SDCL and the CDCL were able to activate the skeletal muscle RyR (Lu et al., 1994). Interestingly, neither peptide activated the purified cardiac muscle RyR (Lu et al., 1994). These results led Lu and collaborators (1994) to suggest that in addition to the DHPR, the RyR may also play a role in determining the type of ECC that exists in muscle.

The phosphorylation state of the II-III loop peptide also dramatically affected its ability to activate the RyR1. Cyclic AMP dependent protein kinase-mediated
phosphorylation of Ser$^{687}$ of SDCL, yielded a protein that failed to activate the RyR, as determined in $[^3H]$ ryanodine binding and single channel measurements (Lu et al., 1995). A mutant SDCL, with a Ser$^{687} \rightarrow$ Ala substitution also failed to activate the RyR, but was able to bind to the RyR protein. Similarly, a Ser$^{813} \rightarrow$ Ala substitution in CDCL yielded a protein that failed to activate the skeletal RyR (Lu, et al., 1995). Hence, Lu and co-workers suggested the loss of activation was due to the formation of an inactive protein and not because of a decrease in binding affinity (Lu, et al., 1995).

Another study that has assessed the effects of the SDCL was conducted in 1998. Binding studies showed that the SDCL double mutant Lys$^{677} \rightarrow$ Glu / Lys$^{682} \rightarrow$ Glu lost most of its capacity to interact with RyR1 (Leong and MacLennan, 1998). This finding suggested an important role for these two positively charged residues in the interaction between the RyR1 and DHPR (Leong and MacLennan, 1998).

In an attempt to localize the exact portion of the SDCL fusion protein that activates RyR1, SDCL was dissected into three peptides, the SDCLf1 (residues 666-726), SDCLf2 (residues 709-766) and the SDCLf3 (residues 733-791) (Lu et al., 1995). Both the SDCLf2 and SDCLf3 did not increase the amount of ryanodine bound to the RyR1. However, the SDCLf1 caused a significant increase in ryanodine binding, to levels virtually identical to that seen with SDCL (Lu et al., 1995). SDCLf1 also bound to the RyR1 in a similar manner to the SDCL (Lu et al., 1995). Thus, it was concluded that the activating ability and the binding site contained within the SDCL peptide was localized in the first 61 amino acids (Lu et al., 1995).
Continuing work with small peptides of SDCL, El-Hayek et al. (1995) identified that peptide ‘A’ (Thr<sup>671</sup>-Leu<sup>690</sup> of the SDCL) was able to increase [<sup>3</sup>H] ryanodine binding to, and induced Ca<sup>2+</sup> release from rabbit skeletal muscle triads. None of the other II–III loop peptides, B (Thr<sup>694</sup> - Val<sup>722</sup>), C (Glu<sup>724</sup> - Pro<sup>760</sup>), C1 (Phe<sup>725</sup> - Gly<sup>743</sup>), C2 (Asp<sup>740</sup> - Pro<sup>760</sup>) or D (Pro<sup>760</sup> - Val<sup>790</sup>), spanning most of the II-III loop, were able to activate the RyR1 (El-Hayek et al., 1995a). Interestingly, when the C peptide and A peptide were investigated simultaneously, the A peptide was no longer able to activate the [<sup>3</sup>H] ryanodine binding in the presence of the C peptide (El-Hayek et al., 1995a). Supporting evidence for the importance of the A region within the II–III loop has come from surface plasmon resonance detection experiments which demonstrated that the presence of the A region inhibits the association of the skeletal DHPR II–III loop with the RyR1, while the presence of the C region had no effect on this interaction (O’Reilly et al., 2002).

In 1998, the region of the A peptide responsible for the activation of the RyR1 was narrowed down to just 10 amino acids (El-Hayek and Ikemoto, 1998). The minimum unit capable of activating the RyR1 was found to be peptide As10 (Arg<sup>681</sup>-Leu<sup>690</sup>), where the increase of ryanodine binding to RyR1 (an indication of the open probability of the channel, as ryanodine binds preferentially to an open RyR1 channel) was stronger than with peptide A (Thr<sup>671</sup>-Leu<sup>690</sup>) (El-Hayek and Ikemoto, 1998). Deletion of one or more amino acid residues from the C-terminus of peptide As10 (beginning with Leu<sup>690</sup>) virtually abolished its activating function (El-Hayek and Ikemoto, 1998). Surprisingly, peptide A was found to have virtually no effect on RyR2 and the RyR1 specificity of peptide activation was retained in peptide As10 (El-Hayek and Ikemoto, 1998). The authors suggested that the region of the II-III loop
corresponding to peptide A could represent a putative trigger of skeletal muscle type ECC. It was proposed that the number and distribution of positive charges within peptide As10 as well as the specific skeletal muscle type sequence of peptide As10 were responsible for its activating function (El-Hayek and Ikemoto, 1998).

Similar results were obtained using the A and As10 peptides on mechanically skinned skeletal muscle fibers (Lamb et al., 2000). In this set of experiments the As10 peptide induced much larger spontaneous responses compared to the A peptide which caused only small spontaneous force responses in approximately 40% of fibers (Lamb et al., 2000). It was found that both peptides potentiated Ca$^{2+}$ release when the voltage sensors were inactivated, indicating a direct action on the RyR1 (Lamb et al., 2000).

A more recent study investigating the effects of truncated peptides derived from the A region of the skeletal DHPR II – III loop have produced conflicting results. Results obtained from single channel and ryanodine binding studies demonstrated that a peptide corresponding to ten amino acids spanning the N-terminal end of the A peptide (residues 671 – 680) activated the RyR1 (Stange et al., 2001). Two other peptides derived from the C-terminal end of the A region (spanning residues 681 – 690 and residues 681 – 685) failed to activate the RyR1 (Stange et al., 2001), contradicting the results previously obtained with the As10 peptide.

Other laboratories have also investigated the effects of peptide A on the RyR1. Peptide A, or derivatives of the peptide A, have been found to be able to: activate a single channel RyR1 incorporated in artificial bilayers (Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Casarotto et al., 2000; Casarotto et al., 2001; Stange et
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al., 2001), cause an increase in ryanodine binding (Gurrola et al., 1999); activate release of calcium from sarcoplasmic reticulum vesicles (Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Green et al., 2003); and potentiate the caffeine-activated force in skinned muscle fibres (Lamb et al., 2000; Green et al., 2003).

In the study conducted by Zhu et al. (1999) it was confirmed that the cluster of positively charged residues from Arg$_{681}$ – Lys$_{685}$, located in the A region of the skeletal DHPR were indispensable for the peptides ability to activate the RyR1. Initially Zhu and collaborators started with a 25 amino acid peptide from the cardiac DHPR II-III loop (Asp$_{788}$ – Pro$_{814}$) that corresponded to the skeletal region (Glu$_{666}$ – Pro$_{691}$) shown to activate RyR1. The 25 amino acid peptide from cardiac muscle was not able to activate the RyR1 (Zhu et al., 1999). Each of the amino acids was then systematically replaced with the corresponding residue from skeletal muscle (Zhu et al., 1999). It was found that the peptide was able to activate the RyR1 only when the cluster of five basic residues of skeletal sequence (Arg$_{681}$ – Lys$_{685}$) was present.

The evidence presented above shows that the cluster of positive charges located within peptide A are crucial for its ability to activate the RyR1. However, the importance of residue Ser$_{687}$, which was crucial for the activating ability of SDCL (Lu et al., 1995), is still unclear. The results obtained with a modified peptide A, termed peptide A2 in which Ser$_{687}$ was replaced by Ala (Casarotto et al., 2000), has produced apparently conflicting results.

Casarotto et al. (2000) used a structure / function study in to determine the activating abilities of three peptides: the 20 amino acid peptide A1 (corresponding to peptide A);
a mutant of peptide A (Ser$^{687}$ → Ala) (A2); and a peptide spanning the previously
determined crucial amino acids (Arg$^{681}$ – Ser$^{687}$) (A9) (Casarotto et al., 2000). It was
discovered that the Ser$^{687}$ → Ala mutation, significantly enhanced the peptides ability
to activate the RyR1, peptide A9 did not significantly activate RyR1 (Casarotto et al.,
2000). The three-dimensional solution structure of each of these peptides was
determined using NMR. It was found that the peptides required both the crucial
sequence of amino acids as well as some form of secondary structure in order to
activate the RyR1 (Casarotto et al., 2000). Peptide A9, which did not contain any
secondary structure and was not able to activate the RyR1, even though it contained
what had been identified as the essential cluster of positive charges and Ser$^{687}$
(Casarotto et al., 2000). Both peptides A1 and A2 contained a large degree of alpha
helical secondary structure and activated RyR1 (Protein Data Bank number for A1:
1DU1) (Casarotto et al., 2000). The helical structure was a significant finding for
peptides of only 20 amino acids in length. The helical portion of the peptides
extended from the N-terminal end through until residue 12-14 (Casarotto et al., 2000).
The secondary structure detected in peptide A2 was more consistent and regular than
that identified for peptide A (Figure 3.1 (b)) (Casarotto et al., 2000). Computational
modeling suggested that the difference between the two peptides occurred because the
–OH group located on Ser$^{687}$ was no longer able to interact with Arg$^{683}$ (Figure 3.1
(b)) (Casarotto et al., 2000). The removal of this interaction reduced the mobility of
the C-terminal portion of the peptide (seen with A1) and allowed the A2 peptide to
retain a longer length of alpha helix (Casarotto et al., 2000).
Figure 3.1 (a) Average structure representation obtained from the 20 lowest energy structures for peptide A1 (left-hand image) and peptide A2 (right-hand image) calculated from NMR data. (b) Predicted structures of the A1 and A2 peptides produced by computational modeling (Casarotto et al., 2000).
Chimera studies indicated that the region critical for skeletal type ECC covers the C region of the II-III loop. Since early studies found that the C peptide was not able to activate the RyR1 (el-Hayek et al., 1995a), but that it blocked the activation caused by peptide A, Saiki and collaborators (1999) investigated the hypothesis that skeletal ECC is regulated by two domains (activating and blocking) of the skeletal DHPR II-III loop (Saiki et al., 1999). They examined conformational changes in the ryanodine receptor induced by synthetic peptides and by T-tubule depolarization (Saiki et al., 1999). Peptide A rapidly changed the ryanodine receptor conformation from a blocked state to an activated state, as did T-tubule depolarisation (Saiki, et al., 1999).

Peptide C, corresponding to the Glu$^{724}$ - Pro$^{760}$ region, blocked both conformational changes induced by peptide A and T-tubule depolarisation (Saiki, et al., 1999). These results are consistent with the model that depolarisation-induced activation of excitation contraction coupling are mediated by the peptide A region and blocking/repriming are mediated by the peptide C region of the II-III loop.

In skinned fibre studies, the C peptide did not cause a release of Ca$^{2+}$, but resulted in a poorly-reversible, use-dependent inhibition of normal ECC (Lamb et al., 2000). More recently, the C peptide has been found to activate Ca$^{2+}$ release from SR vesicles (Yamamoto et al., 2002), purified skeletal RyR channels (Stange et al., 2001) and the native skeletal RyR1 channel complex incorporated in lipid bilayers (Haarmann et al., 2003). In purified single channel bilayer studies, the C peptide (0.3 – 30 µM) activated RyR1 by increasing the number of open events, rather than by increasing open times and activation was approximately 8-fold that at control levels at a holding potential of −40 mV (Stange et al., 2001). In the native RyR1 channel complex, peptide C (0.1 – 50 µM) caused an average 3-fold increase in RyR1 activity.
(Haarmann et al., 2003). The addition of higher concentrations of 50 – 150 µM of peptide C caused inhibition of the RyR1 in the majority of cases (Haarmann et al., 2003). In some cases, addition of 50 µM peptide C caused a short-lived activation of the RyR1 (approximately 50 seconds) before inhibition of the native RyR complex became apparent (Haarmann et al., 2003). Thus, the effects of peptide C on the RyR1, appear to be dependent on the concentration of peptide and whether native or purified channels are used.

Regions from the skeletal DHPR II – III loop are not the only regions of the DHPR found to affect RyR1. A peptide located in the carboxyl terminal region of the DHPR (corresponding to amino acids 1487 - 1506 of the skeletal DHPR and 1612 - 1631 of the cardiac DHPR) inhibits ryanodine binding to both skeletal and cardiac SR membranes and to decrease the activity of the RyR reconstituted in planar lipid bilayers (Slavik et al., 1997). Furthermore, a much longer sequence (residues 1381 - 1627 of the skeletal DHPR) exhibited a similar effect (Slavik et al., 1997). Thus it seems there are several regions of the DHPR which effect the activity of the RyR1 when they are presented in peptide form.
1.3 The Ryanodine Receptor (RyR)

1.3.1 Background information

The ryanodine receptor gained its name because of its high binding specificity with the neutral plant alkaloid, ryanodine (Pessah et al., 1985). At 565 kDa, the ryanodine receptor is the largest ion channel that has been sequenced in the human body (Orlova et al., 1996). The RyR is a cation-selective channel that displays an unusually large ion conductance for monovalent and divalent cations (Sorrentino and Volpe, 1993). Activity of the RyR is modulated by many different ligands and nucleotides including Ca\(^{2+}\), Mg\(^{2+}\) and ATP. Maximum activation of the RyR (open probability close to one) is found in the presence of micromolar Ca\(^{2+}\) and millimolar ATP. In the absence of other regulatory ligands, the RyR exhibits a bell shaped Ca\(^{2+}\) activation curve (Meissner, 1994).

The RyR has three known isoforms in mammals, named skeletal (RyR1), cardiac (RyR2) and brain (RyR3) (Sorrentino and Volpe, 1993). Each of the RyR isoforms are encoded by a separate gene (RYR1, RYR2 and RYR3) and the cardiac and skeletal isoforms share 66% sequence homology (Sorrentino and Volpe, 1993). RyR3 has a sequence homology of approximately 70% compared to both the RyR1 and RyR2 (Sorrentino and Volpe, 1993). The areas of primary sequence not conserved, are consistent between all three isoforms (Sorrentino and Volpe, 1993). RyR1 is the predominant isoform in skeletal muscle (Takeshima et al., 1989), however it has also been discovered in the cerebellum (Kuwajima et al., 1992) and lymphocytes (Grafton and Thwaite, 2001). The cardiac RyR isoform is the major isoform found in heart
muscle (Hakamata et al., 1992) and the brain (McPherson and Campbell, 1993). The distribution of the RyR3 is wide and varied. RyR3 has been identified in: the brain (Hakamata et al., 1992); lymphocytes (Grafton and Thwaite, 2001); smooth muscle (Grafton and Thwaite, 2001); and in skeletal muscle (Conti et al., 1996).

Each of the isoforms performs essentially the same function, acting as the major Ca\(^{2+}\) release channel in the membrane of internal calcium stores. However, subtle structural and functional differences enable the different isoforms to be distinguished. One of the most important differences between the isoforms (see section 1.1.3) is that RyR1 in the whole cell is the only isoform to be activated by the skeletal DHPR in the absence of external calcium.

### 1.3.2 The primary structure of the RyR1

The RyR was first detected by electron microscopy, where it appeared as a rectangular density at regular intervals along the triadic junction (Franzini-Armstrong, 1970). These rectangular structures, initially termed junctional feet, were thought to play a functional role in ECC because of their strategic location in bridging the gap in the triadic junction between the t-tubule membrane and the membrane of the SR. Once the isolated RyR was visualized by electron microscopy it was immediately identified as the junctional foot structure. The RyR was shown to be a cation-selective channel that displayed an unusually high ion conductance for monovalent and divalent cations (Sorrentino and Volpe, 1993).
The sequence and primary structure of the 5037 amino acids that comprise the RyR from rabbit skeletal muscle SR was first deduced from cloning and sequencing the complementary DNA by Takeshima and collaborators (1989). Their predicted structure of the RyR suggested that the calcium release channel activity resided in the C-terminal region of the receptor molecule, whereas the remaining portion constitutes the ‘foot’ structure spanning the junctional gap between the SR and the transverse tubule (Figure 1.8) (Takeshima et al., 1989). The receptor molecule was predicted to have four highly hydrophobic segments with predicted secondary structure in the C-terminal tenth of the RyR (Takeshima et al., 1989). These segments (referred to as M1, M2, M3 and M4), each comprised approximately 20 amino-acids and were thought to represent the membrane spanning regions of the protein. They suggested that the portion of the protein preceding the segment M1, which constituted nine-tenths of the receptor molecule, was on the cytoplasmic side of the SR.

Support for the work by Takeshima et al. (1989), came one year later when cDNA encoding the rabbit and human forms of the skeletal RyR was cloned (Zorzato et al., 1990). It was discovered that the sequence derived from the rabbit and the human were very similar (Zorzato et al., 1990). They found several long amino acid sequences, which exhibited complete homology with the corresponding rabbit sequence (Zorzato et al., 1990). Interestingly their results did not uncover any clear high affinity Ca$^{2+}$ or ATP binding domains in the primary structure, which are expected based on the bell shaped Ca$^{2+}$ activation curve. However, some possible low affinity Ca$^{2+}$ and calmodulin binding sites were noted (Zorzato et al., 1990). From the analysis of the sequence, Zorzato and co-workers predicted 10 potential transmembrane sequences in the COOH-terminal fifth of the molecule, as well as two
additional potential transmembrane sequences nearer to the centre of the molecule, which could contribute to the formation of the Ca\textsuperscript{2+} conducting pore. This is in conflict with the four membrane spanning areas proposed by Takeshima et al. (1989) and the exact number of membrane spanning regions is still an issue today. However, most studies agree on the existence of at least 4 transmembrane spanning regions located in the C-terminal end of the protein.

Figure 1.8 Proposed schematic transmembrane topology of the RyR1 in the triad junction of skeletal muscle. The C-terminal channel region (including the putative transmembrane segments M1-M4), the proposed modulating sites and the large cytoplasmic region are shown. Reprinted with permission (Takeshima et al., 1989).
1.3.3 The three-dimensional structure of the RyR1

There has been an immense amount of work attempting to accurately determine the three-dimensional structure of the RyR. Initially the work started using negative staining electron microscopy techniques allowing Saito and collaborators (1988) to directly visualize the two dimensional structure of both the purified RyR and the feet structures in the junctional face membrane of rabbit fast twitch skeletal muscle in vitro (Saito et al., 1988). Most of their images consisted of square-like structures (=26nm/side), however on occasion the structure would exhibit a rectangular (=26nm*12nm) or a diamond-shaped side view (Saito et al., 1988). The less dense base portion of the diamond appearance was suggested to be the transmembrane region (Saito et al., 1988). The square structures of the RyRs were seen to overlap by approximately 12nm (Figure 1.9) (Saito et al., 1988). Thus, the channels were seen to approximate a square (=23nm/side), enclosing an alternate empty square (=14nm/side) between them (Figure 1.9) (Saito et al., 1988). Their images of purified RyRs identified an outer region and an inner core, each displaying fourfold symmetry (Saito et al., 1988). The inner core was generally more dense and appeared to have a hole at its center (Saito et al., 1988).

Radermacher and co-workers obtained a higher resolution three-dimensional reconstruction of the frozen hydrated RyR using cryo-electron microscopy and a series of random conical tilt images extracted from electron micrographs of isolated detergent-solubilized channels (Radermacher et al., 1994). Their reconstructions showed the RyR consisting of two major components, a larger cytoplasmic assembly
Figure 1.9 A diagrammatic representation of the arrangement of feet structures in the junctional face membrane. Two prominent features are the substantial overlap of the sides of the feet structures (26 nm/side) to enclose alternating spaces (14-nm squares), shown in the T-tubule face (TTF) (f) and the sides of the receptors are tapered so that the terminal cisternae face (TCF) (g) approximates a smaller square (~17 nm), with concomitant larger intervening spaces (Saito et al., 1988). Produced from The Journal of Cell Biology (1988), 107: 211 – 210 by copyright permission of The Rockefeller University Press.

and a smaller transmembrane domain (Radermacher et al., 1992). The larger cytoplasmic assembly was the shape of a square prism (29*29*12nm), as had been noted in earlier two-dimensional studies by Saito et al. (1988). Attached to one of the faces of this cytoplasmic assembly was the protruding mass of the transmembrane domain (Radermacher et al., 1992). The transmembrane assembly was also a square shaped object that tapered as it passed through the membrane, having an edge length of 12nm on the cytoplasmic side and only 6nm at its lumenal end (Radermacher et al., 1994). The total length of the transmembrane assembly was estimated to be 7nm (Radermacher et al., 1994). This is several nanometers more than necessary to
transverse the membrane and implied that the distal portion of the assembly may extend into the lumen of the SR (Radermacher et al., 1994). They noted the existence of a low-density channel of apparent diameter 2-3 nm appearing to run down the center of the transmembrane assembly and suggested this may be the pathway for Ca\(^{2+}\) transversing the membrane (Radermacher et al., 1994). To their surprise they also identified a globular mass of density in the center of the channel that they referred to as the channel plug, as it was 3.0-3.5nm in diameter and appeared to be able to block the central passage (Radermacher et al., 1994) (Figure 1.10). The cytoplasmic assembly comprised more than 50% solvent (Radermacher et al., 1994). Thus the protein appeared to be arranged as domains that are loosely packed together, and they identified and numbered ten of these domains (Radermacher et al., 1994) (Figure 1.10). Numerous spaces that are present within the structure were also identified and it was proposed that would allow a major exit for calcium even if the triad junction was blocked by the channel plug (Radermacher et al., 1994). Internal communication was proposed to occur through structural changes that occur in the cytoplasmic assembly (Radermacher et al., 1994). The authors also proposed that the role of the cytoplasmic assembly was a mechanical one, that is, it serves as a scaffold with the purpose of holding the transverse tubule and the SR membrane in close proximity at the triad junction and also to maintain the junction during the potentially damaging forces generated during cycles of contraction (Radermacher et al., 1994). This hypothesis was shown to be incorrect, when triad junctions were identified in dyspedic mice, lacking the RyR1 (Takekura et al., 1995).

Radermacher and collaborators (1994) also reported that the RyR appeared to be strikingly empty when compared to the three-dimensional structure of the negatively
stained RyR described by Saito et al. (1988). The bottom view of their reconstruction showed an outer square and an inner square, which are rotated with respect to each other over an angle of approximately 40° (Radermacher et al., 1994) (Figure 1.10). The top view, facing the cytoplasm towards the transverse tubule, showed an opening of approximately 50 Å in diameter (Radermacher et al., 1994) (Figure 1.10). The rim of the central channel was seen to form a continuous network that extended to the peripheral mass (Radermacher et al., 1994). The side view of the channel protein has a mushroom-shape with a stem length of = 65 Å (Radermacher et al., 1994) (Figure 1.10). Part of this stem would be expected to contain the membrane spanning domain of the channel protein (Radermacher et al., 1994).

Serysheva et al. (1995) provided the first three-dimensional structure of the Ca\(^{2+}\) release channel in an inactive state, that is, a closed channel (in the absence of Ca\(^{2+}\), using 1mM EGTA in 300mM KCl, 10mM MOPS (pH 7.4)) and concluded that the observed features must be related to this specific chemical state of the protein. It was suggested that the rather hollow and pretzel-like appearance of the putative cytoplasmic portion of the structure may be associated with efficient diffusion pathways for the Ca\(^{2+}\) into the cytoplasm and the binding of different ligands and modulators (Serysheva et al., 1995). The central cavity seen in the top side does not penetrate through the stem of the mushroom, as would be expected for a closed channel (Serysheva et al., 1995). No obvious opening of the Ca\(^{2+}\) release channel on the luminal side of this membrane protein was observed (Serysheva et al., 1995).
Figure 1.10 Surface representation of RyR reconstructed using images from cryo-electron microscopy. Three views are presented: top view showing the surface that would face the cytoplasm (left-hand image); bottom view showing the surface that would interact with the sarcoplasmic reticulum (middle image); and the side view of (right-hand image). The putative structural domains are labeled with numerals. Other abbreviations used are: cc – central cavity; p – plug feature; pc – major peripheral cavity; and ta – transmembrane assembly. Figure reprinted with permission from Radermacher et al. 1994.

Serysheva and co-workers went on to reconstruct the RyR in its open formation (in the presence of Ca\(^{2+}\) and ryanodine) (Orlova et al., 1996) and found that the overall structural features resembled those that they had previously found in the closed state. However, in the open-state reconstruction a central cavity was revealed in the putative transmembrane portion of the channel, whereas there is no apparent hole in the absence of ryanodine and calcium, which were used to activate the channel (Orlova et al., 1996) (Figure 1.11). The channel in their reconstructions had a diameter of 18 ± 7 Å in its narrowest region, had a length of approximately 20 Å, and was located at 20 Å from the lumen face of the channel (Orlova et al., 1996). The reconstructions of the channel in its open states revealed a substantial structural rearrangement in the channel (Orlova et al., 1996). On the SR luminal side, the transmembrane domain in
Figure 1.11 The open and closed states of the RyR1. Top (side facing the t-tubule membrane), side and bottom (side embedded in the SR) views are shown. Important details are marked: the clamp-shaped domain (C), the handle (H), which connects the clamp shaped domain to the central part of the cytoplasmic side (CY) of the tetramer. Printed with permission (Orlova et al., 1996).

The open state is twisted counter clockwise by approximately 4 degrees with respect to its position in the closed state (Orlova et al., 1996). On the cytoplasmic side the opening across the putative membrane region widens to diameter of 30 Å at a distance
of 45 Å from the lumen surface (Orlova et al., 1996). From the side view the channel is also seen to elongate from 190 Å in the closed state to 200 Å when the channel is open (Orlova et al., 1996). They conclude that these changes are global changes rather than local conformational changes. Interestingly, Serysheva and collaborators failed to detect the ‘plug’ found by Radermacher and collaborators (Radermacher et al., 1994).

From all of the structural studies mentioned above there is a clear consensus on the structure of the RyR1. However the resolution obtained in all of the studies has been limited to approximately 27 – 35 Å (Radermacher et al., 1994; Serysheva et al., 1995; Orlova et al., 1996). In order to identify more precise conformation changes within the RyR1 a higher resolution structure will need to be obtained. Even with the current level of resolution obtained, cryo-electron microscopy, through the use of difference studies, has provided a useful tool for investigate the structural motifs of the RyR1. These studies determine the location of ligands or sequence specific anti-bodies by subtracting images in the presence and absence of the ligand or anti-body (see section 1.5).

1.3.4 The role of the skeletal RyR in ECC

Among the three RyR subtypes, RyR1 is the only one that mediates skeletal muscle ECC, whereas RyR2 and RyR3 cannot substitute for RyR1 (Sorrentino and Volpe, 1993). Three regions which have been designated d1 (corresponding to RyR1 residues 4254 - 4631), d2 (RyR1 residues 1342 - 1403) and d3 (RyR1 residues 1872 – 1923) are particularly divergent between RyR1 and RyR2 and it was proposed that the
sequence critical for skeletal type ECC would be located within these regions (Sorrentino and Volpe, 1993).

In 1997 a study investigated the importance of a sequence, based on the D2 region, in eliciting skeletal type ECC (Yamazawa et al., 1997). Yamazawa and co-workers (1997) carried out expression experiments using cultured mutant skeletal myocytes, which lacked RyRs (dyspedic), to study the functional contribution of amino acid residues 1303-1406 in RyR1 (Yamazawa et al., 1997). Expression of RyR1 but not of RyR2 rescued skeletal ECC in the mutant cells (Yamazawa et al., 1997). The deletion of either the entire D2 region or the N-terminal half of RyR1 preserved the function of RyR1 as a calcium release channel but resulted in the loss of ECC (Yamazawa et al., 1997). Substitution of the D2 region for the corresponding sequence of RyR2 had no effect on the function of RyR1 (Yamazawa et al., 1997). These results indicate that the presence of the D2 region is critical for ECC in skeletal muscle, although the D2 region alone cannot determine the functional difference between RyR1 and RyR2. It is quite possible that the mechanisms required to elicit skeletal type ECC are found within a combination of the three divergent regions d1, d2 and d3.
1.4 Functional coupling between the DHPR and RyR

In skeletal muscle the ratio of the RyR / DHPR has been determined to be 0.7 / 0.52, whereas in cardiac muscle the ratio has been found to be 4 – 10 times higher dependent on the mammalian species investigated (Bers and Stiffel, 1993). The organization of the DHPR within the T-tubule membrane is thought to be different in skeletal and cardiac muscle and the organization has been predicted to be a potential reason for the differences noted within skeletal and cardiac type ECC (Protasi, 2002). In skeletal muscle the DHPR has been shown to form tetrads (Block et al., 1988). Tetrads are formed from four DHPRs in a diamond formation corresponding exactly to the position of the subunits of the RyR (Block et al., 1988) (Figure 1.12).

The physical coupling between the RyR and DHPR has been shown to be bi-directional. The two forms of signaling that have been identified have been termed retrograde and orthograde. Orthograde signaling refers to the physical activation of the RyR1 caused by the skeletal DHPR. In ECC this happens immediately following the detection of a depolarization of the surface membrane by the DHPR. The sequence of the DHPR found to be critical for orthograde signaling has been outlined in section 1.2.5.

Retrograde signaling refers to the facilitation of the skeletal DHPR Ca$^{2+}$ current which occurs when RyR1 is expressed in the dyspedic cell line. The notion of retrograde signaling arose from studies using dyspedic myotubes (Nakai et al., 1996). It was discovered that the L-type Ca$^{2+}$ current density from the DHPR was approximately 30
times less in dyspedic myotubes, than in RyR1-expressing myotubes (Nakai et al., 1996). Interestingly, measurements of charge movement indicated that the density of DHPRs was similar in both dyspedic and RyR1-expressing myotubes (Nakai et al., 1996). It was therefore concluded that the presence of a retrograde signal by the RyR1 enhanced the function of the DHPR as a Ca$^{2+}$ channel (Nakai et al., 1996).

In skeletal muscle the primary control of RyR1 during ECC occurs via an interaction with DHPRs. Although previous attempts at demonstrating interactions between purified RyR and $\alpha_1$-DHPR have failed, cross-linking analysis has shown low-level complex formation between the RyR and the $\alpha_1$-DHPR (Murray and Ohlendieck, 1997). After cross-linking of membranes highly enriched in triads with dithiobis-succinimidyl propionate, distinct complexes of more than 3000 kDa were detected (Murray and Ohlendieck, 1997). However, an overlap of immunoreactivity between the receptors was not observed in crude microsomal preparations, indicating that the triad complex is probably of low abundance (Murray and Ohlendieck, 1997). The results of this study do provide some support for the signal transduction hypothesis of a direct physical interaction between triad receptors in adult skeletal muscle.

The effect of RyR1 on DHPR calcium channel activity may not involve a direct interaction with the alpha 1 subunit. For example it was has been proposed that RyR1 could promote the association of alpha 1 with accessory subunits (such as beta 1) that could increase open probability (Nakai, et al., 1996). Alternatively, calcium released from the sarcoplasmic reticulum might feed back during excitation-contraction coupling to potentiate the skeletal L-type current. Freeze fracture studies demonstrate
Figure 1.12 The top (left hand Figure) and side views (right hand Figure) of the proposed physical coupling of the DHPR and the RyR1. The portions of the two channels that are located in the membrane are indicated on the right hand side.

Reprinted with permission from Serysheva et al. (2002)

that junctional tetrads, which seem to be composed of DHPRs, are absent in dyspedic muscle. Conceivably, the absence of RyR1 could impair the biosynthesis or membrane insertion of DHPRs, but the small reduction of $Q_{\text{max}}$ in dyspedic compared with normal myotubes indicates that this is not an important effect (Nakai, et al., 1996). Rather the $Q_{\text{max}}$ values would seem to indicate that DHPRs are present and form low conductance Ca$^{2+}$ channels, but do not organize into tetrads in the absence of RyR1 (Nakai, et al., 1996).

To investigate the molecular mechanism of reciprocal signaling, Nakai et al. (1998) constructed cDNAs encoding chimeras of RyR1 and RyR2 and expressed them in dyspedic myotubes, which lack and endogenous RyR1. They found that a chimera, R10 (RyR1 residues 1635-2636), both mediated skeletal type ECC and enhanced calcium channel function, whereas another chimera R9 (RyR1 residues 2659-3720),
only enhanced calcium channel function (Nakai et al., 1998). This indicated that the orthograde signalling involved in ECC is contained within the region of R10. The demonstration that R9 enhances L-type calcium channel activity without restoring skeletal type ECC indicates that the structures of RyR1 involved in retrograde (channel enhancing) signalling are not identical to those involved in orthograde signaling (Nakai, et al., 1998).

Leong and MacLennan (1998) used protein affinity chromatography to demonstrate that the skeletal DHPR II-III loop interacted with RyR1 residues Leu$^{922}$-Asp$^{1112}$, but not the corresponding fragment from the RyR2. The DHPR II-III loop was fused to glutathione S-transferase and used in a protein affinity column for $^{35}$S-labelled in vitro translated fragments from the N-terminal three-fourths of RyR1. The use of chimeras from RyR1 localised an interaction site to 37 amino acids, Arg$^{1076}$-Asp$^{1112}$. The RyR1 922-1112 fragment did not bind to the cardiac DHPR II-III loop (Leong and MacLennan, 1998). The skeletal DHPR II-III loop double mutant K677E/K682E (residues contained within the A region of the II-III loop) lost most of its capacity to interact with RyR1, suggesting that two positively charged residues are important in that particular interaction between RyR and DHPR (Leong and MacLennan, 1998). It is suggested that interactions between the RyR1 and DHPR are not limited to one site and could possibly involve many sites in the two proteins (Leong and MacLennan, 1998).

Another study which investigated the interaction between regions of the DHPR and the RyR1, was conducted by Proenza et al. (2002). The yeast two-hybrid system was utilized, in which portions of the skeletal DHPR II-III loop were expressed as fusion
proteins with the binding domain of the CAL4 transcription factor (Proenza et al., 2002). Other fusion proteins of approximately 300 amino acids in length, corresponding to portions of the R9 (2659-3720) and R10 regions (1635-2636) of the RyR1 were also expressed (Proenza et al., 2002). Interactions between pairs of these proteins were evident by the formation of colonies after yeast co-transfection (Proenza et al., 2002). Areas proven to have a strong interaction such as the alpha-interaction and beta-interaction domains (see section 1.2.4) showed colony formation after 2-4 days (Proenza et al., 2002). The only segment of the skeletal II-III loop found to interact with the RyR1 through this method was region termed s53, corresponding to residues 719 – 767 of the skeletal DHPR (Proenza et al., 2002). The portion of the RyR1 that the s53 region interacted with was termed the sR16 region (residues 1837 – 2168 of RyR1) (Proenza et al., 2002). It is interesting to note that colonies only appeared after 7-10 days, indicating that the interaction between s53 and sR16 was quite weak (Proenza et al., 2002). Upon testing the functional significance of the sR16 region using a RyR2 chimera with RyR1 sequence in the sR16 region, it was found that replacement of this area with skeletal sequence was able to mediate weak skeletal type ECC (Proenza et al., 2002), indicating that this area was involved in mediating skeletal type ECC to some degree. However it was also found that, in a reverse chimera of RyR1, with the sR16 region replaced with sequence from RyR2, there was no disruption of skeletal type ECC (Proenza et al., 2002). Thus the results show that the region sR16 is involved in skeletal ECC but is not essential for the process.

In conclusion, the precise interaction between the skeletal DHPR and the RyR1, which mediates skeletal type ECC is still unclear. There is substantial evidence that
the II – III loop of the DHPR has considerable involvement, while other subunits, such as the beta subunit and gamma subunit of the DHPR also have a role to play. There is evidence that both the R9 and R10 regions of the RyR1 play an important role (Nakai et al., 1998). Similarly, the region spanning residues Leu$^{922}$ – Asp$^{1112}$ may also contribute to the interaction between the RyR1 and the DHPR. It is most likely that there is a complex series of interactions between several regions on the RyR1, the DHPR complex and several of the accessory proteins located within the triad junction.
1.5 Other important RyR associated proteins found in the Triad Junction

There are several other proteins, located within the triad junction, which interact with the RyR. These include calsequestrin, calmodulin, triadin, junctin, endogenous kinases and FKBP12. FKBP12 has a molecular weight of 12 kDa and for each monomer of the RyR there are four FKBP12 proteins attached (see references included in Wagenknecht et al., 1997). The FKBP12 is an FK-506 binding protein. In 1997, Ahern and co-workers, reported an examination of the gating properties of the RyR1 in the absence of FKBP12. FKBP12 was stripped from the RyR1 using rapamycin, which binds to the FKBP12 protein and dissociates it from the RyR1 (Ahern et al., 1997). Without FKBP12 attached, the coordinated channel openings to maximum conductance were reduced and a larger amount of substate activity was noted (Ahern et al., 1997). FKBP12 stripped channels were activated by 10-fold lower and inhibited by 10-fold higher calcium concentrations compared to the native RyR1 (Ahern et al., 1997). In addition to coordinating channel openings, the FKBP12 has an influence on the gating of the RyR1 (Ahern et al., 1997). In the absence of FKBP12, the open probability of the RyR1 was seen to increase in low calcium concentrations (0.1 - 1.0 mM), but was unaffected at 10 mM calcium (Ahern et al., 1997). The importance of FKBP12 in coordinating channel openings was shown by Marx et al. (1998) who demonstrated that in the presence of FKBP12, several single RyR1 channels incorporated in lipid bilayers exhibited coupled gating while the removal of FKBP12 produced uncoupled channels. It was suggested the role for FKBP12 could
be as a mechanism through which channels not physically coupled with the DHPR can be regulated (Marx et al., 1998).

FKBP12 has also been shown to have a potential role in modulating skeletal type ECC. It was discovered through surface plasmon resonance binding experiments that the A region peptide was the only portion of the skeletal DHPR II – III loop to bind to the RyR1 and that this interaction was potentiated by the presence of FKBP12 (O'Reilly et al., 2002). Similarly, the increase in opening probability of the single RyR1, detected in the presence of peptide A, was abolished in the absence of FKBP12 (Dulhunty et al., 1999; O'Reilly et al., 2002). These results demonstrate a direct interaction, between the RyR1 and a native sequence of the skeletal DHPR, which is dependent on the presence of FKBP12.

FKBP12.6 (cardiac isoform of FKBP12) has also been shown to have a role in regulating the open probability of the RyR2. Phosphorylation of RyR2 by protein kinase A (PKA) caused the dissociation of FKBP12.6 from the RyR2 complex, regulating the open probability of the channel (Marx et al., 2000). This mechanism appears to be involved in failing human hearts, where the RyR2 is hyperphosphorylated by PKA causing FKBP12.6 dissociation and defective channel function, leading to an increased sensitivity to Ca\textsuperscript{2+} induced activation (Marx et al., 2000).

Wagenknecht and co-workers (1997) looked at reconstructions of the RyR with the FKBP12 bound to the channel. They located FKBP12 by subtracting the image of that in the presence of the molecule from that in its absence (Wagenknecht et al., 1997).
FKBP12 was located on each of the four subunits adjacent to region ‘9’ and just above domain ‘3’, 12nm away from the ion channel entrance (see Figure 1.13) (Wagenknecht et al., 1997).

A more complicated interaction is found with several of the other co-proteins of the RyR1: calsequestrin; junctin; and triadin. Calsequestrin (CSQ) is a glycoprotein that binds free calcium within the SR. CSQ monomers form a linear polymer that is anchored to the RyR through the accessory proteins, junctin and triadin (Jones et al., 1995). Junctin and triadin both span the SR membrane and both bind to CSQ and the RyR (Murray and Ohlendieck, 1997; Zhang et al., 1997). Both triadin and junctin share a very similar structure and sequence (Zhang et al., 1997).

Upon binding Ca$^{2+}$, the CSQ monomers undergo a conformation change, resulting in compaction and folding with an increased proportion of alpha helical secondary structure (Wang et al., 1998). Once folded the monomeric CSQ undergoes aggregation into a polymer (Wang et al., 1998). The suggested mechanism for polymerization is induced by Ca$^{2+}$ and involves the compaction of three domains and the associations between calsequestrin monomers due to three types of interactions: N-terminal arm exchange, helix-helix contacts and Ca$^{2+}$ cross bridges (Wang et al., 1998). CSQ is able to bind 40 – 50 mol of Ca$^{2+}$ has a very high capacity to bind Ca$^{2+}$ (MacLennan and Wong, 1971). CSQ has been shown to inhibit native RyR1 channel activity (Beard et al., 2002). There was a 10-fold increase in the duration of open times for single ryanodine receptors incorporated into lipid bilayers when CSQ was dissociated and this was reversed when CSQ was re-associated (Beard et al., 2002).
Calmodulin (CaM) is thought to be able to mediate Ca$^{2+}$ signaling through its interactions with RyR1. CaM has been termed a mediator because the it modifies the overall effect of cytosolic Ca$^{2+}$ on the RyR.

The effects of CaM on the RyR1 are complex. At high Ca$^{2+}$ concentrations (10 µM) the Ca$^{2+}$ saturated CaM (Ca$^{2+}$-CaM) binds directly to the RyR1 with a stoichiometry of one Ca$^{2+}$-CaM per RyR1 subunit (Wagenknecht et al., 1997) and inhibits channel activity (Fuentes et al., 1994; Buratti et al., 1995). However, at low Ca$^{2+}$ concentrations (0.1 µM), Ca$^{2+}$ depleted CaM (apoCaM) activates the RyR1 (Fuentes et al., 1994; Tripathy et al., 1995) and binds four apoCaM per subunit (Tripathy et al., 1995). CaM has also been shown to have a role in controlling the sensitivity of the RyR1 to reduction and oxidation reactions caused by nitric oxide and reactive oxygen species (Eu et al., 2000).

The location of the calmodulin (CaM) binding site on the RyR has been investigated in several studies (Wagenknecht et al., 1994; Wagenknecht and Radermacher, 1995; Wagenknecht et al., 1997; Samso and Wagenknecht, 2002). Ca$^{2+}$-CaM was located in clefts formed by structural domains 3, 4 and 7, approximately 10nm away from the ion channel entrance (see Figure 1.14) (Wagenknecht et al., 1997). The location of apoCaM was determined in 2002 (Samso and Wagenknecht, 2002). It was found that apoCaM was located in a very similar position to that of Ca$^{2+}$-CaM (Samso and Wagenknecht, 2002). Both forms of CaM contacted domain 3 of each of the four RyR1 subunits (Samso and Wagenknecht, 2002). However, the position of apoCaM was closer to the T-tubule face of RyR1 and protruded more from the lateral surface of the structure (Figure 1.14) (Samso and Wagenknecht, 2002). It was thought to be
Figure 1.13 Solid body representations of three-dimensional reconstructions of the RyR1 together with the differences attributed to CaM (yellow) and FKBP (pink). The bottom face, which interacts with the sarcoplasmic reticulum (middle image), the top face, which faces the T-tubule membrane (top image) and side view (bottom image) are shown. Selected domains are indicated by numerals. Reprinted with permission from Wagenknecht et al 1997.

Figure 1.14 Reconstructed views of the RyR1 from the side (left-hand image) and from the T-tubule facing side (right-hand image) with the superposition of the densities attributed to apoCaM (shown in orange) and Ca^{2+} - CaM (shown in yellow) The RyR1 shown in this Figure is a semi-transparent green and the positions of the various CaM can be seen through the image. Reprinted with permission from Samso and Wagenknecht (2002).
unlikely that both of these binding sites would be occupied simultaneously as the volumes detected for the two forms of CaM were partially overlapped (Samso and Wagenknecht, 2002).

Another protein that is thought to modulate the activity of the RyR1 is the endogenous kinase, calmodulin kinase II (CaMKII) (Dulhunty et al., 2001). The presence of CaMKIIβ in SR vesicles was confirmed using immunoblotting (Dulhunty et al., 2001). Activation of the native RyR1 incorporated in a lipid bilayer was detected following the exposure of 2 mM cytoplasmic ATP for 1 minute and this activation was reversible upon ATP washout, whereas, irreversible activation was noted with a 5 – 8 minute exposure of 2 mM cytoplasmic ATP (Dulhunty et al., 2001). The irreversible activation noted with the longer exposure to ATP was reduced by acid phosphatase, indicating that phosphorylation caused the activation (Dulhunty et al., 2001). Both, the CaMKII inhibitor KN-93 and an inhibitory peptide for CaMKII prevented the phosphorylation-induced irreversible activation after application of ATP, suggesting the CaMKII, was the endogenous kinase responsible for the activation noted (Dulhunty et al., 2001).

To summarize it can be seen that the RyR is involved in several protein/protein interactions within the triad junction. The extent to which these proteins contribute to or modify ECC has yet to be completely determined.
1.6 This thesis

In this thesis the structure of several peptides derived from the II – III loop of the skeletal muscle DHPR were investigated using nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD) spectroscopy. Up to this point structural studies on the DHPR have been conducted using cryo-electron microscopy, basic computer modeling and NMR and the studies conducted in this thesis are an extension of the previous findings. Some functional studies were performed using Ca\(^{2+}\) release from SR vesicles. As functional studies of several of the peptides had already been conducted within our laboratory, two chapters are concerned purely with the structure of peptides derived from the A region of the skeletal DHPR, spanning residues 671 – 690. These include several truncated peptides A3, A4, A5, A6 and A9 (chapter 3) as well as a mutated peptide A1(D-R18) (chapter 4). Similarly, a third chapter is concerned only with the structural characteristics of the C peptide (skeletal DHPR residues 720 – 765) and the complete II – III loop (skeletal DHPR residues 666 – 791) (chapter 6). In chapter 5, the structure and functional relationship of the AB peptide (skeletal DHPR residues 671 – 710) and two mutants of this peptide are described. In each results chapter a more detailed introduction, regarding the previous studies of each of the peptides, is presented.
Chapter 2 - Materials and Methods

2.1 Materials

Chemicals and biochemicals were obtained from Sigma-Aldrich (Castle Hill, Australia). All the peptides used in this study, except the SDCL peptide (comprising the sequence of the whole II – III loop), were synthesized using an Applied Biosystems 430A Peptide Synthesiser with purification to 98 to 100% purity using HPLC and mass spectroscopy (solvent used). Precise peptide solution concentrations were determined (Auspep Pty Ltd) using acid hydrolysis followed by a standardized PTC (phenylthiocarbamyl) protocol and analyzed by reverse phase HPLC.

The SDCL was expressed as a 6 x Histidine-Ubiquitin-protein fusion in E.coli cells. The cells were grown at 310 K, to an optical density of ~0.8 and induced by adding 1mM Isopropyl b-D-thiogalactopyranoside (IPTG). The cells were grown for a further 4 hours, spun down and pelleted. The cell pellet was resuspended in phosphate buffer (pH 8.0) and sonicated to lyse the cells. The sonicate was spun down and the supernatant run through a Ni-Agarose column. The column is washed with an excess of 20 mM imidazole to remove non-specific binding. The protein is then eluted with 500 mM imidazole. The eluted protein was incubated with Ubp41 and put through another Ni agarose column to remove the cleaved 6 x Histidine-Ubiquitin-protein tag. The sample was then electrophoresed through a cylindrical non-denaturing gel. Individual components of the sample separate into ring shaped bands and the bands migrate off the bottom of the gel one at a time, where they were collected using a fraction.
Chapter 2 – Materials and Methods

collector. The expression of the SDCL peptide was conducted by Phil Board and Yamuna Karunasekara.

The sequence of the peptides used in this study are listed below (mutations from the native sequence are in bold):

A1: 671TSAQKAKAEERKRKMSRGL 690
A2: 671TSAQKAKAEERKRKMARGL 690
A3: 681RKRRKMSRGL 690
A4: 671TSAQKAKAEED 680
A5: 675KAKAEERKR 684
A6: 678AEERKRKMS 687
A9: 681RKRRKMS 687
A1 (D-R18): 671TSAQKAKAEERKRKMSRGL 690
AB: 671TSAQKAKAEERKRKMSRGLPDTEEKEKSVMKAKLSQKP 710
AB-ch: 671TSAQKAKAEERKRKMSRGLPAKTAVKSMKAKLSQKP 710
AB-link: 671TSAQKAKAEERKRKMSRGLADATEEKEKSVMKAKLSQKP 720
C: 724EFESNVNEVKDPYPADFPGDDEDEPEIPVSPRPRP 760
SDCL: 666EAESLTSAQKAKAEERKRKMSRGLPDTEEEKSVMKAKLSQK PKGEGIPTTAKLKVDEFESNVNEVKDPYPADFPGDDEDEPEIPVSPRPRPLAEQLKEKAVPIPEASSFPFFSPTWKVRVL 791
2.2 Experimental Techniques Used

2.2.1 Solution State Nuclear Magnetic Resonance (NMR) Spectroscopy

2.2.1.1 NMR, what is it?

Nuclear Magnetic Resonance Spectroscopy utilizes intense magnetic fields to irradiate atomic nuclei. The theory of NMR is “based on the fact that atomic nuclei orientated by a strong magnetic field (2-14 Tesla) absorb radiation at characteristic frequencies” (Roberts, 1993). The hydrogen nucleus is most sensitive to detection by NMR, although other nuclei such as $^{13}$C and $^{15}$N can also be detected.

2.2.1.2 Spectra Used

The spectra used in this research include $^1$H TOCSY, $^1$H NOESY, 1H ROESY and $^1$H COSY experiments. Correlated spectroscopy (COSY) spectra were obtained as they provide information regarding protons connected through chemical bonds. COSY spectra are limited as they can only provide information of connectivities between protons separated by two or three bonds. Whereas total correlation spectroscopy (TOCSY) spectra can, under favorable conditions, extend through an amino acid side chain (Roberts, 1993).

TOCSY experiments provide us with information regarding the connections of $^1$H atoms through chemical bonds. This allows us to identify residues based on known chemical shift values for hydrogen atoms located in the side chains of amino acids.

Whereas TOCSY experiments provide information relating to $^1$H atoms connected through chemical bonds, Nuclear Overhauser Effect Spectroscopy (NOESY) spectra
provide information relating to the relative closeness in space of $^1$H atoms. $^1$H atoms closer than 5 Å form cross peaks and the strength of the cross peaks is determined by the closeness of the two atoms. Due to the structural limitations of a peptide backbone there are certain hydrogen interactions that will be present in all NOESY spectra. An intramolecular interaction between the backbone amide hydrogen and $\alpha$ hydrogen of a residue ($i$) (NH$_i$ - $\alpha$H$_i$) will be present. Also the interaction between an $\alpha$ hydrogen ($i$) and the following residues ($i+1$) amide backbone hydrogen ($\alpha$H$_i$ - NH$_{i+1}$) will be evident in all NOESY spectra. In small to medium sized molecules the rotational correlation time (time it takes for a molecule to rotate through an angle of one radian) can correspond to a zero detection rate in NOESY experiments. For this reason another form of NOESY experiment, termed a ROESY experiment, was conducted on peptides in which NH – NH cross peaks were not detected. Hydrogen ROESY spectra also provide information regarding the relative closeness of $^1$H atoms in space, however cross peaks are weaker in these spectra. The advantage of a ROESY experiment is that there is no stage when the signal detection is zero. Thus to confirm that the results obtained in NOESY experiments absent of NH – NH connectivities were due to the presence of random coil secondary structure rather than a zero signal detection, ROESY experiments were performed on peptides without any detectable NH – NH connectivities.

2.2.1.3 Coupling Constants

$^3$J$_{\text{NH-}^1\text{H}}$ coupling constants are measurements relating to the polypeptide backbone dihedral angle ($\phi$) (torsion angle around N - $\alpha$C) (see Figure 2.1) and can provide local structural information complementary to that obtained from NOESY data. In the
case of homonuclear $^1$H experiments $^3J_{\text{NH-CH}}$ coupling constants can be taken from the difference in the double peaks found within a 1-dimensional spectrum or from a COSY spectrum. The measurement itself is a frequency difference (Hz). Individual residues that are involved in alpha helical secondary structure have been found to contain coupling constants of $< 6$ Hz. While residues involved in beta sheet secondary structure have coupling constants $> 8$ Hz. Coupling constants obtained between 6 Hz and 8 Hz are typically from unstructured portions of a protein/peptide.

![Figure 2.1 Illustration of peptide backbone with dihedral angles ($\phi$) between N - $\alpha$C labeled.](image)

2.2.1.4 Temperature Co-efficient

The temperature co-efficient of a residue provides information regarding the stability of its local environment (Deslauriers and Smith, 1980). If a residue is involved in some form of secondary structure, there will be backbone hydrogen bonding present. This hydrogen bonding will present an extra level of stability to the structure of the peptide, even in the presence of additional energy via an increase in temperature. For this study, a value of greater than $-5$ p.p.b.$K^{-1}$ is taken to indicate the presence of
secondary structure. Values obtained that are less than –5 p.p.b.K\(^{-1}\) generally indicate that no secondary structure present.

The temperature co-efficient of each residue was determined by quantifying the difference in amide chemical shift values obtained over a set temperature range. This information is then presented as a difference in parts per billion per degree of temperature change (p.p.b.K\(^{-1}\)). The temperature co-efficient of each residue in the studies presented here, were obtained over a temperature difference of 18-20 degrees.

### 2.2.1.5 Alpha Hydrogen Chemical Shift Index

From an analysis of the \(^1\)H NMR chemical shift assignments and the secondary structure determined for over 70 proteins, it was revealed that all 20 naturally occurring amino acids exhibited a similar shift in \(\alpha\)H values when contained in either alpha helical or beta sheet secondary structure (Wishart et al., 1991). Proteins containing alpha helical secondary structure, exhibited a mean \(\alpha\)-\(^1\)H upfield shift of 0.39 ppm, from the random coil value, while, the \(\alpha\)-\(^1\)H chemical shift was found to move downfield by an average of 0.37 ppm when the residue is contained in a beta-strand or extended configuration (Wishart et al., 1991). Thus we are able to determine from an amino acids \(\alpha\)-\(^1\)H chemical shift the most likely secondary structure present in the peptide / protein. In this study, \(\alpha\)-\(^1\)H CSI (chemical shift index) were measured and are presented as shifts +1 or –1 respectively. An \(\alpha\)-\(^1\)H CSI of –1 refers to a shift of more than 0.1 ppm downfield, while +1 refers to a shift of more than 0.1 ppm upfield, from the assignments determined for random coil structures (Wishart et al., 1991). Values of +1 are suggestive of alpha helical secondary structure, whereas
values of -1 will most likely represent residues participating in beta sheet secondary structure (Wishart et al., 1991).

2.2.1.6 Structure Calculations

The full assignment of chemical shift values for a peptide can be obtained through using the information on TOCSY and NOESY spectra. Once each $^1$H atom can be identified by a chemical shift value the strength of the cross peaks obtained between any two specific $^1$H atoms on the NOESY spectrum can be used to form a distance constraint. The relationship between cross-peak strength and distance for two nuclei with an internuclear distance of $r_{ab}$ is defined as:

$$\frac{f_a(b)}{f_c(d)} = \frac{r_{ab}}{r_{cd}} - 6$$

where $r_{cd}$ is the calibration distance and $f_c(d)$ is the NOE of C upon irradiation of D.

Using the XPLOR program, the known sequence of the peptide is entered and a template is developed. This template consists of inherent limitations that will be present in the peptide purely based on its sequence. The set of distance constraints that have been developed from the NOESY spectrum are then entered together with information regarding the coupling constants of known residues.

The XPLOR program uses all available information to generate a pre-determined number of possible structures for the peptide. In attempting to generate these structures the program will find some distant constraints to be energetically
unfeasible. While still attempting to construct a possible structure using the distance constraint, the structure unfeasible distance constraint may be violated in the possible structure. Information regarding the number of violations for each structure and the number of times a single constraint was violated attempting to produce the pre-determined number of structure are obtained at the end of each attempt. The individual constraints violated are then re-assessed. If a constraint is thought to be incorrect it is removed. However if it appears to be a plausible constraint the intensity of the individual constraint may be lowered or the constraints immediately surrounding it may be checked. Another attempt is then undertaken until the number violations and the energy values obtained for each possible structure has been minimized.

Information regarding hydrogen bonding is obtained in the initial stages of this process. Initially the XPLOR program may not be able to generate the full number of pre-determined structures due to a large number of violations. If this occurs adjustments are made until the full number of pre-determined structures are developed. These structures are then reviewed and information regarding potential hydrogen bonding between backbone residues is obtained. For the studies presented, a hydrogen bond was deemed present if it existed in over 30% of the possible structures developed. This additional information is then added and used by the XPLOR program as a further constraint to develop potential structures.
2.2.1.7 Methodology

Spectra were acquired on either a Varian - Inova 600 or Varian – Inova 500 spectrometer with a spectral width of 6000 Hz or 5000 Hz respectively. Spectra were obtained using a pulse width of 7 µs (90°) and acquisition time of 0.130 s, collecting 4096 data points and 512 increments of 32 transients. NOESY (Kumar et al., 1980) spectra (mixing time of 200-500 ms) and TOCSY (Bax and Davis, 1985) spectra (mixing time of 70 ms) were acquired at either 278 K, 288 K or 298 K and used for the assignment of the $^1$H-NMR resonances. Suppression of the $\text{H}_2\text{O}$ resonance for the NOESY spectra was achieved using pulse field gradients (Piotto et al., 1992) while a presaturation pulse was employed for the TOCSY experiments. Two-dimensional data were processed on an O$_2$ Silicon graphics computer using Felix 98 and Felix 2000 software. Data sets were zero- filled to 4096 by 2048K and multiplied by either a phase-shifted sinebell-squared or gaussian function in both dimensions prior to transformation. $^3J_{\text{NH}-\text{aH}}$ coupling constants were derived from 1-dimensional $^1$H spectra.

Distance constraints were derived manually from a 2-dimensional NOESY spectrum acquired with a mixing time of 200 ms, a pH of 3.0, 5.0 or 6.5 and a temperature of 278 K or 298 K. Upper bounds were derived from NOE crosspeak intensities by counting the number of contour levels of non-overlapped crosspeaks and using distances calibrated from Gln $\gamma$-methylene protons. NOE signals were classified into four categories of upper distance limits of 2.7, 3.5, 4.5 and 5.8 Å, each with a 1.9 Å lower distance limit. Dihedral angle restraints based on the $^3J_{\text{NH-Ha}}$ measurements were included in the final calculations with a tolerance of 20°. Structure calculations
were performed with the X-PLOR program (Brunger et al., 1986; Brunger, 1992), using the topallhdg.pro topology file and parallhdg.pro parameter file, with the simulated annealing protocol on a Silicon Graphics O2 computer. The sum averaging option was used to treat equivalent and non-stereospecifically assigned protons. Hydrogen bonds were simulated by a 3.3 Å restraint between the amide nitrogen and carbonyl oxygen and a 2.3 Å restraint between the amide hydrogen and the carbonyl oxygen in at least 6 out of 20 structures. For superimposed and averaged structures, 100 fully extended structures with random coordinates were generated. Structures were analysed and 20 selected for distance and dihedral constraint violations and low energies. None of the structures obtained had NOE violations >0.5 Å or dihedral violations >5°. For structural comparisons, individual structures were superimposed onto the average structure.

2.2.2 Circular Dichroism

Circular Dichroism was used in this study to monitor the absorbance of a peptide at different wavelengths over a range of 185-250 nm. The absorbance measure is converted to units of molar ellipticity by using the formula below:

\[
\text{Molar Ellipticity} (\theta) = \frac{(3300 \times \Delta \text{Absorbance})}{(\text{concentration (M)} \times \text{cell length (cm)})}
\]

where cell length refers to the length of the Perspex cell used in the experiment.

Previous studies of proteins containing large amounts of alpha helical secondary structure found that certain characteristics are always present. These characteristics include: a maximum at 192 nm; a minimum at 208 nm and a second large minimum at 222 nm (Nakanishi et al., 1994). Spectra obtained from peptides with random coil structure contain a first minimum at 197 nm with the minimum at 222 nm being much
smaller or absent (Nakanishi et al., 1994). Rather than containing a second minima at 222 nm, some spectra in this study were found to contain an inflexion point. The percentage of alpha helical secondary structure present will determine how close the first minimum is located to either 197 nm or 208 nm (Nakanishi et al., 1994).

2.2.3 Sarcoplasmic Reticulum Ca\(^{2+}\) release Studies

2.2.3.1 Methodology

The release of Ca\(^{2+}\) from sarcoplasmic reticulum vesicles was used to assess the functional abilities of the peptides to alter RyR activity. Ca\(^{2+}\) release was measured as described by (Chu et al., 1988; Timerman et al., 1993). The procedure uses intact SR vesicles and the Ca\(^{2+}\) sensitive dye, antipyrilazo III, with absorbance monitored using a CARY spectrophotometer at a wavelength of 710 nm. Calcium that is released from the SR causes an increase in extravesicular Ca\(^{2+}\) concentrations and an increase in the optical density, while Ca\(^{2+}\) uptake into the SR causes a decrease in extravesicular Ca\(^{2+}\) and a decrease in optical density.

SR vesicles were added to a cuvette containing 1600 μl of 125 mM KH\(_2\)PO\(_4\) (pH 7.0); 20 μl of 400 mM MgCl\(_2\); 40 μl of 50 mM Na\(_2\)ATP; 80 μl of 12.5 mM antipyrylazo III; and an amount of double distilled H\(_2\)O. The amount of H\(_2\)O added was dependent on the amount of peptide used in each individual experiment. In order to obtain a final volume of 2 ml in the cuvette, the cumulative total of peptide and H\(_2\)O was to equal 211.5 μL. At time zero, TC vesicles (3.5 μl of 28.5 mg vesicle protein/ml) were added to the cuvette. Vesicles were then partially loaded by four additions of 5 μl of 3 mM CaCl\(_2\) (7.5 μM) added each minute.
After 5 minutes, 4 µl of 100 µM (200 nM) thapsigargin was added to suppress Ca\(^{2+}\), Mg\(^{2+}\)-ATPase activity to prevent further loading of the SR vesicles and to prevent any possible backflux of Ca\(^{2+}\), induced by the peptide, through the Ca\(^{2+}\), Mg\(^{2+}\)-ATPase. In the absence of thapsigargin, a change in absorbance (extravesicular [Ca\(^{2+}\)]) was the net difference between the release of calcium from and the uptake of Ca\(^{2+}\). In the presence of thapsigargin the uptake and backflux of Ca\(^{2+}\) from through the Ca\(^{2+}\), Mg\(^{2+}\)-ATPase is reduced to zero (Kurebayashi and Ogawa, 2001), therefore a change in absorbance (extra-vesicular [Ca\(^{2+}\)]) is due only to a release of Ca\(^{2+}\) through the RyR1. The baseline leak of Ca\(^{2+}\) from the SR was established for approximately 30-45 seconds after the addition of thapsigargin. The peptide was then added and change in absorbance monitored for 135 - 150 seconds. This was followed by the addition of by the RyR1 blocker, ruthenium red (10 µl of 1 mM) after 8 minutes. Ruthenium red was used to confirm that the release of Ca\(^{2+}\) was through the RyR1. To determine the amount of Ca\(^{2+}\) remaining in the vesicles, 10 µl of 600 mg/ml Ca\(^{2+}\) ionophore A23187 (final concentration 3 µg/ml) was added after nine minutes. At the start of each day or when solutions were changed a calibration curve was established by measuring optical density changes in response to five sequential additions of 5 µl of 3 mM CaCl\(_2\) (final concentration of 7.5 µM). The temperature of the cuvette solution was thermostatically controlled at 25°C and the solution was stirred continually with a magnetic stirrer.

The rate of Ca\(^{2+}\) release from SR vesicles is given as nM of Ca\(^{2+}\) / mg protein / min. The rate of release was calculated by subtracting the baseline rate of release (determined after the addition of thapsigargin) from the rate of release obtained after
the addition of the peptide (measured for 5 seconds, 5 – 10 seconds after the addition of the peptide). This measurement was calculated from the Ca\(^{2+}\) calibration curves and the following calculations (Chu et al., 1988):

\[
\text{Slope of Ca}^{2+} \text{ release} = \frac{\Delta \text{ absorbance}}{\text{time (min)}}
\]

\[
\text{Ca}^{2+} \text{ calibration} = 45 \text{ nmoles Ca}^{2+}/ \Delta \text{ absorbance}
\]

\[
\text{Rate of Ca}^{2+} \text{ release} = \frac{\text{(slope of Ca}^{2+} \text{ loading} \times \text{Ca}^{2+} \text{ calibration)}}{\text{protein (mg)}}
\]

I would like to acknowledge the assistance of Jaqui Young and Suzi Pace for assisting with the collection of data at several concentrations for the AB-ch peptide.

2.2.3.2. Vesicle preparation

Native SR vesicles were prepared from the back and leg muscles of New Zealand White rabbits using methods originally described by (Saito et al., 1984) and (Chu et al., 1988). Two aliquots of muscle tissue (50 grams each) was each homogenized in 250 ml homogenizing buffer (20 mM Imidazole, 300 mM sucrose, adjusted to pH 7.4 with 6M HCL) in a Waring blender for a total of 1 minute each. The homogenate was divided amongst three centrifuge bottles and centrifuged at 9000 rpm for 20 min at 277 K (Beckman JA – 14 rotor, Beckman J2 – 21 Centrifuge). The supernatant was removed and each pellet was resuspended in 160 – 170 ml of homogenizing buffer and re-homogenized as before. The homogenate was again centrifuged at 9000 rpm for 20 min at 277 K (Beckman JA – 14 rotor, Beckman J2 – 21 Centrifuge). The
supernatant was then filtered through several layers of cotton gauze, transferred to 70 ml centrifuge tubes and centrifuged at 30000 rpm for 1 – 2 hours at 277 K (Beckman TI – 45 rotor, Beckman L8 – 70 Ultracentrifuge). The pellet was re-suspended in 3 – 4 ml of homogenizing buffer, transferred to a dounce homogenizer and re-suspended in 40 ml homogenizing buffer. Seven milliliters of homogenized vesicles were fractioned on a discontinuous sucrose gradient (4 ml of 45%; 3 ml of 38%; 3 ml of 34%; 3 ml of 32%; 4 ml of 27% weight / volume sucrose in dilution buffer (Imidazole 20 mM, adjusted to pH 7.4 with HCl)) and centrifuged at 20000 rpm, overnight at 277 K (Beckman SW – 28 rotor, Beckman J2 – 21 Centrifuge). The vesicles of the fractionated suspension were collected at the different sucrose density interfaces depending on their sedimentation coefficient. The band formed at the interface between 38 % and 45 % sucrose, termed B4, was collected and slowly diluted at least 2 –fold in dilution buffer. To separate the membrane fractions from the soluble sucrose the suspension of B4 was centrifuged for an hour at 32000 rpm and 277 K. The pellets were re-suspended in approximately 1 ml of re-suspension buffer (homogenizing buffer + protease inhibitors (leupeptin 1 µg / ml; pepstatin A 1 µM; benzamidine 1 mM; and PMSF 0.5 mM) + DTT 2mM). These suspensions were aliquotted into volumes of 15 µl, snap frozen liquid nitrogen and stored at 203 K. Suzi Pace and Joan Stivala prepared the SR vesicles that were used in this study.
Chapter 3 - Peptides A1: Variations and Fragments

3.1 Introduction

The ability of peptide A (derived from the DHPR II-III loop) to activate the RyR1 has been well documented over the past ten years and is described in length in section 1.2.6. The major findings for the region of the DHPR containing the A sequence are as follows. Peptide A, or derivatives of the peptide A: activate a single channel RyR1 incorporated in artificial bilayers (Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Casarotto et al., 2000; Casarotto et al., 2001; Stange et al., 2001), cause an increase in ryanodine binding (Gurrola et al., 1999); activate release of calcium from sarcoplasmic reticulum vesicles (el-Hayek et al., 1995a; Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Green et al., 2003); and potentiate the caffeine activated force in skinned muscle fibres (Lamb et al., 2000; Green et al., 2003).

Truncated peptides derived from the A region of the skeletal DHPR II-III loop have had varying effects on the RyR1. Peptides spanning residues located in the C-terminal end of peptide A (residues 681-690) has been shown to either activate the RyR1 (El-Hayek and Ikemoto, 1998; Lamb et al., 2000) or to have no effect on the activity of the RyR1 (Stange et al., 2001), while another peptide spanning residues 681-686, has been shown to have no effect on the RyR1 (El-Hayek and Ikemoto, 1998; Stange et al., 2001). Yet another peptide spanning the N-terminal end of peptide A (residues 671-680) has been also shown to activate the RyR1 (Stange et al., 2001).

The results obtained with the A1 (native skeletal A sequence) and A2 (Ser^{687} → Ala substitution) peptides indicated that a more structure C-terminal end of peptide A
(achieved through the Ser\textsuperscript{687} → Ala substitution) was responsible for an increase in
the activation of RyR1 (Casarotto et al., 2000). To further investigate the possibility
that RyR1 activation may depend on secondary structure, the structure and function of
five smaller peptides (A3, A4, A5, A6 and A9) derived from the native 20 amino
acids of the A peptide were investigated and the results are described in this chapter.

By dissecting the A peptide into smaller regions we have been able to further
investigate the importance of secondary structure as well as the presence of the five
critical positively charged residues (residues Arg\textsuperscript{681} – Lys\textsuperscript{685}) in the ability of the
peptide to activate the RyR.

Functional studies utilising single channel lipid bilayer and Ca\textsuperscript{2+} release from SR
vesicles techniques were conducted in our laboratory on the smaller A region peptides
(A3: R\textsuperscript{681} – L\textsuperscript{690}, A4: T\textsuperscript{671} – E\textsuperscript{680}, A5: K\textsuperscript{675} – R\textsuperscript{684}, A6: A\textsuperscript{678} – S\textsuperscript{687}, A9: R\textsuperscript{681} – S\textsuperscript{687}) by
Suzanne Curtis and Suzy Pace. From the single channel lipid bilayer studies, it was
determined that: peptides A1, A3, A5 and A6 were able to activate the RyR1 at a
potential of –40 mV when added to the cytoplasmic (cis) side of channel; the mean
current increase by A6 was twofold greater than that induced by peptide A5; the order
of peptides in activating the RyR incorporated in lipid bilayers was A1 > A6 > A5 >
A3 > A9 = A4; a decrease in channel activity at a potential of +40 mV was caused by
peptides A1, A3, A5, A6 and A9; and the order of peptides in blocking RyRs was A3
> A9 > A1 = A6 > A5 > A4. At a potential of +40 mV the direction of cation current
flow is the opposite of that at a potential of –40 mV, i.e. at +40 mV current flows
from cis to trans or cytoplasmic to lumenal side of the channel. In this situation it is
possible for the positively charged peptide added to the cytoplasmic side of the
channel to block the RyR channel by binding to charged residues located in the
channel pore. Studies monitoring Ca\(^{2+}\) release from SR vesicles in our laboratory produced slightly different results to those obtained using single RyR incorporated in lipid bilayers. Small amounts of Ca\(^{2+}\) release were noted with peptides A1, A3, A5 and A6. The order of peptides in releasing Ca\(^{2+}\) from SR was A1 = A6 > A3 > A5 > A4 = A9. This work forms part of two studies published in 1999 and 2000 (Dulhunty et al., 1999; Casarotto et al., 2000). The following studies explore the structural basis for the various functional effects reported.
3.2 Methods and Materials

3.2.1 Peptides Used

The peptides used within this chapter were as follows:

A1: $671\text{TSAQKAKAEERKRRKMSRGL}^{690}$
A2: $671\text{TSAQKAKAEERKRRKMARGL}^{690}$
A3: $691\text{RKRRKMSRGL}^{690}$
A4: $671\text{TSAQKAKAE}^{680}$
A5: $675\text{KAKAERKRR}^{684}$
A6: $678\text{AEERKRRKMS}^{687}$
A9: $681\text{RKRRKMS}^{687}$

3.2.2 NMR Structural Studies

NMR samples were dissolved to ~2 to ~3 mM in an H$_2$O solution containing 10% D$_2$O/90% H$_2$O. Peptides were adjusted to a pH of either 3 or 5 using HCL or NaOH. In the case of peptides A1, A2, A4, A5 and A6, two pH values were used to investigate the importance of the glutamate residues within these peptides. Glutamic acid residues have a pK$_a$ of = 4 and thus, at a pH value of 3, these residues will exist in the protonated form, that is, in a neutral state. At a pH of 5, the glutamate residues will be de-protonated and will contain a negatively charged side chain. Differences between the structural profile at the two different pH values, indicates that the ionization state of the glutamate side chains is involved in the structural stability of the peptide. NOESY, TOCSY and DQF-COSY spectra were obtained for the A3, A4, A5, A6 and A9 peptides at 278 K. Further TOCSY spectra were also obtained at 285
K and 298 K for the A4, A5 and A6 peptides for temperature change studies and ROESY experiments were obtained for the A3 and A9 peptides.

### 3.2.3 Circular Dichroism Studies

Samples were diluted to 25 µM for CD measurements, and the pH values adjusted to 3 and 6.5, with small additions of dilute HCl or NaOH. The various pH values were again used to determine the importance of the ionization state of the glutamate residues (see section 3.2.1). Blank samples containing H₂O were used to form a baseline, which was subtracted from each spectrum.

It should be noted that NMR was conducted at pH values of 3 or 5 and CD studies were conducted at a pH of 3 and 6.5. This was because in NMR, once the pH is raised closer to 7 there is a greater chance of the H⁺ exchanging out of the solution. This presents difficulties with obtaining NMR spectra and thus NMR studies were conducted at a pH of 3 and 5. There is no such consideration required in CD and therefore a higher, more physiological pH of 6.5 was used.
3.3 Results

3.3.1 NMR Structural Studies

3.3.1.1 The A1 Peptide

The amide hydrogen region of the NOE for the A1 peptide is illustrated in Figure 3.2 (Casarotto et al., 2000) and is presented here to enable comparisons between the smaller peptides utilized in this chapter. The presence of strong continuous amide NOE cross peaks between consecutive amino acids (NH$_i$ – NH$_{i+1}$) supports the presence of an alpha helical secondary structure. The A1 peptide contained continuous NH$_i$ – NH$_{i+1}$ NOE cross peaks from residue 1 up to residue 14 (labelled in Figure 3.2) (Casarotto et al., 2000).

Based on these findings and other information obtained from NMR studies the structure of the A1 peptide was found to contain a lengthy helical portion extending from residue 1 through to residue 15 (Casarotto et al., 2000). After residue 15, the A1 peptide becomes disordered (Casarotto et al., 2000). The average structure of peptide A1 is presented in section 3.1.
Figure 3.2 Amide-amide region of the $^1$H NOESY spectrum of peptide A1. Strong and continuous cross peaks between adjacent residues are shown and correspondingly labeled. Reprinted with permission from Casarotto et al. (2000).

3.3.1.2 The A2 Peptide

Similar to peptide A1, the structure of peptide A2 was previously determined using NMR spectroscopy by Casarotto and co-workers (2000). Peptide A2 differs from the A1 peptide by the single mutation of residue Ser$^{687}$→ Ala. Data obtained from NMR structural studies on the A2 peptide revealed that it also contained a large portion of alpha helix (Casarotto et al., 2000). Interestingly the removal of Ser$^{17}$, previously thought to be important in the peptides ability to activate the RyR1 (Lu et al., 1995), actually increased the stability of the helix and promoted a more regular helical shape in the A2 peptide (Casarotto et al., 2000). The 3D solution structure of peptide A2 is also shown in Figure 3.1 (Casarotto et al., 2000). Compared to peptide A1 the alpha
helical portion of peptide A2 is more uniform and regular particularly towards the C-terminal end of the peptide. The helical portion extends to residue 15 in peptide A2.

3.3.1.3 The A4 Peptide

The A4 peptide contains the 10 amino acids from the N-terminal end of peptide A (residues Thr$^{671}$ - Glu$^{680}$), the peptide did not activate or block RyR channels or Ca$^{2+}$ release. In an attempt to investigate the structural importance of two glutamate residues Glu$^{679}$ and Glu$^{680}$, NMR studies were conducted on peptide A4 at two pH values, 3 and 5 (see section 3.2.2). From NMR studies, NH$_i$ – NH$_{i+1}$ NOE cross peaks were evident at both pH 3 and pH 5, indicating the presence of some degree of alpha helical secondary structure within the A4 peptide (Figure 3.3). At pH 3, weak to intermediate NH$_i$ – NH$_{i+1}$ NOE cross peaks were detected between six residues (Figure 3.3 (i)). At pH 5, only 3 NH$_i$ – NH$_{i+1}$ NOE cross peaks were detected and the intensity of these cross peaks was lower than at pH 3 (Figure 3.3 (ii)), indicating a less structured peptide in the presence of negatively charged side chains on residues Glu$^{679}$ and Glu$^{680}$.

Additional information provided from NMR studies provided support for the small amount of alpha helical secondary structural present in the A4 peptide at pH 3. The proton chemicals shift index (CSI) values and coupling constants obtained for the A4 peptide are presented in Tables 3.1 (pH 3) and 3.2 (pH 5). Coupling constants determined at pH 3, did not provide conclusive evidence, but were suggestive of the presence of a small amount of alpha helical secondary structure (Table 3.1). Similarly, temperature change studies over a 20 degree temperature range, suggested the
possibility of some degree of secondary structure towards the C-terminal end of the peptide (Figure 3.4). The deviation of \( \alpha^1H \) chemical shift values from the values obtained for random coil structures were not conclusive in identifying any degree of secondary structure (Figure 3.4).

(i)

![NOESY spectrum of peptide A4 at pH 3](image)

(ii)

![NOESY spectrum of peptide A4 at pH 5](image)

*Figure 3.3 Amide-amide region of the NOESY spectrum of peptide A4 at (i) pH 3 and (ii) pH 5. Strong and continuous cross peaks are shown and correspondingly labeled.*
At a pH of 5, there was no evidence of secondary structure within the A4 peptide.

There were two coupling constants determined with values indicative of random coil structure (Table 3.2). Temperature change studies did not suggest any structural stability in the peptide (Figure 3.4) and the deviation of $\alpha$-H chemical shift values from the values obtained for random coil structures were not conclusive (Figure 3.4).

![Table 3.1 Proton assignment for peptide A4 (pH 3) at 278 °K](attachment:table_3.1.png)

![Table 3.2 Proton assignment for peptide A4 (pH 5) at 278 °K](attachment:table_3.2.png)
Figure 3.4 Summary of the sequential NH$_i$-NH$_{i+1}$ NOE connectivities, $^3J_{\text{NH-alH}}$ coupling constants, $\alpha^1H$ CSI and temperature coefficients at 5°C in 10%/90% D$_2$O/H$_2$O for the A4 peptide at pH 3 (left-hand image) and pH 5 (right-hand image). The NOE connectivities are indicated by horizontal black lines with thickness proportional to NOE intensity. Values of $^3J_{\text{NH-alH}} < 6$ Hz are indicated by ↓. Temperature coefficients $> 5.0$ ppb/k are represented by closed circles.

3.3.1.4 The A5 Peptide

Similar to the A4 peptide, the A5 peptide contained 10 amino acids from the skeletal DHPR. Peptide A5 residues spanned Lys$^{675}$ – Arg$^{684}$ and included 4 of the 5 positively charged residues previously shown to be critical for the activating ability of peptide A (see section 3.1). The A5 peptide caused a moderate activation of RyR channels and a small release of Ca$^{2+}$ from SR vesicles, but did not block the RyR1. Data obtained from NMR studies revealed the presence of a small amount of alpha helical secondary structure at both pH values investigated.
Figure 3.5 Amide-amide region of the NOESY spectrum of peptide A5 at (i) pH 3 and (ii) pH 5. Strong and continuous cross peaks are shown and correspondingly labeled.

The presence of 5 weak NH$_i$ – NH$_{i+1}$ NOE cross peaks was detected on both NMR spectra at pH 3 and pH 5 (Figure 3.5) for the A5 peptide. Proton chemical shift values and coupling constants are presented in Table 3.3 (pH 3) and Table 3.4 (pH 5). Results obtained from monitoring the change in NH chemical shift values over a 20 degree temperature range provided no evidence of secondary structure (Figure 3.6). Similarly, neither the deviation of $\alpha$-$^1$H chemical shifts from the CSI values of random coil structures (Figure 3.6), nor the value of the coupling constants, added any further support for the presence of alpha helical secondary structure. Thus it is concluded that the small amount of alpha helical secondary structure detected in the A5 peptide results from an unstable helical conformation of the peptide.
Figure 3.6 Summary of the sequential NH\textsubscript{1}-NH\textsubscript{1+1}. NOE connectivities, \( ^3 J_{\text{NH-CSI}} \) coupling constants, \( \alpha-^1H \) CSI and temperature coefficients at 5\(^\circ\)C in 10%/90% \( D_2O/H_2O \) for the A5 peptide at pH 3 (left-hand image) and pH 5 (right-hand image).

The NOE connectivities are indicated by horizontal black lines with thickness proportional to NOE intensity. Values of \( ^3 J_{\text{NH-CSI}} \) < 6 Hz are indicated by ↓.

Temperature coefficients > 5.0 ppb/k are represented by closed circles.

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>H(_{\alpha})</th>
<th>H(_{\beta})</th>
<th>Other</th>
<th>( ^3 J_{\text{NH-CSI}} ) (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys(^{675})</td>
<td>8.42</td>
<td>4.20</td>
<td>1.81, 1.76</td>
<td>H(<em>{\gamma}) 1.44/1.35, H(</em>{\epsilon}) 3.00</td>
<td></td>
</tr>
<tr>
<td>Ala(^{676})</td>
<td>8.50</td>
<td>4.27</td>
<td>1.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys(^{677})</td>
<td>8.35</td>
<td>4.21</td>
<td>1.81, 1.75</td>
<td>H(<em>{\gamma}) 1.43, H(</em>{\delta}) 1.67, H(_{\epsilon}) 3.00</td>
<td>6.2</td>
</tr>
<tr>
<td>Ala(^{678})</td>
<td>8.37</td>
<td>4.25</td>
<td>1.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu(^{679})</td>
<td>8.44</td>
<td>4.28</td>
<td>2.07, 1.99</td>
<td>H(_{\gamma}) 2.44</td>
<td></td>
</tr>
<tr>
<td>Glu(^{680})</td>
<td>8.41</td>
<td>4.27</td>
<td>2.00</td>
<td>H(_{\gamma}) 2.41</td>
<td></td>
</tr>
<tr>
<td>Arg(^{681})</td>
<td>8.47</td>
<td>4.27</td>
<td>1.84/1.77</td>
<td>H(<em>{\gamma}) 1.61, H(</em>{\delta}) 3.19</td>
<td>7.8</td>
</tr>
<tr>
<td>Lys(^{682})</td>
<td>8.37</td>
<td>4.25</td>
<td>1.82/1.76</td>
<td>H(<em>{\gamma}) 1.47, H(</em>{\delta}) 1.69, H(_{\epsilon}) 3.00</td>
<td>6.0</td>
</tr>
<tr>
<td>Arg(^{683})</td>
<td>8.42</td>
<td>4.27</td>
<td>1.79</td>
<td>H(<em>{\gamma}) 1.60, H(</em>{\delta}) 3.19</td>
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</tr>
<tr>
<td>Arg(^{684})</td>
<td>8.49</td>
<td>4.28</td>
<td>1.85/1.73</td>
<td>H(<em>{\gamma}) 1.69, H(</em>{\delta}) 3.20</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Proton assignment for peptide A5 (pH 3) at 278 °K.
Chapter 3 – Peptides A1: Variations and Fragments

3.3.1.5 The A6 Peptide

The A6 peptide corresponded to 10 amino acids located towards the C-terminal end of the A region (residues Ala$^{678}$ – Ser$^{687}$) and contained all 5 of the critical positively charged residues as well as Ser$^{687}$, previously shown to be important (see section 3.1).

The A6 peptide activated Ca$^{2+}$ release and single RyR channels and blocked single RyR channels at a holding potential of + 40 mV. The structural information obtained from NMR studies showed that the A6 peptide (at pH 5) contained some degree of alpha helical secondary structure. Ten weak NH$_i$ – NH$_{i+1}$ NOE cross peaks were identified on the NOESY spectrum (Figure 3.7 (ii)) and seven of the coupling constants obtained were < 6.0 (Table 3.6), indicative of alpha helix. Similarly temperature coefficients indicated the presence of secondary structure in seven of the ten amino acids (Figure 3.8) and $\alpha$-1H chemical shift values were suggestive of alpha helical secondary in six residues (Figure 3.8).

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>Hα</th>
<th>Hβ</th>
<th>Other</th>
<th>$J_{(NH_{i-1}H_i)}$ (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys$^{675}$</td>
<td>8.42</td>
<td>4.19</td>
<td>1.80/1.74</td>
<td>H$\gamma$ 1.47, H$\delta$ 1.66, H$\epsilon$ 3.00</td>
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<tr>
<td>Ala$^{676}$</td>
<td>8.52</td>
<td>4.27</td>
<td>1.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys$^{677}$</td>
<td>8.37</td>
<td>4.21</td>
<td>1.81/1.77</td>
<td>H$\gamma$ 1.49/1.45, H$\delta$ 1.68, H$\epsilon$ 3.00</td>
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</tr>
<tr>
<td>Ala$^{678}$</td>
<td>8.41</td>
<td>4.23</td>
<td>1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu$^{679}$</td>
<td>8.51</td>
<td>4.20</td>
<td>2.05</td>
<td>H$\gamma$ 2.41/2.34</td>
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</tr>
<tr>
<td>Glu$^{680}$</td>
<td>8.42</td>
<td>4.19</td>
<td>2.07</td>
<td>H$\gamma$ 2.41/2.34</td>
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</tr>
<tr>
<td>Arg$^{681}$</td>
<td>8.37</td>
<td>4.26</td>
<td>1.81/1.77</td>
<td>H$\gamma$ 1.62/1.68</td>
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<tr>
<td>Lys$^{682}$</td>
<td>8.28</td>
<td>4.24</td>
<td>1.85/1.78</td>
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<tr>
<td>Arg$^{683}$</td>
<td>8.39</td>
<td>4.27</td>
<td>1.87/1.79</td>
<td>H$\gamma$ 1.63/1.70, H$\delta$ 3.21</td>
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<tr>
<td>Arg$^{684}$</td>
<td>8.39</td>
<td>4.27</td>
<td>1.87/1.79</td>
<td>H$\gamma$ 1.63/1.70, H$\delta$ 3.21</td>
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Table 3.4 Proton assignment for peptide A5 (pH 5) at 278 °K
Figure 3.7 Amide-amide region of the NOESY spectrum of peptide A6 at (i) pH 3 and (ii) pH 5. Strong and continuous cross peaks are shown and correspondingly labeled.
In comparison to the results obtained at pH 5, there was little evidence for alpha helical secondary structure in the A6 peptide at pH 3. Two weak NH_{i}−NH_{i+1} NOE cross peaks were identified on the NOESY spectrum (Figure 3.7 (i)) and only three of the coupling constants obtained were < 6.0 (two of these were found to be 5.9) (Table 3.6). Temperature co-efficients indicated the presence of secondary structure in three of the ten amino acids residues (Figure 3.8) and α-1H chemical shift values were not suggestive of alpha helical secondary in six residues (Figure 3.8). Thus the A6 peptide contains more helical structure at a pH of 5 than at pH 3, indicating that the glutamate residues (Glu^{679} and Glu^{680}) are important for stabilizing the secondary structure within the peptide. These structures were the reverse of the A4 peptide where the presence of negative charged side chains on the glutamate residues at pH 5 prevented the peptide from adopting a more alpha helical structure (see section 3.3.1.3).

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>Hα</th>
<th>Hβ</th>
<th>Other</th>
<th>(J_{NH-Hα}(Hz))</th>
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<td>Ala^{678}</td>
<td>8.51</td>
<td>4.15</td>
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<td>Glu^{679}</td>
<td>8.64</td>
<td>4.26</td>
<td>2.08,2.01</td>
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<tr>
<td>Glu^{680}</td>
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<td>4.25</td>
<td>2.08,2.02</td>
<td>Hγ 2.46</td>
<td>5.9</td>
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<td>4.22</td>
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<td>4.24</td>
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<td>1.86,1.78</td>
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<td>1.84,1.79</td>
<td>Hγ 1.64, Hδ 3.20</td>
<td>6.6</td>
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<td>Hγ 1.44/1.48, Hδ 1.70, He 3.00</td>
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<tr>
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<td>2.16,2.03</td>
<td>Hγ 2.56/2.65</td>
<td>6.9</td>
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<tr>
<td>Ser^{687}</td>
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*Table 3.5 Proton assignment for peptide A6 (pH 3) at 278 °K*
Table 3.6 Proton assignment for peptide A6 (pH 5) at 278 °K

<table>
<thead>
<tr>
<th>Residue</th>
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<th>Hα</th>
<th>Hβ</th>
<th>Other</th>
<th>J_{(NH\text{-}aH)} (Hz)</th>
</tr>
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<td>Glu\textsuperscript{686}</td>
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<td>4.16</td>
<td>1.99/2.04</td>
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<tr>
<td>Glu\textsuperscript{680}</td>
<td>8.40</td>
<td>4.15</td>
<td>2.00/2.04</td>
<td>Hγ 2.33</td>
<td>5.8</td>
</tr>
<tr>
<td>Arg\textsuperscript{681}</td>
<td>8.20</td>
<td>4.14</td>
<td>1.84/1.71</td>
<td>Hγ 1.60, Hδ 3.25/3.17</td>
<td>5.4</td>
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<td>Lys\textsuperscript{683}</td>
<td>8.06</td>
<td>4.19</td>
<td>1.89/1.83</td>
<td>Hγ 1.51/1.40, Hδ 1.69, Hε 2.96</td>
<td>5.9</td>
</tr>
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<td>Arg\textsuperscript{685}</td>
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<td>4.21</td>
<td>1.85/1.71</td>
<td>Hγ 1.62, Hδ 3.20</td>
<td>6.6</td>
</tr>
<tr>
<td>Arg\textsuperscript{681}</td>
<td>8.26</td>
<td>4.23</td>
<td>1.83/1.71</td>
<td>Hγ 1.64, Hδ 3.19</td>
<td>5.3</td>
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<td>4.41</td>
<td>3.89/3.89</td>
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Figure 3.8 Summary of the sequential NH\textsubscript{i}-NH\textsubscript{i+1} NOE connectivities, $^3J_{NH\text{-}aH}$ coupling constants, $\alpha$-H CSI and temperature coefficients at 5°C in 10%/90% D\textsubscript{2}O/H\textsubscript{2}O for the A5 peptide at pH 3 (left-hand image) and pH 5 (right-hand image).

The NOE connectivities are indicated by horizontal black lines with thickness proportional to NOE intensity. Values of $^3J_{NH\text{-}aH} < 6$ Hz are indicated by ↓. Temperature coefficients > 5.0 ppb/k are represented by closed circles.
3.3.1.6 The A3 and A9 Peptide

Both, the A3 (Arg^{681} – Leu^{690}) peptide and the A9 (Arg^{681} – Ser^{687}) peptide contained the critical cluster of positively charged amino acids and the peptides were seen to cause the strongest block of the single RyR channel. The A3 peptide caused weak activation of the single RyR channel and Ca^{2+} release from SR vesicles, while the A9 peptide was not able to activate either the single RyR channel or Ca^{2+} release. NMR studies performed on peptide A3 and peptide A9 revealed that neither peptide contained any form of secondary structure. NH_{i} – NH_{i+1} crosspeaks were absent in the NOESY spectrum for both of these peptides. ROESY experiments confirmed these results were due to the presence of random coil secondary structure rather than zero signal detection. Similarly, the coupling constants obtained for both peptides, A3 and A9, indicated the presence of a random coil structure within each peptide (Table 3.7 and 3.8). While the values of the α-1H chemical shifts deviated from those obtained in CSI random coil structures, they did not consistently or conclusively suggest any other form of secondary structure (Figure 3.9).

<table>
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<tr>
<th>Residue</th>
<th>NH</th>
<th>Hα</th>
<th>Hβ</th>
<th>Other</th>
<th>J_{NH1 - H1} (Hz)</th>
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</thead>
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<tr>
<td>Arg^{681}</td>
<td>8.50</td>
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<td>1.81/1.74</td>
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<tr>
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<td>1.80/1.75</td>
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<td>1.76</td>
<td>Hγ 1.63, Hδ 3.21</td>
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<tr>
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<td>4.27</td>
<td>1.76/1.68</td>
<td>Hγ 1.46/1.40, Hδ 1.68, He 2.99</td>
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</tr>
<tr>
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<td>4.55</td>
<td>2.01/2.09</td>
<td>Hγ 2.62/2.55, He 2.00</td>
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</tr>
<tr>
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<td>3.87/3.87</td>
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<td>1.67/1.67</td>
<td>Hγ 1.60, Hδ 0.88/0.93</td>
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</table>

Table 3.7 Proton assignment for peptide A3 (pH 5) at 278 °K
Table 3.8 Proton assignment for peptide A9 (pH 3) at 278 °K

<table>
<thead>
<tr>
<th>Residue</th>
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<th>Hβ</th>
<th>Other</th>
<th>J_{NH\leftrightarrow} (Hz)</th>
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<td>7.0</td>
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</table>

Figure 3.9 Summary of the sequential NH_{i}-NH_{i+1} NOE connectivities, $^3J_{NH\leftrightarrow}$ coupling constants, $^1\alpha$ H CSI and temperature coefficients at 5°C in 10%/90% D_2O/H_2O for the A3 peptide (left-hand image) and the A9 peptide (right-hand image).

The NOE connectivities are indicated by horizontal black lines with thickness proportional to NOE intensity. Values of $^3J_{NH\leftrightarrow} < 6$ Hz are indicated by ↓.

Temperature coefficients > 5.0 ppb/k are represented by closed circles.

3.3.2 Circular Dichroism Studies

3.3.2.1 The A1 Peptide

Circular dichroism (CD) studies were performed on the A1 peptide to confirm the NMR studies previously published. As shown in Figure 3.10, the A1 peptide at both pH 3 and pH 5 contains minima and a maximum at values close to those known for
alpha helical proteins (see section 3.2.1). The A1 peptide at pH 5 contains a maximum at 188nm, a first minimum at 202nm and a second smaller minimum at 220nm. The A1 peptide at pH 3 contains a maximum at 188nm, a first minimum of 200nm and a second shallow minimum of 222nm. The maximum and minimum values and the depth of the second minimum obtained for pH 5 are closer to that seen with alpha helical proteins than the CD spectra at pH 3. At a pH of 5, the residues Glu\textsuperscript{679} and Glu\textsuperscript{680}, are de-protonated and contain negatively charged side chains. It appears that these negatively charged side chains are involved in stabilizing the helical structure, as some secondary structure is lost when they are in a neutral state (pH 3).

![Circular dichroism spectra for peptide A1](image)

**Figure 3.10** Circular dichroism spectra for peptide A1. Shown is a graph of molar ellipticity against wavelength for peptide A1 at pH 3 and pH 5.
3.3.2.2 The A2 Peptide

CD spectra for peptide A2 are shown in Figure 3.11. The first maximum is located between 186 – 190 nm on both of the spectra for peptide A2 at pH3 and pH 5. At a pH of 5, it was found that the A2 peptide contained minima at 203 nm and 220 nm. Similarly at a pH of 3, the A2 peptide contained minima at 203 nm and 219 nm. The depth of the minimum at 220 nm for pH 5 is suggestive a higher percentage of alpha helical secondary structure in the peptide at this pH, however the difference is quite small and the structure appears to be highly conserved for each pH value tested.

Interestingly, the spectra obtained with peptide A2 are closer to the standard values for alpha helical proteins compared to those found in peptide A1 (see section 3.2.1). These results provide more support for the three-dimensional solution structure solved by Casarotto et al. (2000) where the average structure of peptide A2 contained a larger portion of regular alpha helix compared to peptide A1. In contrast to peptide A1, there are only relatively small difference between CD spectra at pH 3 and pH 5 of peptide A2, demonstrating that the alpha helical secondary structure of peptide A2 is more defined at lower pH values compared with peptide A1. It may be that the importance of side chain – side chain interactions involving the glutamate residues are not as critical in alpha helical formation for peptide A2 compared to peptide A1.
Figure 3.11 Circular dichroism spectra for peptide A2. Shown is a graph of molar elipticity against wavelength for peptide A2 at pH 3 and pH 5.

3.3.2.3 The A4 Peptide

The CD spectrum for the A4 peptide (at pH 3) contained a minimum at 199 nm and second small minimum at 222nm, indicating a small proportion of the peptide to be alpha helical in structure (Figure 3.12). At pH 5, the first minimum was located at 197 nm and the second minimum was not as deep as that found for pH 3 (Figure 3.12). These results confirm the information obtained from NMR studies, which indicated the A4 peptide to contain little (pH 3) or no (pH 5) secondary structure.
Figure 3.12 Circular dichroism spectra for peptide A4. Shown is a graph of molar ellipticity against wavelength for peptide A4 at pH 3 and pH 5.

3.3.2.4 The A5 Peptide

The features of the spectra obtained for the A5 peptide did not resemble features obtained for highly structured proteins. This was not surprising, as NMR data had revealed the presence of very little secondary structure in the peptide. The first minimum was located at 197 nm for both pH conditions (Figure 3.13). Similarly the second minimum was not of substantial depth for either pH value (Figure 3.13). Peptides that are random coil in structure have been shown to have minimum at 197 nm, but are absent of the second minimum at 222 nm. Thus presence of the second
minimum does indicate the presence of a small amount of alpha helical secondary structure present at both pH 3 and pH 5.

![Circular dichroism spectra for peptide A5. Shown is a graph of molar ellipticity against wavelength for peptide A5 at pH 3 and pH 5.](image)

Figure 3.13 Circular dichroism spectra for peptide A5. Shown is a graph of molar ellipticity against wavelength for peptide A5 at pH 3 and pH 5.

3.3.2.5 The A6 Peptide

The CD spectrum obtained for the A6 peptide, at high pH, show that the A6 is the most structured of the truncated A region peptides investigated. At pH 5, the A6 peptide contained a positive region, suggestive of alpha helical secondary structure that was not detected with the A3, A4, A5 or A9 peptides. The first minimum was detected at 200 nm and the second minimum is clearly visible (Figure 3.14). These
features, which are prominent in alpha helical proteins, were not evident for the A6 peptide at pH 3. The first minimum was located at 196 nm and the second minimum was not as clearly defined (Figure 3.14).

![Figure 3.14 Circular dichroism spectra for peptide A6. Shown is a graph of molar ellipticity against wavelength for peptide A6 at pH 3 and pH 5.](image)

3.3.2.6 The A3 and A9 Peptide

Further evidence to suggest that peptide A3 and A9 are unstructured comes from the CD studies. Both peptides have a first minimum at approximately 195nm and in both cases there is the absence of any second prominent minimum at higher wavelengths. These results are consistent with peptides containing random coil structure and it implies that structurally these peptides have no helical content (Figure 3.15).
Figure 3.15 Circular dichroism spectrum for peptide A3 and peptide A9. Shown is a graph of molar ellipticity against wavelength for peptide A3 at pH 6.5 and peptide A9 at pH 6.5.
3.4 Discussion

The 3D solution structure obtained for the A1 and A2 peptides (Casarotto et al. 2000) showed the A2 peptide to contain a greater helical content compared to the A1 peptide. In CD studies a more pronounced minimum at 222 nm and a more positive region at 185 nm was observed for the A2 peptide, confirming these findings. However, due to the lack of well-defined helical regions within the A3, A4, A5, A6 and A9 peptides, the 3-D solution structures of the peptides were not determined. Rather a qualitative assessment of the level of helical content was made. The secondary structure possessed by these short peptides (<11 amino acids) was unusual, as most peptides of this length tend to adopt a random coil structure. Both NMR and CD spectra suggest that the A4, A5 and A6 peptides all adopt a nascent helix-type conformation, that is, an inter-converting mixture of random coil and structured peptide. This assertion is based on the presence NH$_i$ – NH$_{i+1}$ connectivities, indicative of alpha helical secondary structure, but the absence of weaker NOE connectivities synonymous with helical structures (that is, NH$_i$ – NH$_{i+2}$, $\alpha$H$_i$ – NH$_{i+3}$, $\alpha$H$_i$ – $\beta$H$_{i+3}$). Other NMR parameters such as $^3$J$_{NH-\alpha H}$ coupling constants, $\alpha$-H chemical shift index, and hydrogen bonding data also support the presence of helical secondary structure. Similarly, CD spectra showed that the A4, A5 and A6 peptides contained characteristics indicative of alpha helical secondary structure. Although the spectrum deviated from the profile obtained for an ideal helix, positive bands at 190 nm, minima at 197 – 200, and second minima at 220 – 222 nm were present in selected spectra. Taken together, NMR and CD indicators provide evidence for the presence of helix like characteristics for the A4, A5 and A6 peptides. From the data obtained at a
pH value of 5, the A6 peptide was seen to contain the largest amount of secondary structure followed by peptide A4 and lastly by the A5 peptide. There was no evidence of any secondary structure determined for the A3 and A9 peptides.

The importance of the glutamate interactions, determined in this study, were not consistent across the five structured peptides (A1, A2, A4, A5 and A6). The importance of the interaction was tested by investigating the structural characteristics of the peptides above and below the pKa value of glutamate (pKa = 4). The effects of pH on the spectra obtained revealed that the glutamate residues (Glu689 and Glu690) aid in the stabilization of the helix at high pH via side chain interactions between the glutamate residues (Glu679 and Glu680) and the lysine/arginine residues (Lys682-Arg684) for the A1, A2 and A6 peptides. The irregular helical structure of the A1 peptide is more susceptible to removal of the side chain-side chain interactions (which occur at pH 3), compared to the more regular helical conformation of peptide A2, suggesting that A1 relies more on these interactions to maintain its helical integrity.

Similarly, in the A6 peptide, the glutamate residues act to stabilize the helical content of the peptide at a high pH, most likely through an interaction with the positively charged residues. In the A4 peptide, the presence of negatively charged side chains on the glutamate residues (at high pH) actually destabilized the formation of secondary structure and a modest amount of helical character was only noted at low pH (pH 3) rather than high pH (pH 5). This may be due to interactions between the negatively charged glutamate side chains and the natural negative dipole of the C-terminal end of the helix (Vieille and Zeikus, 1996). In the A5 peptide, which contained both the glutamate and arginine/lysine residues, the ionized state of the glutamate side chains had no influence on the amount of secondary structure present. This may be explained
by destabilizing interactions between the natural dipole of the helix and the basic residues at the N-terminus of the peptide disrupting the glutamate - lysine – arginine interactions. Similar destabilizing interactions may also account for the absence of secondary structure within the A3 and A9 peptides. While the absence of glutamate residues in both peptides A3 and A9, may also lower the probability of these peptides adopting an alpha helical secondary structure.

Functional studies on these peptides have shown that the ability of a peptide to activate the RyR can vary slightly with the technique used to investigate the effect of the peptide. This is illustrated by the slightly different order of activation determined with Ca\(^{2+}\) release from SR vesicle and single channel lipid bilayer studies (Casarotto et al., 2001) (For channel activation A1 > A6 > A5 > A3 > A9 = A4, while for SR Ca\(^{2+}\) release A1 = A6 > A3 > A5 > A4 = A9). In Ca\(^{2+}\) release from SR vesicles studies, the A6 peptide and A1 peptide were equal in activating Ca\(^{2+}\) release and more effective than A3, which caused a greater activation than the A5 peptide (Casarotto et al., 2001). The A4 and A9 peptides did not cause any additional release of calcium above the baseline (Casarotto et al., 2001). From single channel lipid bilayer studies the A1 peptide was more effective at activating the RyR1 than then A6 peptide, the A6 peptide was > 2-fold more effective than peptide A5 and the A5 peptide was more effective than peptide A3. No increase in activity was noted with the A4 and A9 peptides (Casarotto et al., 2001). The differences noted between the two techniques are confined to those peptides that were able to initiate some form of activation of the RyR1. Nevertheless, the results from the two techniques were in agreement in that A1 and A6 were the most active peptides, while A4 and A9 were the least active peptides.
Results for the functional ability of the As-10 peptide (which is identical to the A3 peptide) have come from studies conducted by El-Hayek and Ikemoto (1998), Lamb et al. (2000) and Strange et al (2001). In these studies the As-10 peptide produced either a larger increase in the activity of the RyR1 compared to the A peptide (corresponding to the A1 peptide) (El-Hayek and Ikemoto, 1998; Lamb et al., 2000), or did not cause any activation of the RyR1. Both studies differ from our finding that A3 caused moderate activation of the RyR. Just as varying results have been obtained here through the use of varying techniques, some of the variation in the results obtained with peptides corresponding to the A3 peptide (Thr$^{671}$ - Glu$^{680}$) may also potentially be attributed to the different techniques used. El-Hayek and Ikemoto (1998) used ryanodine binding, Lamb et al. (2000) used mechanically skinned fibers and our laboratory used single channel bilayer studies. However, both El-Hayek and Ikemoto (1998) and our laboratory used Ca$^{2+}$ release from SR vesicles and conflicting results were obtained, although the stopped flow technique used by El-Hayek et al., differed from our spectrophotometric technique. Therefore preparation of SR vesicles, the Ca$^{2+}$ sensitive indicator and the spectrophotometric techniques differed and may have potential contributed to the discrepancies reported. Variations obtained from single channel bilayer studies performed by Stange et al. (2001) and in our laboratory may be explained by the amounts of free Ca$^{2+}$ present in each experiment. Single channel bilayer experiments conducted in our laboratory were in the presence of 100 µM free Ca$^{2+}$. Activation of the RyR1, after the addition of the peptide corresponding to the A3 peptide, was noted by Stange et al (2001) when using maximally activating Ca$^{2+}$ levels (20 µM). However, when using sub-maximally activated channels (in the presence of 0.1 µM Ca$^{2+}$), application of the peptide corresponding to peptide A3 had no effect on increasing the activation of the RyR1.
A block of RyR1 incorporated in lipid bilayers at a holding potential of +40 mV was caused by peptides A1, A3, A5, A6 and A9 (Dulhunty et al., 1999; Casarotto et al., 2001). Strongest block was seen with peptides A3 and A9, while a lesser degree of block was caused by peptides A1 and A6. The lowest level of block detected was from the A5 peptide, while the A4 and A7 peptide were not seen to block the RyR1 to any detectable extent. Thus, all peptides containing an overall positive charge blocked the RyR1 channel at +40 mV.

To identify the characteristics necessary to activate the RyR1, the structural and functional results are combined. The A6 peptide, which at pH 5 contained the largest amount of helical content of the truncated peptides investigated and the critical cluster of five positively charged residues, was the most active peptide. The A4 peptide, which was found to contain a small amount of helical structure but not any of the critical positively charged residues, did not cause any activation of the RyR1 over the concentration range tested. Peptide A5 contained a very small amount of alpha helical secondary structure and four of the five critical positively charged residues, was able to cause a small amount of activation. The A3 peptide contained all five of the critical positively charged residues but no secondary structure was still able to activate the release of Ca\(^{2+}\) through the RyR1. Similar structural characteristics found with the A9 peptide, which also contained the five critical positive charges located in a 7 amino acid peptide, were not seen to activate the RyR1. Thus from these correlations it can be concluded that activation of the RyR1 requires some portion of the critical cluster of positively charge amino acids; is decreased as fewer of these critical positive
Charged residues are present; is increased in the presence of a more structured peptide and requires a peptide of > 7 amino acids in length.

The functional / structural relationship, for the peptides shown to block the RyR1, show that the strongest block was found with the A3 and A9 peptides, which both contained five positively charged residues in an unstructured manner. Lower levels of block were detected with the A1 and A6 peptides, which both included five positively charged residues but restricted in some degree of secondary structure. The A5 peptide, which contains four of the five positive residues, displayed less block compared to the A1 and A6 peptides (Casarotto et al., 2001). No block was detected with the A4 or A7 peptides, which both contain only two positively charged residues (Casarotto et al., 2001). Similar to the activation of the RyR1, the block of the RyR1 appears to be dependent on the presence of several positively charged residues. However, in contrast to activation, block is enhanced in the presence of an unstructured peptide. It is thought that the block of the RyR1 may occur through the formation of multiple random interactions between positively charged residues and scattered negative charges located near the RyR pore (Dulhunty et al., 1999), first suggested for other positively charged K+ channel blockers by Mead et al. (Mead et al., 1998).

The results obtained confirm those of previous studies, which have suggested that the cluster of five positively charged residues (Arg<sup>681</sup> – Lys<sup>685</sup>) are critical for a peptides ability to activate the RyR1 (El-Hayek and Ikemoto, 1998; Dulhunty et al., 1999; Zhu et al., 1999). In addition to this, the structural studies that have been performed on the peptides derived from the skeletal DHPR II – III loop A region, have further revealed several important facts. Information from CD studies supported the previously
determined structures for the A1 and A2 peptides. For the truncated peptides, derived from the A region of the skeletal DHPR, the presence of some form of alpha helical secondary structure increased the ability of the peptide to activate the RyR1. In some cases (A1, A2 and A6), the formation of alpha helical secondary structure was assisted by the presence of Glu$^{679}$, Glu$^{680}$ / Lys$^{682}$ – Lys$^{685}$ interactions, suggesting an important role for the glutamate resides within these peptides. Block of RyR1 is a result of the positive charge density of the peptide and is more potent for unstructured peptides.
Chapter 4 - The A1(D-R18) Peptide

4.1 Introduction

Previously, the A region of the skeletal muscle DHPR II – III loop has been shown to be a strong activator of RyR1 (see section 1.2.6). In chapter 3, it was shown that a cluster of positively charged amino acids were critical for a peptide, derived from the A region of the skeletal DHPR, to activate the RyR1. In addition to this, the presence of alpha helical secondary structure increased the activation caused by the peptide.

Two 33 amino acid peptides from the venom of the scorpions, Imperatoxin A (IpTxa) and maurocalcine (Mca), has been used as an experimental probes for RyR activity (Figure 4.1), (el-Hayek et al., 1995b; Zamudio et al., 1997; Gurrola et al., 1999; Fajloun et al., 2000; Green et al., 2003). IpTxa is a very useful tool as it contains an amino acid sequence similar to the A region of the II-III loop of the skeletal DHPR (El-Hayek, et al., 1995a). At nanomolar concentrations, IpTxa increased the binding of ryanodine to skeletal SR (El-Hayek, et al., 1995b). In single channel lipid bilayer studies IpTxa increased the open probability of rabbit skeletal muscle RyRs by increasing the frequency of open events and decreasing the duration of the closed lifetimes (el-Hayek et al., 1995b; Zamudio et al., 1997; Gurrola et al., 1999; Green et al., 2003). This activating effect of IpTxa was dose-dependent, had a fast onset, and was fully reversible. The activating effect of IpTxa on the skeletal SR was calcium dependent and independent of other modulators of RyRs (el-Hayek et al., 1995b) and 1 µM was shown to elicit an average of 889 ± 87 nmol of Ca$^{2+}$ / mg of protein /min
(Green et al., 2003). However, IpTx₄ had negligible effects on tissues where the expression of skeletal type RyR isoforms were, small or altogether absent, outlining that its effects are specific for the RyR1 (el-Hayek et al., 1995b) and similar to those of peptide A (see section 1.2.6).

In 1999, the effects of IpTx₄ were investigated in conjunction with a synthetic peptide, corresponding to a segment of the skeletal II-III loop region (Glu⁶⁶⁶-Leu⁶⁹⁰). It was shown that both activated the RyR1 in a similar manner (Gurrola et al., 1999).

Mutations of the residue corresponding to Ser⁶⁸⁷ (Figure 4.1), previously found to be critical for activation elicited with the recombinant skeletal DHPR II-III loop (SDCL) (Lu et al., 1995), within either, IpTx₄ or the peptide segment Glu⁶⁶⁶ – Leu⁶⁹⁰, caused a decrease in affinity of binding and a decrease in the activation for both peptides, even though both peptides caused some activation of the RyR1 (Gurrola et al., 1999).

Another mutation within the critical cluster of positively charged amino acids resulted in even more dramatic effects. When the mutation Arg → Glu was performed in the residue corresponding to Arg⁶⁸⁴ (Figure 4.1), the activating ability of, IpTx₄ and the peptide segment Glu⁶⁶⁶ – Leu⁶⁹⁰, was completely abolished (Gurrola et al., 1999). Interestingly when a similar mutation (Lys → Glu) was performed on the residue corresponding to Lys⁶⁷⁵, activation was relatively unchanged (Gurrola et al., 1999), leading to the conclusion that the dramatic effect of the mutation within the critical cluster of basic amino acids (Arg⁶⁸⁴) was not solely attributable to a change in electrical charge of the peptides (Gurrola et al., 1999). Thus it appears that both peptides bind to RyRs via a structural domain consisting of a cluster of basic amino acids (Arg⁶⁸¹-Lys⁶⁸⁵ of the II-III loop and Lys¹⁹-Arg²⁴ of IpTx₄) followed by a hydroxylated amino acid (Ser⁶⁸⁷ of the II-III loop and Thr²⁷ of IpTx₄). Binding studies
and functional studies, indicated that both IpTx$_a$ and the peptide segment Glu$^{666}$ - Leu$^{690}$ were most likely competing for the same binding site on the RyR1 (Gurrola et al., 1999; Green et al., 2003).

The location of the binding site for IpTx$_a$ on the RyR1, was determined by Samso et al (1999). Difference mapping cyro-electron microscopy, showed that IpTx$_a$ binds to the RyR1, 11 nm away from the transmembrane pore, in between the handle and clamp regions (Figure 4.2) (Samso et al., 1999).

Since the discovery of IpTx$_a$, there has been another scorpion toxin identified which activates the RyR1. The second scorpion toxin shares an 82% homology to IpTx$_a$, also contains 33 amino acids and is known as maurocalcine (Figure 4.1) (Fajloun et al., 2000). Similar to IpTx$_a$, maurocalcine activates the RyR1 in single channel bilayer experiments (Fajloun et al., 2000). The three-dimensional solution structure of maurocalcine has been solved by $^1$H NMR (Mosbah et al., 2000). Interestingly, maurocalcine was the first scorpion toxin found to exhibit the Inhibitor Cystine Knot fold (ICK) which is present in numerous toxic and inhibitory peptides (Mosbah et al., 2000). It was shown that the structure consisted of a compact disulfide-bonded core from which emerged loops and the N-terminal end of the toxin (Figure 4.3) (Mosbah et al., 2000). Residues 20-23 and 30-33 were involved in a double-stranded antiparallel beta-sheet (Mosbah et al., 2000). Another extended strand spanning residues 9-11 was shown perpendicular to the beta-sheet (Figure 4.3) (Mosbah et al., 2000). The location of the cluster of positively charged amino acids (Lys$^{19}$ – Arg$^{24}$) was found to be along an externally exposed surface of a region involved in beta sheet secondary structure (refer to Figure 4.3).
A Region: \textsuperscript{666}EAESLTSAQKAKKEERK-RRKMSRGL\textsuperscript{690}

Iptx: \textsuperscript{1}GDCKPHLRCKADN-DCCG\textsuperscript{KK}KR\textsuperscript{RR}G\textsuperscript{TNAEKRCR}\textsuperscript{33}

Mca: \textsuperscript{1}GDCLPHLK\textsuperscript{LCK-ENKDCCSKK}KR\textsuperscript{RR}G\textsuperscript{TNI}EKR\textsuperscript{CR}\textsuperscript{33}

Figure 4.1 The amino acid sequence of the peptide segment from the A region of the DHPR used by Gurrola et al. (1999), IpTx\textsubscript{a} and maurocalcine. Areas of the sequence that are common to both toxins are in bold font. Critical cluster of positively charged residues are in red and residues corresponding to Ser\textsuperscript{687} are in blue.

Figure 4.2 The three-dimensional location of IpTx\textsubscript{a} on the RyR1. Images are shown from the T-tubule facing side (left-hand image), the SR-facing side (middle image) and the side view (right-hand image). Reprinted with permission from Samso et al. (1999).

A recent study conducted by Chen et al. (2003) investigated the hypothesis that maurocalcine and peptide A were acting at a common site on the RyR1. Maurocalcine (5 – 30 nM) released Ca\textsuperscript{2+} from SR vesicles, while peptide A (0.5 – 40 µM) enhanced Ca\textsuperscript{2+} loading into the SR and fully inhibit release of Ca\textsuperscript{2+} induced by either Ca\textsuperscript{2+}, caffeine or maurocalcine (Chen et al., 2003). Interestingly, these SR Ca\textsuperscript{2+} release studies were performed without the addition of thapsigargin, which has been shown to
block the Ca\(^{2+}\), Mg\(^{2+}\)-ATPase activity, thus making it very difficult to separate the effects of the reagents on the specific proteins. In the same study peptide A was shown to increase ryanodine binding demonstrating that it is increasing the open probability of the RyR1 (Chen et al., 2003). The mode of activation determined for maurocalcine and peptide A in Ca\(^{2+}\) release studies from SR vesicles, single channel bilayer studies and ryanodine binding assays were shown to be different in each case (Chen et al., 2003). Also, results obtained from surface plasmon resonance analyses indicated that the peptides did not interact by simple competition for a single class of mutually exclusive sites on RyR1 (Chen et al., 2003). Thus it is currently unclear if peptide A and the scorpion toxins, IpTx\(_3\) and maurocalcine, activate the RyR1 through binding to the same or different sites.

Figure 4.3 The 25 best molecular backbone structures of maurocalcine superimposed for best fit (left-hand side). The molscript ribbon drawing of the averaged minimized maurocalcine structure (right-hand side). Amino acids involved in disulphide bridges are depicted by ball and stick molecules and are numbered accordingly. Figures reprinted with permission from Mosbah et al., 2000.
However, as with the A peptide, it can be concluded that the ability of IpTxₐ and maurocalcine to activate the RyR₁ is dependent on a cluster of positively charged amino acids. The presence of the residue corresponding to Ser⁶₈⁷, has been shown to be not as critical for RyR activation as the cluster of positively charged residues (Dulhunty et al., 1999; Gurrola et al., 1999) and its importance appears to be directly correlated with the length of the peptide. It was found that the presence of Ser⁶₈⁷ decreased the activating ability of the 20 amino acid A peptide and increased the activation caused by peptides ranging in length from 25 – 33 amino acids. The presence of Ser⁶₈⁷ was found to be essential for activation by the full length II–III loop recombinant protein (126 amino acids) (Dulhunty et al., 1999; Lu et al., 1995; Gurrola et al., 1999).

Although IpTxₐ and a peptide derived from the A region of the skeletal DHPR have been shown to bind to the same site on the RyR (Gurrola et al., 1999; Green et al., 2003), IpTxₐ activates RyR₁ at nanomolar concentrations, while peptide A requires micromolar concentrations. Therefore the aim in this section was to modify the positively charged residues on peptide A₁ to resemble the electrostatic surface properties, found in the scorpion toxins, to create a more potent RyR activator. It has been shown that the Ser⁶₈⁷ → Ala mutation within peptide A₁ (to yield peptide A₂) has stabilized the helical structure of the peptide (see section 3.3.2.2) and modestly enhanced the peptide’s ability to activate the RyR₁ (Casarotto et al., 2000). It was thought that this enhancement occurred via an increased alignment of positively charged residues along one surface of the helix which occurred because of the more regular helical formation towards the C-terminal end of the peptide (Casarotto et al.,
2000). Thus to mimic the charged surface of the scorpion toxins the chirality of Arg<sup>688</sup> was altered, from the L - isomer in peptide A1 to the D - isomer, to produce a new peptide named A1(D-R18). The residue Arg<sup>688</sup> was targeted because its position was three residues downstream from the cluster of critical positively charged residues and therefore after modulation, could potentially be located on the same surface as several of the positively charged residues spanning Arg<sup>680</sup> - Lys<sup>685</sup>. Functional studies investigating the ability of the A1(D-R18) peptide to activate the RyR1 have been conducted in our laboratory. It was found that the A1(D-R18) peptide is substantially more active than either the A1 or A2 peptides (Green et al., 2003). The A1(D-R18) peptide was shown to activate single RyR1 channel incorporated in lipid bilayers (holding potentials of −40 mV and +40 mV) at nanomolar concentrations (Green et al., 2003). The addition of 33 µM peptide A1(D-R18) caused an average release of 332 ± 87 nmol of Ca<sup>2+</sup> / mg / min from the SR vesicles (see Figure 4.3A) and 10 µM peptide A1(D-R18) potentiated the caffeine induced force response from skinned muscle fibers to near maximal force in every fiber (Green et al., 2003).

![Figure 4.3A](image-url) *Figure 4.3A Average initial rates of Ca<sup>2+</sup> release (nmol / mg of protein in the SR vesicle / per minute) induced by the A1 and A1(D-R18) peptide at various concentrations.*
4.2 Methods and Materials

4.2.1 The peptide used

A1 (D-R18): $^{671}$TSAQKAKAEERKRKMSR$^{690}$

4.2.2 NMR Structural Studies

The A1(D-R18) peptide was dissolved to ~2 to ~3 mM in an H$_2$O solution containing 10% D$_2$O/90% H$_2$O and adjusted to a pH of 5. NMR studies were performed at 278 K and 298 K. Two-dimensional NOESY, DQF-COSY and TOCSY spectra were obtained and the 3D solution structure of the A1(D-R18) peptide was determined.

4.2.3 Circular Dichroism Studies

The A1(D-R18) peptide was diluted to 25 μM for CD measurements, and the pH values adjusted to 5, with small additions of dilute HCl. Studies were conducted at 298 K. Blank samples containing H$_2$O were used to form a baseline, which were then subtracted from each spectrum.
4.3 Results

4.3.1 NMR Structural Studies

The three-dimensional solution structure of the A1(D-R18) peptide revealed a substantial increase in helical content compared to the A1 peptide (see section 3.1). The NH – NH and NH - αH regions of the NOESY spectrum obtained for the A1(D-R18) peptide are shown in Figure 4.4, the fingerprint region of the TOCSY spectrum are shown in Figure 4.5 and the chemical shift values, temperature coefficients and \(^3J_{\text{NH-αH}}\) coupling constants are presented in Table 4.1. From the NOESY spectrum, NH\(_i\)-NH\(_{i+1}\) NOE connectivities can be traced throughout the whole length of the peptide (see Figure 4.4). This information, coupled with a large number of other NOE connectivities indicative of alpha helical secondary structure (including αH\(_i\) – NH\(_{i+1}\), βH\(_i\) – NH\(_{i+1}\), NH\(_i\) – NH\(_{i+2}\), αH\(_i\) - βH\(_{i+3}\), αH\(_i\) – NH\(_{i+3}\) (Figure 4.6), outline the large percentage of helical structure present in the A1(D-R18) peptide. Further support for the presence of a large amount of helical structure comes from other NMR data. Chemical shift values of αH’s were found to be representative of alpha helical structures in 7 out of the 20 amino acids (Figure 4.6), while coupling constants < 6 Hz were determined for 10 residues (Table 4.1 and Figure 4.6) and temperature coefficients containing a chemical shift of > -5 ppb/K were identified in 14 residues (Table 4.1 and Figure 4.6). It should be noted that the temperature coefficients determined in the first three residues of the N-terminal or C-terminal end of a peptide containing alpha helical secondary structure are not always accurate indicators of the secondary structure. This could be largely due to the presence of a single set of hydrogen bonds in the first three residues, while other residues forming part of the helical structure will have two sets of hydrogen bonds, both upstream and downstream.
Figure 4.4  NH - αH region of the NOESY spectra (a) and NH –NH region of the NOESY spectra (b) for the A1(D-R18) peptide. Cross peaks corresponding to adjoining amino acids have been shown and labeled.
Figure 4.5 Finger Print region of the TOCSY spectra for the A1(D-R18) peptide.
Table 4.1: Proton assignment (p.p.m.), coupling constants (Hz) and Temperature coefficients (p.p.b./K) for the A1(D-R18) peptide at pH 5.0 and 278 K.

The 222 NOE cross peaks detected within the NOESY spectrum for the A1(D-R18) peptide were used together with the known sequence to construct a series of 100 possible 3D structures for the peptide and the 20 lowest energy structures were selected from these. The summary of constraints, structural statistics and atomic root mean squared deviations (rmsd) for the family of 20 lowest energy structures are given in Table 4.2. The number of NOEs determined for the A1(D-R18) peptide was very similar to that of the A1 peptide, while the atomic rmsd for all residues in the ensemble compared to the average and the Xplor energies, were both seen to be lower in the A1(D-R18) peptide, compared to the A1 peptide (Table 4.2) (Casarotto et al., 2000).
Figure 4.5 Summary of the sequential NH$_2$-NH$_{i+1}$, NOE connectivities, $^3J_{\text{NH-aH}}$ coupling constants, $\alpha$-$^\text{II}$ CSI and temperature coefficients at 5°C in 10%/90% D$_2$O/H$_2$O A1(D-R18) peptide. The NOE connectivities are indicated by horizontal black lines with thickness proportional to NOE intensity. Values of $^3J_{\text{NH-aH}} < 6$ Hz are indicated by ↓. Temperature coefficients $>5.0$ ppb/K are represented by closed circles. For the CSI, a value of +1 or −1 is assigned for residues whose $\alpha$-H chemical shift deviates from tabulated random-coil values by more than 0.1 ppm, downfield and upfield, respectively.

The violations of constraints and root mean square deviations for each of the 20 lowest energy structures obtained for the A1(D-R18) peptide are shown in Table 4.3. Only 5 of the 20 lowest energy structures produced violations of NOE constraints and all of the lowest energy structures had rmsd’s of < 190 kcal mol$^{-1}$ (Table 4.3).
<table>
<thead>
<tr>
<th>Restraints</th>
<th>Peptide A1-D18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NOEs</td>
<td>222</td>
</tr>
<tr>
<td>Intra</td>
<td>108</td>
</tr>
<tr>
<td>$i-(i+1)$</td>
<td>58</td>
</tr>
<tr>
<td>$i- (i&lt;1)$</td>
<td>56</td>
</tr>
<tr>
<td>$^3J_{\text{NH-Ha}}$</td>
<td>12</td>
</tr>
<tr>
<td>Hydrogen Bonds</td>
<td>5</td>
</tr>
<tr>
<td>Atomic rmsd backbone</td>
<td></td>
</tr>
<tr>
<td>(ensemble versus average)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>2.66 ± 0.56</td>
</tr>
<tr>
<td>Residues 671-686</td>
<td>2.35 ± 0.61</td>
</tr>
<tr>
<td>Residues 674-690</td>
<td>4.58 ± 0.86</td>
</tr>
<tr>
<td>rmsd from experimental restraints</td>
<td></td>
</tr>
<tr>
<td>NOE</td>
<td>0.022 ± 0.013</td>
</tr>
<tr>
<td>Dihedral restraints</td>
<td>0.33 ± 0.32</td>
</tr>
<tr>
<td>rmsd from idealized covalent geometry</td>
<td></td>
</tr>
<tr>
<td>Bonds</td>
<td>0.0020 ± 0.001</td>
</tr>
<tr>
<td>Angles</td>
<td>0.51 ± 0.058</td>
</tr>
<tr>
<td>Improper dihedral angles</td>
<td>0.41 ± 0.080</td>
</tr>
<tr>
<td>Xplor energies (kcal mol$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>$E_{\text{tot}}$</td>
<td>37.56 ± 7.45</td>
</tr>
<tr>
<td>$E_{\text{NOE}}$</td>
<td>3.22 ± 1.73</td>
</tr>
<tr>
<td>$E_{\text{CDIH}}$</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Ramachandran analysis</td>
<td></td>
</tr>
<tr>
<td>Most favoured regions (%)</td>
<td>76.5</td>
</tr>
<tr>
<td>Additionally allowed regions (%)</td>
<td>17.6</td>
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<tr>
<td>Generously allowed regions (%)</td>
<td>5.9</td>
</tr>
<tr>
<td>Disallowed regions (%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.2 Summary of restraints, structural statistics and atomic root mean square deviations for the family of 20 calculated lowest energy structures of the A1(D-R18) peptide. Where applicable means ± SD are shown.
Table 4.3 Violations of constraints and the root mean square deviation (kcal mol\(^{-1}\)) for the family of 20 calculated lowest energy structures of the A1 – D18 peptide.

The 20 lowest energy structures were overlaid in various formations (Figure 4.7) and were used to construct an average structure for the A1(D-R18) peptide (Figure 4.8).

The conformation of the average structure was seen to contain and high degree of alpha helical secondary structure, as was expected from the derived NMR data. Using the Insight II program (accelrys), it was possible to overlay particular residues from the 20 lowest energy structures to identify structural characteristics consistent across the lowest energy structures. When the first 16 residues for the peptide were overlaid there was a remarkable similarity in the structure between residues 4 – 16, while the C-terminal end of the peptide appeared to be unrestrained (Figure 4.7). These findings are not dissimilar to those obtained with the A1 peptide, however, the C-terminal end of the A1 peptide appears to be even less restrained than that seen with the A1(D-R18) peptide (Casarotto et al., 2000). When residues 4-20 were overlaid the consistent helical portion of the peptide was seen in all of the structures and appeared to extend from residues 6 - 19, while the N-terminal end of the peptide that had not been overlaid adopted several different conformations (Figure 4.7). From the average...
structure of the A1(D-R18) peptide presented in Figure 4.8 it can be seen that the alpha helical region in the A1(D-R18) peptide extends virtually the whole length of the peptide compared to only to residue 15 in the A1 peptide (see section 3.1). This difference between the conformation of the two peptides is quite remarkable considering the only difference is the substitution of residue D-Arg^{688} for L-Arg^{688}.

![Figure 4.7 Best fit superposition of heavy backbone atoms of the 20 lowest energy structures for A1-d18 for (a) residues 1-16 and (b) residues 4-20.](image-url)
4.3.2 Circular Dichroism Studies

Circular Dichroism studies provided additional support for the NMR 3D structure obtained for the A1(D-R18) peptide. The spectrum contained a large positive band at approximately 185 – 192 nm, a minimum at 204 nm and a second large minimum at 222 nm, all indicators of alpha helical secondary structure (Figure 4.9). The spectrum obtained for the A1 peptide at pH 5 (see section 3.3.2.1) has also been plotted on Figure 4.9 as a comparison. By comparing the two spectra it is evident, from the strength of the positive band between 185 – 192 nm, the shift of the first minimum

Figure 4.8 The representative average structure for the A1-D18 peptide, derived from the 20 lowest energy structures with the crucial positively charged residues in green.
from 202 nm – 204 nm and the depth of the second minimum, that the A1(D-R18) peptide contains a larger portion of alpha helical secondary structure compared to the A1 peptide (see section 2.2.2).

![Circular Dichroism spectrum for A1 and A1(D-R18) at pH 5 and 298 K.](image)

*Figure 4.9 Circular Dichroism spectrum for A1 and A1(D-R18) at pH 5 and 298 K.*
4.4 Discussion

The A1(D-R18) peptide was developed in an attempt to mimic the positively charged surface found with the scorpion toxins IpTx₄ and maurocalcine and contains the sequence of the A1 peptide with the D-, rather than L-, isomer for Arg₆₈₈. The structural studies performed on the A1(D-R18) peptide have revealed a large percentage of alpha helical secondary structure, extending the full length of the peptide, indicating the substituted Arg₆₈₈ residue is now an active part of the helix. In contrast, the A1 peptide was found to contain a helical portion extending from residues 1 – 15 (Casarotto et al., 2000). These findings provide evidence for significant secondary structural effects that can be caused by very small changes in primary structure. Structural changes resulting from the Ser₆₈₇ → Ala mutation of the A1 peptide (that is, peptide A2) have already been described in sections 3.3.1.2 and 3.3.2.2, but appear small compared with those seen with the A1(D-R18) peptide. In structural studies involving alpha helical peptides, the substitution of D-amino acids towards the center of the peptides has been shown to destabilize the secondary structure, while substitution towards the termini have had a lesser effect (Hong et al., 1999). In this study, the substitution of the D-isomer for Arg₆₈₈, had the unusual effect of stabilizing the helix at the C-terminal end of the peptide. It is known that the presence of positively charged residues located towards the C-terminal end of a helical peptide will stabilize the helical nature of the peptide due to the interaction between the positive amino acid charge and the negative dipole inherent in the helix (Vieille and Zeikus, 1996). Therefore, it is quite conceivable that altering the
stereochemistry of Arg$_{688}$, located close to the C-terminus, has promoted the extension of the helical region of the peptide.

Functional studies investigating the ability of the A1(D-R18) peptide to activate the RyR1 have been conducted in our laboratory. It was found that the A1(D-R18) peptide is substantially more active than either the A1 or A2 peptides (Green et al., 2003). The A1(D-R18) peptide was shown to activate single RyR1 channel incorporated in lipid bilayers (holding potentials of $-40 \text{ mV}$ and $+40 \text{ mV}$) at nanomolar concentrations (Green et al., 2003). The addition of 33 µM peptide A1(D-R18) caused an average release of $332 \pm 87 \text{ nmol of } \text{Ca}^{2+}/\text{mg/ min}$ from the SR vesicles and 10 µM peptide A1(D-R18) potentiated the caffeine induced force response from skinned muscle fibers to near maximal force in every fiber (Green et al., 2003).

A number of findings from the single channel studies with peptide A1(D-R18) and IpTx$_a$ have confirmed the engineering of a peptide which mimics the activation of the RyR1 caused by the scorpion toxins, IpTx$_a$ and maurocalcine. The gating of single RyR channels incorporated in lipid bilayers ($-40 \text{ mV}$ and $+40 \text{ mV}$) in the presence of peptide A1(D-R18) (100 nM – 10 µM) were compared to those caused by IpTx$_a$ (10 – 100 nM) and in both cases there was a large 10 – 100 fold reduction in the mean closed time with only a small (approximately 2 fold) increase in mean open time (Green et al., 2003). Similarly, the addition of IpTx$_a$ to single channels, which were already activated by the addition of 100 nM peptide A1(D-R18), did not have any additive effect on channel activity (which would be expected if the peptide and toxin activated the RyR by binding to different sites) even though channel activity in all
experiments remained low ($P_0$ was well below 1.0 and mean current was well below the 12 - 15 pA expected if $P_0$ were close to 1.0) (Green et al., 2003).

Figure 4.10 Ball representation of the positively charged side chains of residues spanning Lys$^{677}$ - Arg$^{688}$ for the 20 lowest energy NMR structures of the A1, A2 and A1(D-R18) peptides. The Figure shows side (left-hand images) and top (right-hand images) views. The color code is as follows: Lys$^{677}$ – brown; Arg$^{681}$ – blue; Lys$^{682}$ – green; Arg$^{683}$ – yellow; Arg$^{684}$ – black; Lys$^{685}$ – purple and Arg$^{688}$ – red.
From the studies described in chapter 3 of this thesis, it is clear that the ability of peptides derived from the A region of the DHPR II-III loop to activate the RyR1 increased with the degree of alpha helical secondary structure, as long as the peptide contained the cluster of 5 critical positively charge residues. The experiments described in this chapter show that A1(D-R18) has a greater amount of helical structure than A1. This is consistent with experiments done by others in the laboratory, which show that the modified peptide has a stronger activating action. By investigating the alignment of the positively charged residues (spanning Lys$^{677}$ – Arg$^{690}$) in the 20 lowest energy structures of the A1, A2 and A1(D-R18) peptides, it was discovered the A1(D-R18) peptide contained a more consistent and regular alignment of positive charges compared to the A1 and A2 peptides. This can be seen in Figure 4.10 where the positively charged side chains of residues Lys$^{677}$ – Arg$^{688}$ for the 20 lowest energy structures are represented by colored balls. The top view of the A1(D-R18) peptide clearly identifies a hollow passage through the center of the peptide, an indication of the regular alpha helical secondary structure found in the peptide. This clear central pore is not found in the A1 or A2 peptides (Figure 4.10). From the side view it can be seen that the positively charged side chains are aligned along one edge on the A1(D-R18) peptide, whilst being more evenly distributed around the whole surface of the A1 and A2 peptides.

Thus by substituting the L-isomer for the D-isomer of Arg$^{688}$ a peptide was created that appears to mimic the activation of the RyR1 caused by the scorpion toxins, IpTx$_3$ and maurocalcine. This occurred through the increased stability of the secondary structure of the peptide and the alignment of a series of positively charged residues.
along one surface of the peptide. The similarity of IpTxα and the A1(D-R18) peptide were confirmed with single channel bilayer studies, which demonstrated that both peptides activated the RyR1 in a very similar fashion. Also the absence of additive activation affects indicated that both, IpTxα and the peptide A1(D-R18) were activating the RyR1 at the same binding site.
Chapter 5 - The AB peptide

5.1 Introduction

In the previous chapters the ability of the A peptide derived from the skeletal muscle DHPR to activate the RyR1 has been outlined (chapters 1, 3 and 4). Briefly, it has been found that the A peptide, or derivatives of the A peptide, are able to: (i) activate a single channel RyR1 incorporated in artificial bilayers (Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Casarotto et al., 2000; Casarotto et al., 2001; Stange et al., 2001); (ii) cause an increase in ryanodine binding (el-Hayek et al., 1995b; El-Hayek and Ikemoto, 1998; Gurrola et al., 1999); (iii) activate release of calcium from sarcoplasmic reticulum vesicles (el-Hayek et al., 1995b; El-Hayek and Ikemoto, 1998; Dulhunty et al., 1999; Zhu et al., 1999; Green et al., 2003); and (iv) potentiate the Ca$^{2+}$ activated force in skinned muscle fibers (Lamb et al., 2000; Green et al., 2003).

In contrast to the A region, the B region of the skeletal DHPR II-III loop has not been studied in any great detail. The name “B region” was coined by El-Hayek et al. in 1995 and refers to residues Thr$^{694}$ - Val$^{722}$ of the skeletal DHPR. It was found that the B peptide did not cause calcium release from rabbit skeletal muscle triads, nor increase ryanodine binding (el-Hayek et al., 1995b). Also, in the presence of the A peptide, the B peptide did not reduce or interfere with the activation elicited by the A peptide (el-Hayek et al., 1995b). However the SDCLf1 peptide, comprising the A region and B region plus an additional 9 residues (Glu$^{666}$ - Glu$^{726}$) increased [$^3$H] ryanodine binding (Lu et al., 1995).
Another region that has been investigated, the s31 region (Glu$^{666}$ - Pro$^{709}$) (Proenza et al., 2002) is of great interest to the peptide studies described in this chapter, as it spans virtually the identical region to the AB peptide. Proenza et al (2002) found that there was no significant interaction between the s31 region and R10 (residues 1633-2635) or R9 (2636-3720) of the RyR1 (Proenza et al., 2002). In that study they utilized the yeast two-hybrid system, where portions of the skeletal DHPR II-III loop were expressed as fusion proteins with the binding domain of the CAL4 transcription factor (Proenza et al., 2002). Other RyR fusion proteins of approximately 300 amino acids in length, corresponding to portions of the R9 (2659-3720) and R10 regions (1635-2636) of the RyR1 were also expressed (Proenza et al., 2002). Interactions between pairs of these proteins were evident by the formation of colonies after yeast co-transfection (Proenza et al., 2002). Areas proven to have a strong interaction such as the alpha-interaction and beta-interaction domains showed colony formation after 2-4 days (see section 1.2.4) (Proenza et al., 2002). The only segment of the skeletal II-III loop found to interact with the RyR1 using this method was the s53 region (residues 719 - 767). The portion of the RyR1 that the s53 region was found to interact with is the sR16 region (residues 1837 - 2168) (Proenza et al., 2002). It is interesting to note that colonies only appeared after 7-10 days, indicating that the interaction between s53 and sR16 is relatively weak (Proenza et al., 2002).

Investigations into the relationship between structure and function can provide information regarding the mechanism underlying many biological processes. In the case of ECC the ultimate goal is to assess the structure / function relationship of the whole II – III loop, which has been shown to be critical for eliciting skeletal type ECC
(see section 1.2.5). Since the structure / function relationship of the A region had been
determined (chapters 3 and 4), it was a logical step to assess the structure / function
relationship of the A region plus the next 20 amino acids downstream of the A region,
by synthesizing the AB peptide (spanning DHPR residues 671 – 709). The next 20
amino acids were chosen as a peptide of 40 amino acids is close to the limit of \(^1\)H
NMR and of peptide synthesis. The structure of the II–III loop will be investigated in
the future when successful labeling of \(^{13}\)C and \(^{15}\)N within the 126 residue recombinant
peptide is obtained and is beyond the scope of this thesis.

Initial functional studies using the AB peptide showed that it was surprisingly inactive
on the activity of the RyR1 and two mutant peptides were designed to determine the
reason for this inactivity and also to reveal the structure / function relationship of
these peptide. Thus two mutant peptides were the AB-link and AB-ch. In the AB-ch
peptide four of the negatively charged residues from the C-terminal half of the AB
peptide were replaced with neutral residues (AB-ch mutations: Asp\(^{692}\) \to\) Ala; Glu\(^{695}\)
\to\) Ala; Glu\(^{696}\) \to\) Val; Glu\(^{697}\) \to\) Ala). The second mutant, AB-link, was designed to
restrict the flexibility found within the linker region (see section 5.3.1.1). The
flexibility of the linker region was reduced to remove a potential masking of
positively charged residues that may occur because of interaction with negatively
charged residues in the native AB peptide (AB-link mutations: Pro\(^{691}\) \to\) Ala and
Lys\(^{693}\) \to\) Ala). The ability of the AB, the AB-ch and the AB-link peptides to activate
RyR channels was determined by examining Ca\(^{2+}\) release from SR vesicles and the
structure of the peptides determined by structural NMR and CD.
5.2 Methods

5.2.1 Materials

The sequence of the AB, AB-ch and AB-link peptides used in this chapter are as follows (mutations of the native sequence are in bold):

The AB peptide

\[ ^{671}\text{TSAQKAKAEERKRRKS}^{710}\text{RLPDKTEEEKSVM}^{710}\text{AKKL}^{710}\text{SQPK}\]

The AB-ch Peptide

\[ ^{671}\text{TSAQKAKAEERKRRKS}^{710}\text{RLP}^{710}\text{AKTAV}^{710}\text{KSVM}^{710}\text{AKKL}^{710}\text{SQPK}\]

The AB-link Peptide

\[ ^{671}\text{TSAQKAKAEERKRRKS}^{710}\text{RL}^{710}\text{AD}^{710}\text{A}^{710}\text{TEE}^{710}\text{KSVM}^{710}\text{AKKL}^{710}\text{SQPK}\]

5.2.2 NMR Structural Studies

The AB peptide was dissolved at ~2-3 mM in either 10\% D\textsubscript{2}O/90\% H\textsubscript{2}O or 18 \% trifluoroethanol (TFE) \((v/v)\) and pH adjusted to a value of 5. For the AB peptide, TOCSY spectra were acquired at 278 K, 288 K or 298 K while NOESY spectra were only obtained at 298 K. For the AB-ch and AB-link peptide TOCSY and NOESY spectra were obtained at 298 K.

TFE was used to assist in stabilizing the secondary structure within the AB peptides. TFE has been shown to stabilize a variety of secondary structures in peptides.
including: beta sheet (Mutter et al., 1985); beta-turn (Blanco et al., 1994); and alpha helix (Lu et al., 1984; Nelson and Kallenbach, 1989; Segawa et al., 1991). The dielectric constant of TFE is approximately one-third that of water and the stabilization of secondary structure is due to the decreased hydrogen bonding of amide protons to the solvent, resulting in increased intra-molecular hydrogen bonds (Nelson and Kallenbach, 1986; Sonnichsen et al., 1992). However, caution should be taken with the use of TFE as high concentrations, (> 30 % v/v) have been shown to induce new conformations in peptides (Wray et al., 1998; Demarest and Raleigh, 2000; Salinas et al., 2002). TFE has also been shown to denature quaternary structures by disrupting hydrophobic protein interactions (Lau et al., 1984; Slupsky et al., 1995a). The use of TFE at low concentrations (< 20 % v/v) stabilizes the secondary structure of peptides without causing substantial structural changes (Buck et al., 1993; Slupsky et al., 1995a; Slupsky et al., 1995b).

Due to the high degree of overlap of chemical shift values produced from $^1$H NMR studies with peptides of over 35 amino acids, the investigation of the AB peptides using NMR was assisted by the addition of 18 % TFE (v/v). As discussed above, this percentage of TFE assists in stabilizing the secondary structure that is present in a peptide without inducing new conformations. This increased stability of the secondary structure present in the AB peptide created a greater dispersion of cross peaks with less overlap and assisted in the assignment of residues.
5.2.3 Circular Dichroism Studies

Samples were diluted to 25 µM for CD measurements. Two samples were made up for each peptide, one containing 18% TFE (v/v) and one without TFE. Blanks, containing either H₂O or H₂O and 18% TFE, were used to obtain a baseline which was subtracted from each spectrum.

5.2.4 SR Vesicle Release Studies

The effects of the AB peptide were assessed at 20 µM, and 60 µM. The AB-link peptide was tested at 10 µM, 30 µM and 60 µM, while a larger range of concentrations including, 0.5 µM, 1.0 µM, 5 µM, 20 µM, 30 µM and 60 µM were used to examine the AB-ch peptide. Five experiments were conducted at each peptide concentration for the AB-ch and AB-link peptides. As experiments investigating the AB peptide had previously been performed by others members of the laboratory, only 2 experiments were conducted at each concentration to confirm past results. In order to assess the statistical significance of differences between various concentrations of peptide AB a two-tailed students t-test was used (p<0.05 considered to be significant).
5.3 Results

5.3.1 The AB peptide

5.3.1.1 NMR Structural studies

The 40 amino acid ‘AB’ peptide comprises the 20 amino acids spanning the previously described A region (residues 671-690 of the skeletal muscle DHPR) together with the adjoining 20 amino acids from the B region (residues 691-710).

Some of the NMR spectra used to determine the 3-dimensional structure of the AB peptide are shown in Figures 5.1, 5.2 and 5.3. $^1$H NOESY spectra provide information about the relative proximity of $^1$H atoms in space. As the AB peptide contains 40 amino acids, it was expected there would be some degree of overlap between the chemical shift values obtained and this was the case (Figure 5.1 and 5.2(a)). The amide-amide region of the AB peptide NOESY in the absence of TFE is shown in Figure 5.1. Evidence indicating the presence of alpha helical secondary structure are seen with crosspeaks between adjacent amide backbone protons. Clearly, assignment of this spectrum would be challenging, therefore the assignment of the chemical shift values for the AB peptide was greatly assisted by the addition of 18 % TFE (Figure 5.2). This not only resulted in an increase in spectral resolution but also in an increase in the dispersion of the peaks. The crisp nature of the spectrum is evident in both the NOESY and TOCSY experiments (Figure 5.2 and 5.3).

Cross peaks between the amide $^1$H atoms of adjoining residues $(i - i+1)$ or for residues two positions downstream $(i - i+2)$ are indicative of alpha helical secondary structure. Figure 5.2 (b) shows a large number of cross peaks evident in the amide-
amide region of the NOESY spectrum. Peak connectivities between adjoining residues are shown and correspondingly labeled. The large portion of alpha helix, spanning residues 1-14 that was previously shown in the A peptide (Casarotto et al., 2000) was not disrupted by the addition of 20 downstream residues the AB peptide. The chemical shift values obtained at 278 K for the first 15 residues were very similar to those published by Casarotto et al. (2000). The results of the present study indicated that the 20 amino acids attached to the C-terminal end of the A peptide also contained some secondary structure. Analysis showed that this secondary structure was alpha helical in nature. The helix in the C-terminal portion of the peptide approximately spanned residues 23-36.

Evidence for the structure of the AB peptide can also be found from: (i) coupling constants, (ii) $\alpha$-H chemical shift values, (iii) mapping of NOE connectivities, and (iv) temperature coefficients (Table 5.2). Coupling constants were derived from one-dimensional 1H spectra where possible and/or fitted from one-dimensional slices extracted from DQF-COSY spectra. Coupling constants which could be accurately determined were located either within the first 12 residues or spanning residues 23-33 (Table 5.1 and Table 5.2) and were $\leq 6.1$ Hz indicating the presence alpha helical secondary structure within these regions. Similarly the $\alpha$-H chemical shifts obtained for residues 1-14 and 23-33 deviated from those that have been established for peptides with random coil structure (Table 5.2) (Wishart et al., 1991). It can be seen that connectivities indicative of alpha helical secondary structure are located between residues 1-14 and 23-37 (Table 5.2).
Figure 5.1 NH - $\alpha$H region of the NOESY spectrum (a) and NH – NH region of the NOESY spectrum (b) for the AB peptide at pH 5 and 278 K.
Figure 5.2 NH-αH region of the NOESY spectrum (a) and NH–NH region of the NOESY spectrum (b) for the AB peptide in 18% TFE, pH 5 and 278 K. Cross peaks corresponding to adjoining amino acids have been shown and labeled.
Figure 5.3 Finger Print region of the TOCSY spectrum for the AB peptide in 18 % TFE.
Further evidence to support the large amount of secondary structure in the AB peptide comes from temperature co-efficient determined for each amino acid. The differences in amide $^1$H chemical shifts were taken over a temperature change of 20 degrees and a co-efficient of greater than $-5$ ppb / K indicate of the presence of secondary structure. The data presented in Table 5.1 show that residues located within the two portions of alpha helix, have a co-efficient greater than $-5$ ppb / K (Table 5.2). This is true except for residues Thr$^{671}$, Ala$^{673}$, Lys$^{703}$ and Glu$^{706} - $ Lys$^{710}$. A temperature co-efficient of less than $-5$ is expected for the residues at the beginning of helical sections (see section 1.6.1.5). However, the value of $-7.1$ obtained for Lys$^{703}$ may indicate a less stable portion of helix contained within the C-terminal end of the peptide while the C-terminus is most probably unstructured.

Using a total of 414 NOE constraints and the known sequence of the AB peptide a series of 100 possible 3-D structures was obtained. From the 100 structures obtained, 20 of the lowest energy structures were overlaid (Figure 5.4). The number of NOE restraints used to construct the 20 lowest energy structures for the AB peptide are presented in Table 5.3. When the 20 lowest energy structures were overlaid it was apparent that two regions of alpha helical secondary structure existed and these were linked through an unstructured flexible linker region, spanning residues 17-22.
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<th>Hβ</th>
<th>Other</th>
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*Table 5.1: Proton assignment (p.p.m.), coupling constants (Hz) and Temperature coefficients (p.p.b./K) for the AB peptide in 18% TFE, pH 5.0 at 25°C*
Table 5.2 Summary of the sequential and medium-range NOE connectivities, $\alpha H$ CSI, $\gamma$NH-Ha coupling constants and temperature coefficients for the AB peptide at 298 K. NOE connectivities are indicated by black lines with thickness proportional to NOE intensity. Values of $\gamma$NH-Ha $< 6.1$ Hz are indicated by ■. Temp coefficients $> 5.0$ p.p.b./K are represented by o and those $< -5.0$ p.p.b./K by •. Deviations from random coil CSI tables by $> 0.1$ ppm are represented by ▲, values $< -0.1$ ppm by ▼.
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<tr>
<td>$i - (i+1)$</td>
<td>106</td>
</tr>
<tr>
<td>$i - (i&lt;1)$</td>
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<tr>
<td>Hydrogen Bonds</td>
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Table 5.3 Summary of NOE restraints for the AB peptide

When the first 14 residues of the 20 lowest energy structures are overlaid, it is possible to see the formation of a conserved alpha helix at the N-terminal end of the AB peptide (Figure 5.4(a)). Similarly, when the residues spanning 23-36 are overlaid it is also possible to see a conserved alpha helical region contained near the C-terminal end of the peptide (Figure 5.4(b)). From both images in Figure 5.4 it is clear that once the structures are no longer restrained (at the end of the overlaid portions) there is little similarity between the 20 lowest energy structures and due to the ‘flexible linker’ area each helix appears to be able to move independently of the other.

The sequence of the AB peptide is such that the peptide contains two areas of opposite charge. Six positively charged amino acids were located between residues 11 and 18 while six negatively charged amino acids were located within residues 22 and 29. Therefore the AB peptide contained two oppositely charged alpha helical regions joined by a flexible linker. It is therefore possible that the peptide could fold back onto itself. Interestingly, when the set of NOEs used for the AB peptide contained constraints holding the two helical regions together (i.e. NOEs between Gln$^{674}$ and Lys$^{708}$) it produced 3D structures with substantially higher energy values ($= 2 - 3$ fold
higher) than in the absence of these constraints. Similarly, none of the low energy structures produced in this study provided any conclusive evidence for the interaction of the two helical regions. It is still possible that once bound to the RyR such a conformation may be adopted. Equally as feasible is the possibility that, due to the high dynamic nature of the peptide, the NOEs may be averaged out. That is, the helical regions do interact, but only do so for only a small portion of the time.

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Table 5.4 Table of violations of constraints and the root mean square deviation (kcal mol⁻¹) for the family of 20 calculated lowest energy structures of the AB peptide

Figure 5.5 illustrates the average structure of the AB peptide, obtained from the 15 lowest energy structures. The presence of the two alpha helical regions is clearly illustrated in this average structure. The formation of the helix located at the N-terminal end (spanning residues 1-16) is more regular and uniform compared to the helix found in the C-terminal end (spanning residues 23-36). The unstructured flexible linker is also clearly seen.
Figure 5.4 Best fit superposition of backbone heavy atoms of residues Gln\textsuperscript{671} - Arg\textsuperscript{684} (a) and Lys\textsuperscript{693} - Gln\textsuperscript{707} (b) of the 20 lowest energy structures for the AB peptide.
Figure 5.5 The average structure, determined from the 20 lowest energy structures of the AB peptide. Labeled are regions corresponding to the alpha helical portions of the A region and B region and the flexible linker which joins the two regions.

5.3.1.2 Circular Dichroism Studies

Circular Dichroism studies were performed in order to confirm the secondary structure of the AB peptide. Experiments were performed in the presence and absence of 18% TFE. TFE is often used in NMR studies to help stabilize secondary structures that may be present in smaller peptides and was used in the NMR study of the AB peptide (see section 5.2.2). Thus, it was important to confirm that the AB peptide contained secondary structure in the absence of TFE. Therefore experiments were conducted in its presence and absence.

As mentioned previously (Section 2.2.2), molecules containing large portions of alpha helical secondary structure produce specific a maximum and minima in circular dichroism spectra. These include a maximum at 196nm, a minimum at 208nm and a second minimum at 222nm.
In the absence of TFE, the AB peptide exhibited some of the traits expected for a molecule containing alpha helical secondary structure. The spectrum possessed a minimum at 199 nm and an inflexion point at 223 nm (Figure 5.6), indicating a small degree of secondary structure (see 2.1.2). The presence of alpha helical secondary structure is clearly more evident after the addition of 18% TFE. The addition of TFE produced a maximum at 188 nm, a minimum at 203 nm and a deeper minimum at 219 nm (Figure 5.6), characteristics indicating a larger degree of alpha helix than in the absence of TFE. Thus not surprisingly, the secondary structure in the AB peptide is stabilized by the presence of TFE.

Figure 5.6 Circular Dichroism spectrum for the AB peptide in the presence and absence of 18% TFE.
5.3.1.3 SR Vesicle Release Studies

The AB peptide (20-60 µM) did not cause any significant increase in Ca$^{2+}$ released from SR vesicle indicating that it did not activate the RyR1. Three different concentrations of the AB peptide (20, 30 and 60 µM) were tested. Normal calcium loading was seen in all experiments and the baseline leak of calcium was noted after the addition of thapsigargin (before the addition of peptide). The addition of the AB peptide failed to cause any further release of calcium from the SR vesicles above the baseline thapsigargin release. A typical experiment with the addition of 20 µM of the AB peptide is illustrated in Figures 5.7.

Figure 5.7 SR calcium release experiment assessing the activating ability of the AB peptide (20µM).
It was surprising that the AB peptide did not release Ca$^{2+}$ from the SR since the structure of the A region was very similar to that of the A peptide which does release Ca$^{2+}$ (Dulhunty et al., 1999). It was possible that (a) the flexibility of the linker region allowed the negative charges in the B region (see section 5.2.1) to bind to the positive charges in the A region that are important for activation (El-Hayek and Ikemoto, 1998; Dulhunty et al., 1999; Zhu et al., 1999) and thus prevent them from interacting with the RyR or (b) that the negatively charged residues in the B region masked the active site in the A region or prevented it from binding to the RyR. Therefore we constructed 2 mutants of the AB peptide to test these possibilities. The AB-link mutant was designed for reduced flexibility, while the AB-ch peptide was designed to reduce the negative charges in the B region. Unlike the AB peptide, full NMR evaluations of the mutant peptides were not performed. Instead the structural comparisons were based on the NOE profiles obtained for the peptides.

5.3.2 The AB-link peptide

5.3.2.1 NMR Structural Studies

The AB-link peptide was designed to investigate one of the possible mechanisms preventing the AB peptide from activating the RyR1. The AB-link peptide contained the following mutations, Pro$^{691} \rightarrow$ Ala and Lys$^{693} \rightarrow$ Ala. It was used to test the hypothesis that flexibility of the native AB peptide allowed the negatively charged residues located in the C-terminal end of the peptide to mask the critical positively residues located in the N-terminal end of the peptide. We anticipated that mutating Pro$^{691}$ and Lys$^{693}$ may alter the flexibility of the link region and prevent such masking.
The AB-link peptide contained more secondary structure than the AB peptide. NMR structural studies indicated a larger number of NOE cross peaks between adjoining residues in the amide-amide region of the NOESY spectrum. As mentioned previously in this and previous chapters, cross peaks between adjoining residues located in this area of the spectrum are indicative of alpha helical secondary structure. In the AB-link peptide there were a total of 32 NH$_i$ – NH$_{i+1}$ NOE cross peaks. It was found that successive cross peaks could be traced from the beginning of the peptide through to residue 23, these adjoining cross peaks are indicated and labeled accordingly in Figure 5.8 (b). A second portion of the peptide contained several other NH$_i$ – NH$_{i+1}$ cross peaks. This second portion stretched from residues 24 to 36 (Figure 5.8(b)). Further support for these findings comes from the chemical shift values obtained for the AB-link peptide. The amide chemical shift was again very similar to those obtained for the AB peptide, indicating a similar degree of secondary structure in each peptide. The information obtained from $\alpha$-$^1$H CSI values supported the finding from the NOE spectrum. The AB-link peptide was found to have $\alpha$-$^1$H values consistent with alpha helical structure in residues 1 – 35.

The TOCSY spectra for the AB-link peptide (Figure 5.9) did not contain the efficient magnetization transfer found with the AB peptide. Magnetization transfer refers to the transfer of signal from one spin to another on the molecule. It is used in magnetic resonance imaging as an additional way to generate unique contrasts within spectra. The decreased efficiency of magnetization transfer in the AB-link peptide TOCSY spectrum resulted in a lengthy assignment process as many of the cross peaks originating from the backbone amide protons to the beta, gamma and delta $^1$H's were
not present in the spectra obtained. The chemical shift values were nonetheless obtained and are shown in Table 5.5.

Figure 5.8 (a) Amide-Alpha region of the NOESY spectrum for the AB-link peptide (b) Amide-Amide region of the NOESY spectrum for the AB-link peptide. Cross peaks corresponding to adjoining amino acids have been shown and labeled.
Figure 5.9 Finger Print region of the TOCSY spectrum for the AB-link peptide in 18% TFE, pH 5, 278 K.
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Table 5.5: Proton assignment and α<sup>1</sup>H CSI information (▲ = alpha helical, ▼ = beta sheet) for the AB-link peptide in 18% TFE, pH 5.0 at 278 K.
5.3.2.2 Circular Dichroism Studies

Similar to the AB peptide, the CD studies for the AB-link peptide supported the NMR data. The CD studies for the AB-link peptide show that the peptide contained a large amount of alpha helical secondary structure, especially in the presence of 18% TFE. In the absence of TFE, the CD spectrum produced a minimum at 200 nm and an inflexion point at 218 nm, evidence for a small amount of alpha helical secondary structure (Figure 5.10). The appearance of a maximum at 188 nm and minima at 205 nm and 219 nm in the presence of 18% TFE, are clearly shown in Figure 5.10. The position of the maximum and minima, as well as the depth of the minimum at 219 nm, suggests the AB-link peptide contains the largest amount of alpha helical secondary structure out of the three AB peptides. This information also supports the NMR studies, which showed that the AB-link peptide was the most structured of the three peptides.

![Figure 5.10 Circular Dichroism Spectrum of the AB-link peptide in the presence and absence of 18% TFE.](image-url)
5.3.2.3 SR Vesicle Release Studies

The AB-link peptide (20-60 µM) did not cause any significant increase in Ca\(^{2+}\) release from SR vesicles indicating that it did not activate the RyR1. Three different concentrations of the AB-link peptide (10, 30 and 60 µM) were tested. The addition of the AB-link peptide failed to cause any further release of calcium from the SR vesicles above the baseline release in the presence of thapsigargin. A typical experiment with the addition of 30 µM of the AB-link peptide is illustrated in Figure 5.11.

![Figure 5.11 SR calcium release experiment assessing the ability of the AB-link peptide (30µM) to activate the RyR1.](image-url)
5.3.3 The AB-ch peptide

5.3.3.1 NMR Structural Studies

The AB peptide and the AB-link peptide were both functionally inactive (see sections 5.3.1.3 and 5.3.2.3). This may have been due to the negative charge density present in the C-terminus of the peptides. To test this possibility the AB-ch peptide was designed by removing several of the negatively charged residues in the B region of the AB peptide (Asp$^{692}$ → Ala; Glu$^{695}$ → Ala; Glu$^{696}$ → Val; and Glu$^{697}$ → Ala). A full 3-dimensional structure was not determined for the AB-ch peptide, rather we opted for a qualitative study to determine whether the helical structure was maintained after the change in amino acid composition. Chemical shifts values were assigned using the TOCSY and NOESY spectra. From the NOE studies performed on the AB-ch peptide there is evidence to suggest that a large amount of alpha helical structure is still present in this peptide. From the amide-amide region of the NOESY spectrum for the AB-ch peptide there were 25 detectable cross peaks (Figure 5.12). Comparatively, in the AB peptide there were 24 detectable cross peaks. Thus, it appears that the distribution of NOEs in this portion of the spectra for the AB-ch peptide has changed little following the mutations of residues Asp$^{692}$, Glu$^{695}$, Glu$^{696}$, and Glu$^{697}$.

The amide $^1$H backbone NOE connectivities between adjoining residues have been identified and labeled in Figure 5.12. As with the AB peptide, it is possible to trace connectivity's from the beginning of the peptide through to residue 14, corresponding to the presence of an alpha helix located at the N-terminal end of the AB-ch peptide. Similarly connectivity's can be traced through the later half of the peptide (residues 22 and 36) indicating a helical portion located near the C-terminal end of the peptide.
Figure 5.12 (a) NH - aH region of the NOESY spectrum (a) and NH – NH region of the TOCSY spectrum (b) for the AB-ch peptide in 18 % TFE. Cross peaks corresponding to adjoining amino acids have been shown and labeled.
Figure 5.13 Finger Print region of the TOCSY spectrum for the AB-ch peptide in 18% TFE, pH 5 at 278 K.
There were no NOEs observed for residues 17-22 that would indicate secondary structure. From this it is concluded the AB-ch peptide also retains the ‘flexible linker’ seen in the AB peptide. Further, the NH chemical shift values obtained for the majority of residues in the AB-ch peptide (Table 5.6) were very similar to those obtained for the AB peptide, implying that the secondary structure is reasonably well conserved between the two peptide. The deviation of $\alpha-^1H$ from random coil CSI values are illustrated in Table 5.6 (upward arrowhead indicating alpha helical secondary structure, downward arrowhead indicating beta sheet) and show that it is most likely that alpha helical secondary structure is present between residues 4 – 17 and 22 – 33.

Similar to the ABlink peptide, the TOCSY spectrum for the AB-ch peptide (Figure 5.13) did not contain the efficient magnetization transfer found for the AB peptide. The differences obtained with the three peptides can be seen by comparing Figure 5.3, Figure 5.9 and Figure 5.13.
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<td>1.69/1.59</td>
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<td>1.61/1.80</td>
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<td>2.44/2.42</td>
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<td>4.62</td>
<td>1.87/1.80</td>
<td>Hγ 1.53, Hδ 1.72/1.74, He 3.05, ΖΝΗ 7.58</td>
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<td>2.34</td>
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<td>Hγ 1.94, Hδ 3.67/3.87</td>
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<td>4.27</td>
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<td>Hγ 1.52, Hδ 1.73, He 3.04, eNH&lt;sub&gt;3&lt;/sub&gt; 7.61</td>
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Table 5.6: Proton assignment and α<sup>1</sup>H CSI information (▲ = alpha helical, ▼ = beta sheet) for the AB-ch peptide in 18% TFE, pH 5.0 at 278 K.
5.3.3.2 Circular Dichroism Studies

The CD studies for the AB-ch peptide provided further evidence to support the results obtained using NMR spectroscopy, that the AB-ch peptide contained a considerable amount of alpha helical secondary structure. The amount of alpha helix, is increased with the addition of 18% TFE. The notable features of the spectrum obtained for the AB-ch peptide in the absence of TFE (Figure 5.14) are the minimum at 198 nm and a small inflection point at 218 nm. 18% TFE altered the spectrum so that there was a maximum at 188 nm and minima at 204 nm and 218 nm. The maximum, the movement of the first minimum closer to 208 nm and the depth of the second minimum all suggest of an increased amount of alpha helical secondary structure in the presence of 18 % TFE. These results are very similar to those seen with the AB peptide.

![Figure 5.14 Circular Dichroism spectrum for the AB-ch peptide in the presence and absence of 18% TFE.](image-url)
5.3.3.3 SR Vesicle Release studies

Unlike the AB and AB-link peptides, the AB-ch peptide was able to activate $\text{Ca}^{2+}$ release from the SR. Activation was seen at concentrations as low as 0.5 $\mu$M. The rate of release of $\text{Ca}^{2+}$ through RyR1 continued to increase as the concentration of the AB-ch peptide was increased. Maximal activation, recorded in this study, was produced with the addition of 60 $\mu$M of the AB-ch peptide.

A typical SR vesicle release trace (20 $\mu$M) has been illustrated in Figure 5.15. The traces presented in Figure 5.16 are representative of the results obtained from SR vesicle release studies for varying concentrations of the AB-ch peptide. Each trace starts approximately 1-3 seconds after the addition of thapsigargin. The arrows indicate the addition of peptide at the labeled concentrations. The rate of release is dependent on the initial baseline release of $\text{Ca}^{2+}$ at the beginning of each trace. It should be noted that, even though it appears the addition of 0.5 $\mu$M of the AB-ch peptide has caused a larger release of $\text{Ca}^{2+}$ from SR vesicles compared to the addition of 1 $\mu$M, it is actually not the case on average. Once the baseline values have been subtracted from the rate of release after the addition of the AB-ch peptide there was actually a greater release with the addition of 1 $\mu$M of the AB-ch peptide.
Figure 5.15 SR calcium release experiment assessing the ability of the AB-ch peptide (20µM) to activate the RyR1.

The average rate of release of Ca$^{2+}$ from SR vesicles is presented in Figure 5.17. There were statistically significant differences between the rate of release obtained for the 0.5 µM and the 1.0 µM additions of the AB-ch peptide (p = 0.03). Similarly, significant differences were obtained between the additions of 5 µM and 20 µM (p < 0.01), 5 µM and 30 µM (p < 0.001), 5 µM and 60 µM (p < 0.001), and between 20 µM and 60 µM (p = 0.02). There were no significant differences in the initial rate of calcium release once the concentration of the AB-ch peptide was in excess of 30 µM (30 µM and 60 µM, p = 0.29).
Figure 5.16 SR calcium release experiments assessing the activating ability of the AB-ch peptide at various concentrations. Baseline levels were achieved by the addition of 200 nm thapsigargin and are indicated by dotted lines. Arrows indicate the addition of the labeled amount of AB-ch peptide. Peptides were added approximately 30 to 45 seconds after the baseline was established.
Figure 5.17 Average initial rates of Ca\(^{2+}\) release (nmol/mg of protein in the SR vesicle / per minute) induced by the AB-ch, A1 and A1(D-R18) peptide at various concentrations. Data for the AB-ch peptide are presented as means ± SEM.
5.4 Discussion

The mutant peptides used in the experiments reported in this chapter have provided insight into why the AB peptide failed to activate Ca\(^{2+}\) release through the RyR1. It was expected that the AB peptide would have retained the activating abilities noted with the A peptide (details in chapter 2.1) because the structure of the A region was retained. However, the addition of another 20 amino acids from the native sequence of the skeletal DHPR was enough to extinguish the activating ability of the peptide.

The structural profiles determined from both NMR and CD spectra, showed each of the AB peptides contained large portions of alpha helical secondary structure. Two helical portions were detected in all peptides, one located in the first 13-16 amino acids and one located between residues 24-36. In each of the peptides the two helical portions are separated by what has been termed a ‘flexible linker’. Although all of the AB peptides contained very similar regions of secondary structure there were a few differences in the predicted length of the helical portions. The AB-link peptide contained the largest amount of alpha helical secondary structure. While the AB-ch peptide contained more alpha helical secondary structure than the AB peptide.

All peptides adopted a more stable secondary structure in CD studies the presence of 18% TFE (v / v). As each of the NMR studies was conducted in the presence of 18% TFE, it should be noted that the structures determined with TFE are likely to be the most stable conformation of the peptides. When the stability of the secondary structure of a peptide is increased, it generally increases the dispersion and decreases the line width of the NMR signal by decreasing the amide hydrogen interactions with...
the solvent (see section 5.2.2). This helps to provide highly resolved spectra, which assists in the assignment of chemical shift values for the peptides. However there is a catch 22 because TFE can also induce unreal peptide conformations. Another potential problem that can be caused by the addition of TFE is an increased difficulty in obtaining the NMR spectra. Two of the steps involved in obtaining an NMR spectrum are shimming and locking of the sample. Locking involves locking onto the deuterium resonance frequency and using this signal to "tune" the magnet in order to obtain a more homogeneous magnetic field. Shimming is conducted to increase the homogeneity of the magnetic field. Effective shimming gives rise to narrow and symmetrical peak shapes which results in improved spectral resolution. These steps are more difficult in the presence of TFE and may be a reason for the poor spectral quality obtained for the AB-ch and AB-link spectra (see section 5.3.1.2 and 5.3.1.3).

The peptides ability to activate the RyR1 was determined using SR vesicle Ca\(^{2+}\) release. Both the AB and the AB-link peptides were unable to activate Ca\(^{2+}\) release through the RyR1. Interestingly, the AB-ch peptide caused activation, measured by the initial rate of Ca\(^{2+}\) release from the SR vesicles through the RyR1, over a concentration range of 500 nM – 60 \(\mu\)M. Maximal activation occurred at 60 \(\mu\)M and was approximately 20 fold faster than at 500 nM. The rate increase in activation with peptide concentration appeared to decrease between 30 and 60 \(\mu\)M and the AC\(_{50}\) was approximately 10 to 15 \(\mu\)M.

The average initial rates of release achieved with 30 \(\mu\)M and 60 \(\mu\)M of the AB-ch peptide were 443.07 ± 44.89 (n=11) and 520.41 ± 52.84 (n=7) nmoles of Ca\(^{2+}\)/mg of protein/min respectively. These values are higher than the values that have previously
been reported for peptides from the A region of the skeletal DHPR. A graph published by Dulhunty et al. (1999), suggested that 20 μM of the A1 and A2 peptides released approximately 50 and 140 nmoles of Ca^{2+}/mg of protein/min respectively (see also Figure 5.17). Even the A1(D-R18) peptide, which has been shown to be the most potent activator of the A peptides, in excess of 30 μM released 300-325 nmoles of Ca^{2+}/mg of protein/min (Green et al., 2003). The activating ability of the AB-ch peptide appears to be between the A1(D-R18) and the scorpion toxin, IpTxα, but remains closer to A1(D-R18). One micromolar IpTxα induces initial rates of Ca^{2+} release from SR vesicles in excess of 400 nmoles of Ca^{2+}/mg of protein/min, while 30 μM increased the rate to ≈ 900 nmoles of Ca^{2+}/mg of protein/min (Green et al., 2003). While the AB-ch peptide does not activate Ca^{2+} release through the RyR with the same high affinity of IpTxα, it caused average initial releases that were substantially greater than those caused by any other A peptide.

These results show that the AB-ch peptide activates the RyR1 at a faster rate than the most potent A region peptide, the A1(D-R18) peptide (section 4.3.3). Most importantly though it is more than 5 times more potent than the A1 peptide (Casarotto et al., 2000), whose sequence is contained in the N-terminal half of the peptide.

The fact that a crucial determinant in the ability of the A peptide to activate the RyR1 is the presence of several positively charged residues has been discussed in Chapters 2 and 3. Further to this, the positive charges needed to have an alpha helical secondary structure. It was therefore surprising that the AB peptide, which contained the required positively charged residues, as well as the necessary secondary structure, did not activate RyR1. Removal of negative charges in the B region allowed peptide AB-
ch to activate the RyR1, but lowering the flexibility of the linker region in AB-link did not. Since the secondary structural elements of the three AB peptides were so similar it would be highly unlikely that the minor structural differences noted would be responsible for the large functional differences observed.

It has been noted that the B region of the AB peptide contains a large number of negatively charged residues and that once the structure of the AB peptide was determined it was thought that these negative charges, located in the second helical portion of the peptide, could possibly interact with the cluster of positive charges seen in the A region of the peptide. There was no evidence from NMR to suggest that this was the case. Also, because it is not energetically feasible for the ‘flexible linker’ region to form such a tight turn so that the two helical portions can interact. An alternative possibility was that the overall charge of the AB peptide was enough to remove the activating ability of the A region. That is, that the A region may no longer be able to bind at the same site of activation with so many negatively charged residues present in the B region.

In fact this appears to have been the case. Removal of four of the negatively charged residues located in the C-terminal half of the AB-ch peptide restored the RyR1 activating ability. Further support for the overall charge hypothesis came from the results obtained with the AB-link peptide. It also contained a very similar overall structure to that of the AB and AB-ch peptides. However the AB-link peptide contained the negatively charged B region and it was not able to activate the RyR1. Interestingly, the recombinant peptide derived from the whole II – III loop, SDCL, activates the RyR1 even though it contains the sequence of the AB peptide (see
section 6.4). Therefore within the SDCL protein, either the A region does not contribute to activation or the negative charges are somehow modulated in the full loop by other charge or geometric constraints.

The reasons why the AB-ch peptide is a more potent activator of RyR1 than the A peptide remains unclear. Similar to the A peptide, IpTx₃ requires a string of positive charged amino acids located in the C-terminal end of the toxin (residues 19-24) to activate the RyR1 (Gurrola et al., 1999). A closer inspection of the sequence contained in the B region of the AB-ch peptide reveals that 5 out of the 13 amino acids in the C-terminal end of the peptide are positively charged. It could be possible that by eliminating the negatively charged residues in AB-ch we have actually created a peptide with positive charges at both ends, that is able to activate the RyR1 via either of its positively charge helices. This possibility could be investigated by removing some of the positively charged residues from the C-terminal end of the AB-ch peptide, whilst still attempting to retain the peptide’s structure.

By removing the negatively charged residues from the B region of the peptide, the B region of the peptide could bind to a different site than the original A peptide. The positively charged B region of the AB-ch peptide may act as an anchor to secure the AB-ch peptide in the immediate vicinity of the A regions activating binding site. With the B region bound, the A region of the peptide may become a more potent activator as it would be present at the binding site for longer periods of time. Binding studies using protein affinity columns and RyR fragments could potentially be used to investigate the site of activation of the A and AB-ch peptides.
Chapter 5 – The AB peptide

The results in this section show that the mutation of 4 amino acids can dramatically affect the functional effects of a peptide. This is not a novel finding, even for a single point mutation within a whole protein. It has been shown that a single point mutation within the III-IV loop of the DHPR is responsible for symptoms of malignant hypothermia (Jurkat-Rott et al., 2000).

One finding is very important for studies using either peptides or protein fragments. We added 20 amino acids of native sequence to a peptide and abolished it activating ability. By dissecting a peptide or peptide into smaller pieces one can never be certain that the dissection is not destroying a potential binding site, or in fact, developing a completely new one that would not be present in the whole protein. On the other hand, it is possible to abolish activity by adding residues to a simple peptide. Also it is not possible to conclude that the structure present in the peptide fragment is retained in the whole protein.

In light of these findings it is of interest to review the previous literature for the area surrounding the AB peptide and the importance of the A and B regions of the skeletal DHPR II-III loop. It was initially found that a 61 amino acid peptide, SCDLfl (residues 666-726) was able to bind to and activate RyR1 (Lu et al., 1995). The sequence from the A region (residues 671-690) also activated Ca\textsuperscript{2+} release from skeletal SR (el-Hayek et al., 1995a; Dulhunty et al., 1999; Gurrola et al., 1999; Lamb et al., 2000). It was then discovered that s31 region (residues 666-709) did not interact with either the R9 or R10 regions of the RyR1 (Proenza et al., 2002). This is particularly relevant and strikingly similar to the present finding that the AB peptide (residues 671-710) was not able to activate the RyR1. In other words, the A region
can activate the RyR1. Once 20 amino acids of native sequence downstream are added, the peptides ability to activate is abolished. Addition of another 16 residues of native sequence downstream and 5 residues upstream, the peptide is again able to activate the RyR1 (Lu et al., 1995). Therefore if Proenza and co-workers (2002) had used either residues 671 – 690 or residues 666-726, they may have identified an interaction with the R9 and R10 regions of RyR1.

In conclusion, the inability of the AB peptide to activate Ca\(^{2+}\) release from SR vesicles is unlikely to be due to the size or binding limitations of the peptide. But is more likely to be due to the presence of several negatively charged amino acids in the B region of the peptide preventing the peptide from binding to its site of activation.
Chapter 6 - The C peptide and preliminary studies of the SDCL

6.1 Introduction

Numerous chimera and deletion studies have shown that the C region of the skeletal DHPR II – III loop (Glu$^{724}$ – Pro$^{760}$) is the critical site for eliciting skeletal type ECC (see section 1.2.5). In addition, the C peptide has been shown to have various effects on the RyR1 including activation and inhibition (see below and section 1.2.6). The recombinant protein derived from the sequence of the II – III loop of the skeletal DHPR (SDCL) has been shown to activate the RyR1 (Lu et al., 1994) (see section 1.2.6).

The initial study investigating a peptide composed of the C region (Glu$^{724}$ – Pro$^{760}$) (peptide C) of the skeletal DHPR II – III loop found that neither peptide C, peptide C1 (residues Phe$^{725}$ – Gly$^{743}$) nor C2 (residues Asp$^{740}$ – Pro$^{760}$), which were the two subdomains of peptide C, increased the amount of $[^3]$H ryanodine bound to the RyR1 (el-Hayek et al., 1995a). However, it was noted that peptide C significantly suppressed the activation of the RyR1 caused by peptide A (el-Hayek et al., 1995a). More recently, the C peptide has been found to activate Ca$^{2+}$ release from SR vesicles (Yamamoto et al., 2002), purified skeletal RyR channels (Stange et al., 2001) and the native skeletal RyR1 channel complex incorporated in lipid bilayers (Haarmann et al., 2003). The C peptide caused activation and inhibition of the native RyR1 complex depending on the concentration used (Haarmann et al., 2003).
The structural characteristics of several peptides derived from the II – III loop have been examined in chapters 3, 4 and 5. The functional ability of these peptides to activate the RyR1 has been correlated with the structural characteristics in an attempt to determine key structural features critical for a peptides to activate RyR1. The secondary structure in the II – III loop of the DHPR is of critical importance for understanding the protein – protein interaction that occurs between the DHPR and the RyR1 in skeletal type ECC. However, due to the limitations of $^1$H NMR, the 3D solution structure of the SDCL peptide will not be determined until it is successfully labeled with $^{13}$C and $^{15}$N, which has now been achieved in our laboratory. In this chapter preliminary studies are described which determined whether SDCL contained any secondary structure. In addition to this, the secondary structure of the C peptide has been investigated in an attempt to correlate structural and functional properties of the peptide and to structurally characterize an important portion of the skeletal II-III loop.
Chapter 6 – The C peptide and SDCL

6.2 Material and Methods

6.2.1 The peptides used

C: \textsuperscript{724}EFESNVNEVKDPYP\textsuperscript{760}SADFPGDDEDEPEIPVSPRP

II – III loop: \textsuperscript{666}EAESLTSQKAKAEERKRRKMSRGLPDKTEEKSVMAKKLSQK PKGE\textsuperscript{791}GIPTTAKLVDEFSNVNEVKDPYP\textsuperscript{808}SADFPGDDEDEPE IPVSPRPRPPLAELQLEKAVPIPEASSFFIFSPTWKVRVL

6.2.2 NMR Structural Studies

The C peptide and the recombinant II – III loop (SDCL) were dissolved to a final concentration of \( \approx 2 \) mM in either 10\% D\textsubscript{2}O / 90\% H\textsubscript{2}O or 18\% TFE / H\textsubscript{2}O and adjusted to a pH of 5.0. Two-dimensional NOESY spectra were produced at 278 K and used to investigate the secondary structure present in the peptide.

6.2.3 Circular Dichroism Studies

The C peptide was adjusted to a concentration of 25 \( \mu \)M in H\textsubscript{2}O or a solution containing 18\% TFE / 82\% H\textsubscript{2}O. The recombinant II – III loop was diluted to a concentration of 25 \( \mu \)M in H\textsubscript{2}O. Both peptides were adjusted to a pH of \( \approx 5 \). CD spectra were obtained at 298 K.
6.3 Results

6.3.1 Peptide C

6.3.1.1 NMR Structural Studies

Neither the NOESY spectrum obtained for the C peptide, in H$_2$O or 18 % TFE, revealed evidence for the presence of any alpha helical secondary structure. The NH – NH and αH - NH regions of the C peptide (in 18 % TFE) NOESY spectrum are illustrated in Figure 6.1. The absence of alpha helical secondary structure was indicated by the absence of any NH$_i$ – NH$_{i+1}$ NOE connectivities in both spectra (in the presence and absence of TFE). This was confirmed by the simplicity of the cross peaks located in the αH - NH region. Because of the structural limitations of the peptide backbone, αH$_i$ – NH$_{i+1}$ connectivities should be found in this region for all NOE spectra. However, additional NOE connectivities, indicative of alpha helical secondary structure (for example: αH$_i$ – NH$_{i+2}$ or αH$_i$ – NH$_{i+3}$), would also appear in this region if alpha helical secondary structure was present. In the absence of these connectivities, it is concluded that the C peptide contains a random coil secondary structure.

6.3.2.2 Circular Dichroism Studies

Circular Dichroism studies of peptide C confirmed a random coil secondary structure. The spectrum obtained for the C peptide is shown in the presence and absence of 18 % TFE (Figure 6.2). The absence of a positive maximum at 185 – 192 nm, the presence of a minimum at 197 nm and the absence of a second minimum at 222 nm
are all indicators of random coil secondary structure (Figure 6.2). Eighteen percent TFE did not induce any non-native secondary structure in peptide C.

Figure 6.1 NH - αH region of the NOESY spectrum (a) and NH –NH region of the NOESY spectrum (b) for the C peptide in 18 % TFE at pH 5 and 278 K.
Figure 6.2 Circular Dichroism spectrum for the C peptide, in the presence and absence of 18% TFE at pH 5 and 298 K.

6.3.2 The recombinant II – III loop peptide

6.2.3.1 NMR Structural Studies

The II – III loop has been shown to be an integral part of the skeletal DHPR in eliciting skeletal type ECC. In this preliminary study using $^1$H NMR we have investigated the presence of secondary structure within the skeletal DHPR II – III loop (SDCL). Due to the large overlap of cross peaks present within the NH – NH and NH - $\alpha$H region of the NOE spectrum (Figure 6.3) it was hard to identify distinct connectivities. The presence of weak cross peaks in this region are suggestive of secondary structure within the SDCL. The assignment of chemical shift values for the SDCL was not possible due to the large degree of overlap within the spectrum and
without the chemical shift values it is impossible to determine the nature of the secondary structure that may be present.

Figure 6.3 NH - αH region of the NOESY spectrum (a) and NH - NH region of the NOESY spectrum (b) for the SDCL peptide in at pH 5 and 278 K.
6.3.2.2 Circular Dichroism Studies

CD results from the SDCL demonstrated that, at least a small percentage of the protein fragment, contains alpha helical secondary structure. The spectrum obtained for SDCL has been plotted on the same graph as the A1 peptide (Figure 6.4). The maximum and minima detected at 188 nm, 200 nm and ≈ 222 nm, respectively, are suggestive of alpha helical structure (see section 2.2.2), however the proportion of alpha helical secondary structure in SDCL is not as high as that seen for the A1 peptide.

![Figure 6.4 Spectrum from Circular Dichroism for the recombinant DHPR II – III loop (SDCL) and the A1 peptide at pH 5 and 298 K.](image-url)
6.4 Discussion

Structural studies using NMR and CD techniques have revealed that peptide C has a random coil secondary structure. Peptide C does not contain the cluster of positively charged residues that are found to be critical for peptides, derived from the A region of the II – III loop, or scorpion toxins, to activate the RyR1. Therefore it is likely that the C peptide does not activate the RyR1 via the same binding site as either the peptides derived from the A region of the skeletal DHPR II – III loop or the scorpion toxins (lpTx4 and Mca).

The ability of peptide C to activate the RyR1 has recently been demonstrated using several different experimental techniques. The C peptide activated Ca\(^{2+}\) release from SR vesicles ([10 – 30 nM]) (Yamamoto et al., 2002), the purified RyR1 channel ([0.3 – 30 µM]) (Stange et al., 2001) and the native RyR1 channel ([0.1 – 50 µM]) (Haarmann et al., 2003) incorporated in lipid bilayers. The C peptide was also shown to inhibit RyR1 activity in 25 % of channels when the concentration was 1 – 30 µM and in the majority of channels when the concentration was 50 – 150 µM (Haarmann et al., 2003). In the study of the effects of peptide C conducted in our laboratory, there was no correlation between the degree of activation and inhibition of the RyR1 and in several instances, either activation or inhibition was absent. Therefore it was concluded that activation and inhibition of the native RyR1 occurred through independent mechanisms (Haarmann et al., 2003). The structural findings of this chapter support the conclusions either (a) that peptide C can activate or inhibit RyR1 through occasionally adopting the conformation necessary to bind to the activation or
inhibition sites of (b) that it may bind to the one site, causing activation or inhibition depending on the conformation obtained.

The unstructured nature of the C region has recently been shown to be critical for its ability to elicit skeletal type ECC (Flucher, B. Biophysics abstracts, 2003). Various constructs were inserted into the Musca II – III loop (see section 1.2.5) and expressed in dysgenic myotubes. The minimal skeletal sequence needed to elicit skeletal-type ECC were residues 734 – 748 and this region had a predicted random coil structure. When five residues were replaced with cardiac sequence the predicted structure of the C region was altered from random coil to alpha helical and cardiac-type Ca$^{2+}$ transients were recorded in most cases (Flucher, B. Biophysics abstracts, 2003). These results again demonstrate that the C region is responsible for the tissue specific effects of ECC. It also demonstrates a potential important role for the unstructured C region. This is not a new discovery as intrinsically unstructured proteins (IUPs) have now been shown to have various physiological roles. In a recent review of the importance of IUPs it is stated that the number of full length proteins and domains that have been identified as IUPs has already exceeded 100 and their functions can be classified in 28 distinct categories (Tompa, 2002).

Initial studies into the activity of peptide C suggested that there was no effect on the activity of RyR1 (indicated by ryanodine binding studies), but the peptide could decrease the activation of the RyR1 caused by peptide A (el-Hayek et al., 1995a). Considering that peptide A contains an overall positive charge and peptide C contains an overall negative charge, a plausible hypothesis for the combined effects of peptides A and C would be that the two peptides interact with each other so that peptide A is
no longer able to activate the RyR1 in the presence of peptide C. However, it was discovered that the chemical shifts values corresponding to several of the positive charged residues in peptide A were unchanged, that is, no difference in chemical shift values or line broadening with the addition of peptide C (Haarmann et al., 2003). Similarly, sedimentation equilibrium experiments failed to detect the presence of a complex corresponding to the molecular weight of peptides A and C (Haarmann et al., 2003). Thus, it appears that the ability of peptide C to inhibit the activation caused by peptide A is not due to the interaction of the two peptides.

NMR studies show that SDCL contained weak NH – NH NOE connectivities, suggestive of secondary structure. The nature of the CD spectrum obtained for SDCL indicates that a portion of this secondary structure is likely to be alpha helical and that some less structured regions exist. A better understanding of the structural / functional relationship of the SDCL will be obtained, now that the peptide has been successfully labelled with $^{13}$C and $^{15}$N and the complete 3D solution structure can be determined. Preliminary examinations suggest that the A, B and D regions of the SDCL contain alpha helical secondary structure whilst the C region is random coil. This preliminary data is supportive of the structures that have been determined for the peptides covering the A, B and C regions of the SDCL.

The studies described in this chapter suggest that SDCL contains secondary structure, with portions of this likely to be alpha helical. The C peptide contains random coil secondary structure, but is still able to activate and inhibit the RyR1 through occasionally obtaining the necessary structure. The mechanism through which the C
peptide activates and inhibits the RyR1 is most likely different to the A region peptides, IpTx, and Mca.

Future work on this area may involve investigating the effects of a mutant peptide C containing a stable secondary structure. By mutating residues that are thought to increase the stability of the secondary structure of peptide C as determined by Kugler et al (2004), it may be possible to create a more structured version of this peptide. The effects functional effects of the ‘stable’ C peptide could then be investigated and compared to the original peptide C. In this way, the importance of the unstructured nature of the protein could be investigated.

Chapter 7 – General Discussion

7.1 The control of skeletal type ECC

Insights into the protein – protein interactions that occur between the skeletal DHPR and the RyR1 in skeletal type ECC have been provided through the use of chimera, deletion and peptide studies. From chimera and deletion studies it has been shown that the region most critical for eliciting skeletal type ECC is located between residues 720 – 765. The involvement of the A region of the skeletal DHPR in eliciting skeletal type ECC appears to be very minimal as skeletal type ECC has been shown to exist with a scrambled A region (Proenza et al., 2000), a deleted A region (Ahern et al., 2001) or an altered A region sequence (Wilkens et al., 2001). However, as discovered by Ahern et al. (2001) the C region does not contain all of the pieces in the puzzle concerning skeletal type ECC (see section 1.2.5). This was highlighted in a deletion study when both the A and the C regions were deleted from the DHPR, moderate voltage evoked ECC was restored (Ahern et al., 2001). In the absence of the A region alone, skeletal type ECC was observed, but skeletal type ECC was no longer present in the absence of the C region alone.

In past studies peptides corresponding to regions of the DHPR II-III loop have been used to examine interactions between the A and C regions of the skeletal DHPR II – III loop and the RyR. These findings have revealed several interesting findings. The A peptide is a high affinity activator of the RyR (Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Casarotto et al., 2000; Lamb et al., 2000; Casarotto et al., 2001; Stange et al., 2001; Green et al., 2003). Initially the C peptide was shown to
inhibit the activating ability of the A peptide (see section 1.2.6). As these two peptides contained a net overall charge difference (peptide A was positively charged, the C peptide negatively charged) it was possible that the two peptides were simply interacting and this prevented peptide A from activating the RyR1. Through the use of co-sedimentation studies it was shown that this was not the case (Haarmann et al., 2003) and the precise mechanism responsible for the C peptides ability to inhibit the activation of the A peptide is still unknown. More recently, the C peptide has also been shown to produce moderate activation or inhibition of the RyR1 incorporated in lipid bilayers depending on the concentration used (see section 6.1). The peptide comprised of the residues spanning the C region was found to contain a random coil structure (see section 6.3). It is likely that when this region is incorporated within the complete DHPR it may contain a more stable conformation and that this may enhance its ability to activate a release of Ca\(^{2+}\) through the RyR1. Preliminary studies of the entire II – III loop suggests that the C region is random coil (unpublished observations). This agrees with structure prediction studies which suggests that the skeletal C region is random coil, while the cardiac C region is more helical and that mutations that increase the helical nature of the C region abolish skeletal ECC (Flucher, B. Biophysics abstracts, 2003). The mechanism of activation is likely to be different from that with the A region peptides, as the C region does not contain the cluster of positively charged residues found to be critical for activation of peptides derived from the A region of the skeletal DHPR II – III loop (see section 3.4). Further to this, when the C region is contained within the complete DHPR there may be other portions on the cytoplasmic loops and other targeting factors/proteins which assist in positioning the C region close to its activation site.
As the results obtained from peptide studies have varied in some respects from those expected from chimera and deletion studies, there is caution required when assessing the physiological significance of such findings. The results obtained from studies using peptide probes presented in this thesis have identified at least one mode of activation of the RyR1. The sequence of the peptides used were identical or closely resemble the native sequence of the skeletal DHPR II – III loop and it is therefore possible that the II – III loop may activate the RyR1 via the same mechanism. The activating ability of a peptide derived from the A region of the skeletal DHPR II – III loop has been shown to be determined by the number of critical positive amino acids (Arg$^{681} -$ Lys$^{685}$) and the amount of secondary structure present (see chapters 3 and 4). As several of the peptides investigated in this thesis have adopted some form of secondary structure, it is likely that the secondary structure determined is energetically favorable and that it will be maintained within the tertiary structure of the II – III loop (Casarotto, unpublished observations) and the presence of these regions may be involved in interactions between the II-III loop and the RyR (these interactions may or may not be associated with ECC per se). These findings have increased the possibility that the A region within the II – III loop may play a role in the interaction between the DHPR and RyR1 in vivo.

As it appears the A region does not play a direct role in eliciting skeletal type ECC it may have an alternative role in manipulating the RyR1. If in the complete DHPR, the A region of the II – III loop is able to make contact with its site of activation on the RyR1 it may possibly be one mechanism modulating the release of Ca$^{2+}$ through the RyR1. As the sequence of the cardiac and skeletal A regions are quite similar (the C-terminal end of the cardiac A region, residues 803 - 812 is able to activate the RyR1
(Lamb et al., 2000)) there is a possibility that the A region plays a non-tissue specific function. The potential role of the A region as a non-tissue specific regulator of the RyR would be dependent on a physical coupling existing between the RyR and DHPR in cardiac muscle. As there is still controversy surrounding this physical interaction in cardiac muscle and the ratio of DHPRs to RyRs in cardiac muscle is about 1 to 10 (Bers and Stiffel, 1993), this potential role for the A region is unclear. Coupled with the controversy surrounding the coupling of the cardiac DHPR with RyR2 is the fact that neither the SDCL or CDCL peptides, derived from the skeletal and cardiac II – III loops, were able to activate the purified RyR2 (Lu et al., 1994). However recent studies show that SDCL and CDCL interact functionally with native RyR2, containing its associated proteins (Dulhunty, unpublished observations). This observation supports a non-tissue specific function of the A region of the II – III loop. Nevertheless, the physiological importance of the A region from the skeletal DHPR II – III loop is still unclear. However it is becoming increasingly clear that the A peptides have useful roles as probes of RyR function (El-Hayek and Ikemoto, 1998; Dulhunty et al., 1999; Gurrola et al., 1999; Zhu et al., 1999; Casarotto et al., 2000; Lamb et al., 2000; Casarotto et al., 2001; Stange et al., 2001; Green et al., 2003).

The ability of the A peptides, IpTx₃ and maurocalcine to activate the RyR1 has been shown to be dependent on the presence of a cluster of several positively charged residues with similar alignment along one face of the molecule (see section 1.2.6 and section 4.1) Thus it has been suggested that these molecules may all be activating the RyR1 via a common binding site. Another critical residue in each of these molecules has been found to be the Ser or Thr molecule located two residues upstream of the cluster of positively charged residues. The presence of this residue has been shown to
be important in the ability of the peptides to modulate Ca$^{2+}$ release through the RyR1. In the case of the recombinant SDCL, IpTxA and maurocalcine the removal of this critical Ser or Thr residue has decreased the peptides ability to activate the RyR1 (see section 1.2.6 and section 4.1). In contrast to this, the removal of the corresponding Ser residue from the A peptide (corresponding to the A2 peptide) resulted in a more active peptide and the secondary structure of the A2 peptide was found to contain a more regular helical structure compared to the A peptide. Within all of the studies conducted in this thesis an increased alpha helical secondary structure in a peptide comprised of the complete A region peptide, has produced a more active peptide, potentially explaining the increased activity seen with the A2 peptide. However, in the case of SDCL, the removal of the critical Ser residue did not alter the ability of the peptide to bind to the RyR1 (Lu et al., 1995) and therefore a reduced amount of secondary structure may be responsible for the decreased activation seen in its absence. Alternatively a structural change may have allowed negatively charged residues in other regions of the loop (of which there are many) to mask the activating positive charges in the A region.

The tertiary structure of maurocalcine has been shown to be very stable due to several intermolecular disulphide bonds (Mosbah et al., 2000) and it would be predicted the structure for IpTxA would be very similar as these molecules share an 83 % homology. The cluster of positively charged residues critical for the activating ability of the molecule were shown to be located on a portion of double stranded anti-parallel beta sheet. In the very stable tertiary structure present in maurocalcine it seems implausible that the removal of the Thr residue would significantly decrease the amount of secondary structure present in the toxin and therefore a potential role for the critical
Thr residue maybe to facilitate binding of the toxin to its binding site. If this was the case then it would appear that despite the similar functional requirements for the A peptide and IpTx₅ and maurocalcine, i.e. a cluster of positively charged amino acids, they could potentially activate at different sites. Further evidence supporting this theory has been provided in recent findings by Chen et al. (2003) who identified distinct gating properties of single RyR1 channels incorporated in lipid bilayers induced by peptide A and maurocalcine (see section 4.1).

In our laboratory it has recently been shown that IpTx₅ has three independent actions on the RyR1. IpTx₅ was shown to produce the prolonged substate levels of activity, to increase transient opening (concentrations between 200 pM and 10 nM) and to decrease transient openings (concentrations between 100 nM and 100 µM) (Dulhunty et al., 2004). Further to these findings it was shown that peptide A compete with IpTx₅ for activation of transient openings (Dulhunty et al., 2004). These findings indicate some form of competition between IpTx₅ and the A peptide for at least one of the effects of IpTx₅ and provide support for the two molecules interacting with the RyR1 via a single site.

The functional differences noted between the scorpion toxins (IpTx₅ and maurocalcine) and the A1, A1(D-R18) and AB peptides are most likely explained by the structural stability of the molecules. Molecules possessing the acquired number and spacing of positively charged amino acids and a high degree of structural stability are the most potent activators of the RyR1. It is also possible that these peptides activate the RyR1 via different sites of activation. These two activation sites on the RyR1 are activated by peptides containing an exposed positively charged segment.

The experimental data indicates that both IpTx₅ and peptide A have multiple sites of activation including low and high affinity sites. Results from single channel bilayer (Dulhunty et al., 2004) and [³H] ryanodine binding (Gurrola et al., 1999) experiments have both indicated some form of competition between IpTx₅ and peptide A induced activation of RyR1. Thus it seems likely that IpTx₅ and peptide A share at least one common binding site, however, the physiological importance of the shared binding site(s) is still unclear.

The highest affinity binding site for IpTx₅ was identified by Samso et al (1999) and was 11 nm away from the transmembrane pore, in between the handle and clamp regions of the RyR1 (see Figure 4.2). Given the location of the highest affinity IpTx₅ binding site, the length of the II-III loop (126 amino acids) and the dimensions of the triadic junction (approximately 12 nm across), the tertiary structure of the II-III loop would need to be stretched or elongated in order to traverse the triadic junction and bind to the IpTx₅ binding site on the RyR1. In addition to this the sequence of the A region has been found to be located only 5 amino acids away from what is thought to
decrease transient openings (concentrations between 100 nM and 100 μM) (Dulhunty et al., 2004). Further to these findings it was shown that peptide A competed with IpTx4 for activation of transient openings (Dulhunty et al., 2004). These findings indicate some form of competition between IpTx4 and the A peptide for at least one of the effects of IpTx4 and provide support for the two molecules interacting with the RyR1 via a single site."

The functional differences noted between the scorpion toxins (IpTx4 and maurocalcine) and the A1, A1(D-R18) and AB peptides are most likely explained by the structural stability of the molecules. Molecules possessing the acquired number and spacing of positively charged amino acids and a high degree of structural stability are the most potent activators of the RyR1. It is also possible that these peptides activate the RyR1 via different sites of activation. These two activation sites on the RyR1 are activated by peptides containing an exposed positively charged segment.

The experimental data indicates that both IpTx4 and peptide A have multiple sites of activation including low and high affinity sites. Results from single channel bilayer (Dulhunty et al., 2004) and [3H]ryanodine binding (Gurrula et al., 1999) experiments have both indicated some form of competition between IpTx4 and peptide A induced activation of RyR1. Thus it seems likely that IpTx4 and peptide A share at least one common binding site, however, the physiological importance of the shared binding site(s) is still unclear.

The highest affinity binding site for IpTx4 was identified by Samso et al (1999) and was 11 nm away from the transmembrane pore, in between the hand e and clamp regions of the RyR1 (see Figure 4.2). Given the location of the highest affinity IpTx4 binding site, the length of the II-III loop (126 amino acids) and the dimensions of the triadic junction (approximately 12 nm across), the tertiary structure of the II-III loop would need to be stretched or elongated in order to traverse the triadic junction and bind to the IpTx4 binding site on the RyR1. In addition to this the sequence of the A region has been found to be located only 5 amino acids away from what is thought to be a transmembrane domain of the DHPR, i.e. very close to the beginning of the transmembrane spanning segment between repeats II and III of the skeletal DHPR. Therefore, if peptide A does have a binding site of physiological significance, it is highly unlikely that it is located at the IpTx4 binding site identified by the Samso et al. (1999). Further difference mapping cryo-electron microscopy studies investigating the binding location(s) of IpTx4 and peptide A will resolve the question regarding a common physiological meaningful site of activation for IpTx4 and peptide A.

restrained in a certain spacing and structure. The possibility of two activation sites could also potentially explain the large difference in activation levels obtained with the A1, A1 (D-R18) and AB peptides and maurocalcine and IpTx\textsubscript{a}. Strong activation of Ca\textsuperscript{2+} release is noted with nanomolar concentrations of maurocalcine and IpTx\textsubscript{a} while micromolar concentrations of the A1 peptide are required to produce a small to moderate increase in RyR1 activity.

The A1(D-R18) peptide was a more potent activator of Ca\textsuperscript{2+} release through the RyR1 compared to the A1 peptide and it was shown to contain an alignment of positively charged residues along one surface of the peptide with similar spacing to maurocalcine and this can be seen in Figure 7.1. A plausible explanation for the increased activity noted with the A1(D-R18) peptide is because of the increased secondary structure found within the peptide. An alternate hypothesis could take into account the possibility of two binding sites for the molecules concerned (see above). As the spacing of the positively charged residues now mimics that of maurocalcine, the possibility that the A1(D-R18) peptide now activates the RyR1 via the same activating site as maurocalcine, is increased. An interesting experiment would be to gauge the effects of the Thr and Ser residue previously found to be important in the activating ability of IpTx\textsubscript{a} and maurocalcine in the A1(D-R18) peptide. Would the removal of this residue increase the activating ability of the peptide, as with the A1 peptide, or decrease the activating ability of the peptide as shown with IpTx\textsubscript{a}? Considering the Arg residue in position 18 of the A1(D-R18) peptide has been implicated in significantly increasing the activating ability of the peptide (see section 4.4), it is unlikely that the Ser residue would have the same effect it had in maurocalcine, as it is actually located within the cluster of critical positively charged
Figure 7.1 Average 3D solution structures determined by $^1$H NMR for the A1 peptide, the A2 peptide, the A1(D-R18) peptide, the AB peptide and maurocalcine. Positively charged side chains are shown using 'stick and ball' models.

residues and in alpha helical secondary structure. In maurocalcine, the critical Thr residue is located two residues downstream of the cluster of positively charged residues and contained within beta-sheet secondary structure. As the A1(D-R18)
peptide contains similarly spaced positively charged residues without a Ser or Thr residue located downstream, it is possible that it is now acting in a similar manner to that shown with IpTx₉ in the absence of the critical Thr residue, i.e. still able to activate the RyR1, but not as potently as in the presence of the Thr residue.

It was discovered that the AB peptide was not able to activate the RyR1 due to the overall charge of the peptide. When several of the negatively charged residues located in the C-terminal end of the peptide were mutated out (forming the AB-ch peptide), the peptide was converted into a potent activator of the RyR1 (see section 5.4). The large number of positively charged side chains present on the AB peptide can be seen in Figure 7.1. There is suitable evidence from NMR and CD studies to suggest that the secondary structure present within the AB-ch peptide is very similar to that contained within the AB peptide. Thus in the case of the AB-ch peptide, the average structure present in Figure 7.1 can be used as a fairly accurate description of the positively charged side chains present in the peptide, which now contains two positively charged helical regions rather than a positive and negative helical region in the AB peptide. In the presence of the flexible linker region, found in the AB peptide, the AB-ch peptide could adopt conformations with positively charged side chains in positions similar to those identified in maurocalcine. This could be a potential explanation for the increased activation seen with this peptide compared to the Al peptide. The functional differences noted between the AB-ch peptide and maurocalcine (activation is noted at much lower concentrations with maurocalcine) may again be a result of the increased structural stability present in maurocalcine. Studies investigating the gating mechanisms of the Al(D-R18) and AB-ch peptides
and the scorpion toxins will shed more light on the possibility that these peptides may activate the RyR1 via two activation sites.

The studies conducted in this thesis have confirmed that peptides derived from the native sequence of the skeletal DHPR can be useful in investigating the mechanisms responsible for activation of the RyR1. It was found that peptides derived from within the A region of the skeletal DHPR II – III loop required the presence of several positively charged residues as well as some degree of secondary structure to activate the RyR1. The substitution of the D-isomer rather than the L-isomer in the Arg residue (position 18) of the A1 peptide created a significantly more potent activator of the RyR1, potentially by mimicking the mechanism of activation seen with the scorpion toxins, IpTx4 and Maurocalcine. The addition of 20 amino acids, derived from the native sequence of the DHPR, to the C-terminal end of the A1 peptide created a peptide which was unable to activate the RyR1 (the AB peptide), despite the secondary structure of the N-terminal end of the peptide remaining unchanged. Mutations of several negatively charged residues in the C-terminal end of the AB peptide resulted in a peptide that was able to cause a potent release of Ca\(^{2+}\) through the RyR1. These findings have identified that the structural stability of a peptide containing the required array of positively charged amino acids is highly correlated with its ability to activate the RyR1.

Although it has been suggested, based on chimera studies in myocytes, that the A peptide region of the skeletal muscle DHPR does not play an important physiological role, the actual physiological importance of the A region in ECC is still uncertain. It is possible that mutation of the A peptide region of the II – III loop by sequence scrambling, replacement to non-functional sequence, or deletion, causes some compensatory mechanism(s) to activate ECC, so that the A region is seemingly unimportant. Therefore the true role of the A region in the full DHPR protein ECC in a functioning muscle cell remains unclear.
Chapter 8 - References


