Production of ryanodine receptor calcium release channel ATP-binding site mutants

Catarina Cutter

Dr. Edward Balog – Ryanodine Receptor Lab
Georgia Institute of Technology

Abstract

Ryanodine receptors (RyRs) are a class of mammalian ion channels which are the primary efflux pathways for the release of Ca^{2+} from the sarcoplasmic reticulum. They play a critical role in muscle excitation-contraction coupling (ECC). Because it is the largest known ion channel, the mechanisms for its activation are not fully understood. ATP is a well characterized channel activator. However, its mechanism of activation has not been determined and the importance of ATP regulation of RyRs *in vivo* is not clear. In 2016, des George, *et al*. published a structure of RyR1 with ATP bound. The adenosine group of ATP is contained within a hydrophobic cleft while the triphosphate tail is extended and interacts with positively charged residues. The goal of this study was to identify residues important for ATP binding to the channel. Site-directed mutagenesis of the receptor was used to substitute specific residues in order to change their size and or charge. After transfection with recombinant DNA, HEK293 cells were harvested for isolation of microsomal membranes. Two of the largest hydrophobic residues of the cleft were replaced with alanine with the goal of drastically reducing or abolishing ATP binding to RyR1. The selected mutations F4960A and L4985A were expected to impair channel activation by both ATP and adenosine. After initial verification of wild type channel expression in HEK293 cells, later transfections with wild type and mutant RyR1 DNA failed to produce detectable amounts of protein. Low DNA transfection efficiency combined with the low yield of microsomal membrane likely contributed to the inability to detect channels in these preparations. Optimizing DNA transfections and scaling up the cell culture may increase the likelihood of successful protein production.

Introduction

Ryanodine receptors (RyRs) are a class of intracellular ion channels which mediate the efflux of calcium (Ca^{2+}) from intracellular stores. They are the largest intracellular ion channels known. There are three isoforms: RyR1 is predominant in skeletal muscle cells, RyR2 is predominant in cardiac muscle cells, and RyR3 is found at low levels in numerous cell types, generally with one or more of the other isoforms (Fill & Copello 2002). Because of their significant physiological function, germ-line mutations in RyRs cause multiple serious medical conditions. Mutations in RyR1, for example, may lead to malignant hyperthermia (MH), a potentially fatal adverse reaction to volatile anesthetics (Litman, 2018).

In cardiac and skeletal muscle, RyRs are essential for excitation-contraction coupling (E-C coupling), the process by which an action potential triggers Ca^{2+} from the sarcoplasmic reticulum (SR). E-C coupling is the process by which transverse-tubule (t-tubule) depolarization is linked to Ca^{2+} release from the SR. In skeletal muscle, depolarization of the t-tubule causes a reorientation of L-type Ca^{2+} channels, which in turn opens RyR1 in the SR via a physical coupling between the two channels (Meissner, 1994). ATP has been shown to be both an energy source and regulatory molecule for E-C coupling *in vivo*. In a study of fast-twitch muscles in rats, Dukta and Lamb exhibited that lowering the concentration of ATP below ~0.5 mM reduced the depolarization induced calcium release (Dukta, Lamb 2004). This study concluded that ATP is a limiting factor for normal activation of RyR with low concentrations of it inhibiting E-C coupling. S-Adenosyl-L-methionine (SAM) has been used to probe the adenine nucleotide binding site of RyR2, showing a drastic reduction in channel openings and conductance (Kampfer, Balog 2021).

In vitro, RyRs are regulated primarily by Ca^{2+}; they are activated by micromolar Ca^{2+} and inhibited by millimolar Ca^{2+}. RyR Ca^{2+} sensitivity can be modulated by numerous intracellular constituents. Previously, research into the physiology of RyRs has focused on discerning the effects of various ligands on the channel. ATP has been widely studied as a ligand that affects the ability of the channel to open, duration of channel opening, etc. The efficacy of ATP in activating ryanodine receptors has been widely studied. It was found that the adenine ring structure was necessary for the activation of RyR2, the cardiac ryanodine receptor (Chan *et al*., 2000). This was determined because the addition of GTP lead to no activation of channel gating. This study also determined that the large electrostatic force created by the phosphate groups attached to adenosine play a role in the strength of the activation. The conclusion was that less phosphate groups on the adenine-binding ligands (ATP > ADP > AMP) means a less efficient activation.

Further work compared various adenosine-binding ligands by studying the activation of the channel with multiple ligands present. One study showed that ATP and ADP compete for the same binding site, and ATP is the better competitor (Kermode, 1998). A later study confirmed the earlier assumption that the large electrostatic field around the phosphate groups of ATP stabilized the open state of the channels (Chan *et al*., 2003). Although ATP is the most effective ligand at recruiting long open states, this study proved that the structural properties for tight binding at the activation site are different from those required for efficient channel opening. There are questions left about the mechanisms of ATP binding as this study showed that the electrostatic field of the phosphate tail is not the only determining factor.

While RyRs are regulated primarily by Ca^{2+}, Ca^{2+} activation of the channel is modulated by other ions including Mg^{2+} and H^{+}, small molecules such as adenine nucleotides, proteins such as the Ca^{2+}-binding protein calmodulin and posttranslational modifications including phosphorylation, nitration, S-nitrosylation, and oxidation (Meissner, 1994). Research on RyR's has focused on primarily establishing what ligands affect receptor activation and elucidating the protein's structure. ATP is a well-researched ligand that acts to recruit long open states (Chan et al., 2003). Significant research has determined the efficacy of ATP in comparison to other agonists/ligands (Chan et al., 2000; 2003; Kermode, 1998), but research has yet to define the specific mechanisms by which ATP acts on the RyRs. Germ-line mutations in RyRs are linked to multiple serious medical conditions including potentially lethal cardiac arrhythmias (RyR2) and adverse reactions to anesthetics (RyR1) (Litman, 2018). Elucidating the specific regulatory sites that control channel opening, as well as their mechanisms, will help determine if those sites can be targeted for pharmacological treatments.

In recent years, improvements in biotechnology have allowed researchers to elucidate the structure of the RyR protein at resolutions allowing visualization of amino acid sidechains. Specifically, the cryo-EM techniques are used to form images of the ryanodine receptor due to its large size and constant motion (Efremov *et al*., 2015). One of the earlier studies using this technique took images of closed-state receptors only (no activating ligands were present) (Zalk *et al*., 2015). This study focused on defining the transmembrane pore, and it also defined interactions between transmembrane helices and paired EF-hands. The more relevant study was done in 2016. Cryo-EM techniques were used again, but this time with a focus on determining the structural basis for channel opening of the RyR1 (des Georges *et al*., 2016). This research compared activated and non-activated receptor images to determine the structural changes that take place during activation. The comparison also located the binding sites for RyR activating ligands such as calcium, caffeine, and ATP. The composite images, which can be analyzed in a protein modeling application, express

interaction between the ligands and specific residues in the ligand's binding site. These interactions are mostly theoretical based on the program analyzing many images of "frozen" receptors, but they provide a starting point as to what residues are more likely to be interacting with the ligands than others. Examination of the ATP-binding site showed the adenosine moiety in a hydrophobic cleft formed by Met4954, Phe4959, Thre4979 and Leu4985. ATP's phosphate tail interacted with three positively charged residues Lys4211, Lys4214 and Arg4215. These specific residues now have sufficient evidence of interaction with the activating ligands to allow for research into whether they are involved with the mechanism of channel opening.

Methods

This project focused on the RyR1 isoform, which is found predominantly in skeletal muscles. The pClneo vector containing a full-length RyR1 insert, pClneo_RyR1 [Figure 1] was a gift from Paul Allen & Robert Dirksen (Addgene plasmid # 75113 ; http://n2t.net/addgene:75113 ; RRID:Addgene_75113). The ATP binding site is composed of two non-contiguous regions, an adenosine-binding site and a tri-phosphate interacting region (Figure 1 highlighted in blue). The adenosine-binding site is flanked by BstZ17I and XbaI restriction sites.

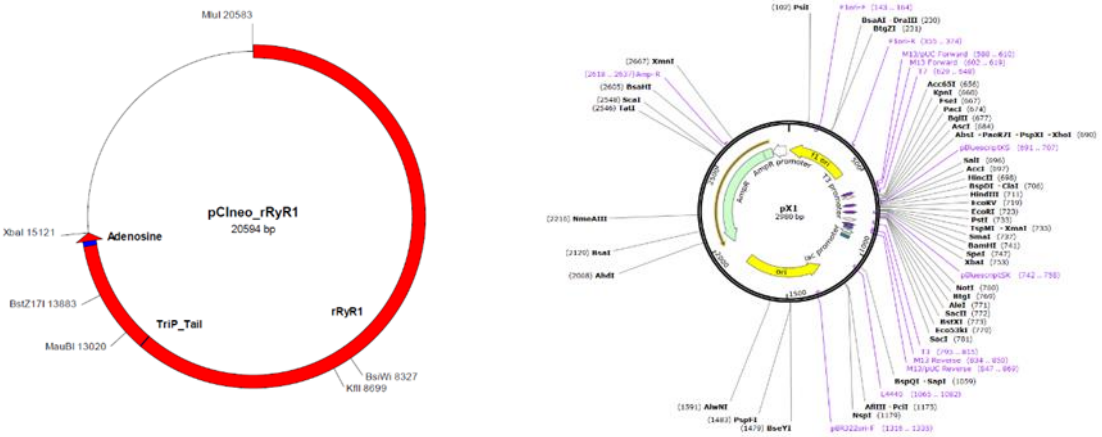

Figure 1, Vectors: These are models of the pClneo_rRyR1 (left) and pX1 (right) vectors that show the relative locations of their restriction sites. This image does not include BstZ17I as this was added later to the multiple cloning site). The section in blue on the left image is the target DNA for the adenosine binding pocket. Both images from AdGene.

The first goal was to subclone the BstZ17I-XbaI fragment of RyR1 coding for adenosine binding region of the ATP binding site into pX1 [Figure 1]. The pX1 plasmid was a gift from Monica Hollstein (Addgene plasmid # 46848 ; http://n2t.net/addgene:46848 ; RRID:Addgene_46848). This plasmid contained the XbaI restriction site. The BstZ17I restriction site was added to pX1 by the lab. Both the pX1 plasmid and the pClneo_rRyR1 plasmids were separately digested at the given restriction sites. The digest products were run via gel electrophoresis to separate the fragments. These target fragments were stained with ethidium bromide so they could be visualized under UV light [Figure 2]. This figure shows the digested pCIneo in lanes 2 and 3. After locating the target fragments on the gel based on expected relative size and location, the fragments were cut out of the gel and recovered using the Zymoclean Gel DNA recovery kit.

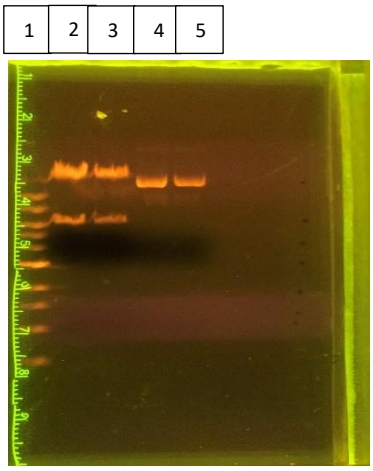

Figure 2: This gel exhibits the separation of PClneo_rRyR1 from the target fragment, which is the smaller and further along product in wells 2 and 3 (around mark 4.5).

Once both fragments were separated and purified, the pCIneo_RyR1 adenosine fragment from Bst17I to XbaI was subcloned into the fragment of pX1 to produce a functional pX1 plasmid containing the inserted adenosine binding DNA fragment.

The target residue within the fragment was selected based on des Georges research into the gating and activation of RyR1 (2016). des Georges hypothesized numerous residues to be involved with binding of adenosine to RyR1. Previously in this project, the mutation F4960A was created in the same target region. L4985 was selected as the second target for manipulation due to its large size and hydrophobic qualities. Primers to be used for the mutagenesis were designed using PrimerX (https://www.bioinformatics.org/primerx/) [Figure 3]. The figure containing the

primers exhibits the particular mutation that converted the Leucine reside to Alanine: AG1774GC (noted by the asterisks *).

```
Primer pair 1
                                **
    Forward: 5' GCTAGAGGAGCACAATGCGGCCAATTACATGTTC 3'
    Reverse: 5' GAACATGTAATTGGCCGCATTGTGCTCCTCTAGC 3'
                                **
     GC content: 50.00%           Location: 131-164
     Melting temp: 76.1°C         Mismatched bases: 2
     Length: 34 bp                Mutation: Substitution
     5' flanking region: 16 bp    Forward primer MW: 10475.93 Da
     3' flanking region: 16 bp    Reverse primer MW: 10408.88 Da
```

Figure 3, Primers: Details of the primers ordered as calculated on the website PrimerX

Site-directed mutagenesis was performed using the QuikChange II kit and the designed primers on the pX1 containing the previously created F4960A mutation in the adenosine binding site. The DNA was sequenced to determine the accuracy of the mutation produced [Figure 5A]. XL1Blue E. coli were transformed with the pX1 containing the double mutant fragment. Large quantities of pX1 were produced by expanding these cultures through a MaxiPrep protocol. The pX1 was extracted from the cultures and purified using the Qiagen DNA purification kit. The double mutant fragment was then cut out of the pX1 plasmid and ligated back into the pClneo_rRyR1 so that the whole ryanodine receptor could be produced with the two mutations present.

Both wild type and F4960A /L4985A double mutant RyR1 channels were expressed in mammalian HEK293 cells. These cells underwent plasmid transfection using the calcium phosphate precipitation method (Chen and Okayama, 1987). HEK293 cells were plated in 8-75 cm^2 flasks at 4 X 10^6 cells per flask 24 hrs prior to transfection. Cells were transfected with 5 μg DNA per 1 X 10^6 cells and incubated for 24 hrs. Media with fresh complete Dulbecco's modified Eagle's medium containing 2 mM sodium butyrate to enhance protein expression. After 24 hours of incubation, cells were harvested for microsome membrane preparations. The cell pellet was resuspended a buffer containing 20 mM tris-malate buffer pH 7.4, 1 mM EDTA and protease inhibitor. Cells were passed a 23-gauge needle 20X and homogenized by 30-40 strokes of a glass homogenizer. The homogenate was diluted with and equal volume of buffer and homogenized a second time. After centrifugation at 4000Xg the supernatant was centrifuged at 40,000Xg and the pellet resuspended in 0.2 mL buffer containing 250 mM sucrose, 10 mM MOPS pH 7.4, 1 mM EDTA and 1 mM dithiothreitol. With these samples collected, a western blot was performed with RyR1

specific antibodies (34C mouse anti-RyR monoclonal IgG; obtained from the Developmental Studies Hybridoma Bank) to confirm the presence and relative concentration of RyR1 channels in the samples [Figure 8].

Single channel electrophysiology was performed as previously described (Kampfer and Balog, 2021). Briefly, planar lipid bilayers were formed by painting a lipid mixture (phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine) across a 100 - 250 μm aperture in a Delrin cup. Microsome vesicles were added to the cis chamber which contained 250 mM cesium methanesulfonate (CsMs), 10 mM MOPS pH 7.0, and 1 mM $CaCl_2$. The trans chamber contained 50 mM CsMs and 10 mM MOPS pH 7.0. After incorporation of channels into the bilayer, the osmotic gradient was abolished by raising the concentration of CsMs in the trans chamber to 250 mM and the cis chamber Ca^{2+} concentration adjusted by adding small aliquots of concentrated EGTA and $CaCl_2$. Voltage was controlled and channel currents were recorded using an Axoclamp 200B patch clamp amplifier (Molecular Devices) with the trans chamber held at virtual ground. Currents were low-pass filtered at 1 kHz with an 8-pole Bessel filter, monitored on an oscilloscope, and digitized 5 kHz. Channel data were collected using a pulsing protocol in which the potential was held at 0 mV for 120 ms between 2-s duration steps to negative and positive potentials, generally -50 and + 50 mV (CLAMPEX program, pCLAMP 9.2 software, Molecular Devices, Sunnyvale, CA).

Results

Based on the research by des Georges, the adenosine binding site on the RyR1 channels was visualized with the ATP molecule inside the cleft. Using the interaction theorized by this study, mutations F4960A and L4985A were selected as targets in the adenosine binding site. These residues in the wild type RyR1 are large hydrophobic proteins whereas alanine (the substitute) is small and relatively neutral. Figure 4 shows the adenosine head in blue and green in close contact with the L4985 residue.

Figure 4, ATP binding visualiztion: L4985 is highlighted in magenta, showcasing its proximity to the adenosine head of ATP in the binding site (blue and green striped)

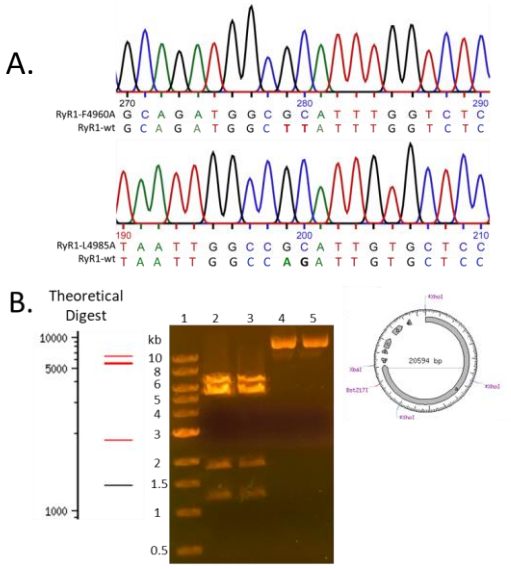

Figure 5: Production of the F4959A/L4985A double RyR1 mutation.
A. Chromatograph showing mutations pX1$_{Bstz17I\text{-}XbaI}$ TT1696GC and AG1774GC.
B. Gel showing fragments resulting from Xhol, BstZ17I and XbaI digest of wt or TT1696GC/AG1774GC RyR1 cDNA in pCIneo. Lane 1, std; lane 2: digested pCIneoRyR1$_{wt}$, lane3: digested pCIneoRyR1$_{TT15468GC/AG15547GC}$, lane 4: undigested pCIneoRyR1$_{wt}$, lane5: undigested pCIneoRyR1$_{TT15468GC/AG15547GC}$.

After site-directed mutagenesis of the single mutations in the pX1 plasmids, both mutations were individually confirmed through sequencing. The chromatograph shown in figure 5A confirms that the individual mutations TT1696GC to produce F4960A and AG1774GC to produce L4985A were successfully completed in the samples. After this confirmation, the double mutant pX1_RyR1 was produced containing both F4960A and L4985A. The BstZ17I-XbaI fragment containing the two mutations was subcloned from pX1 back into pCIneoRyR1. Subcloning was verified by diagnostic digest using the restriction enzymes Xhol, BstZ17I and XbaI [Figure 5B]. This digest confirmed without sequencing that no major cuts or rearrangements occurred during the subcloning of the fragments since the WT and the mutant pCIneo_RyR1 plasmids all appeared the same after digestion.

The wild type and double mutant channels were expressed in HEK293 cells, and a microsome prep protocol extracted the desired proteins from these cells. These extractions were fairly low-yield, with an average of 8-10 T-75 flasks of confluent cells only producing 200 µl of microsomes.

A protein assay for the purified WT protein completed on the microsome preps [Figure 6]. The results from this assay are shown in Figure 7 and allowed for an approximation of the concentration of protein present in the sample.

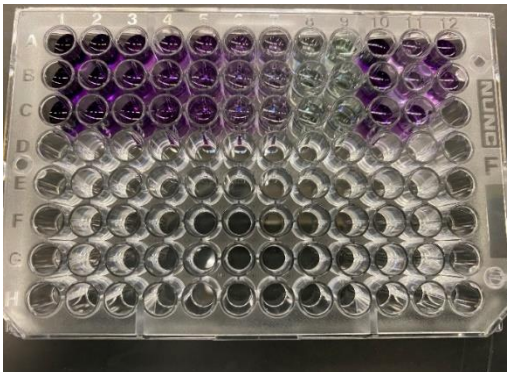

Figure 6, Protein Assay: This gel assay visually confirms the presence of protein in the sample. The control is lane 9, which is DI water.

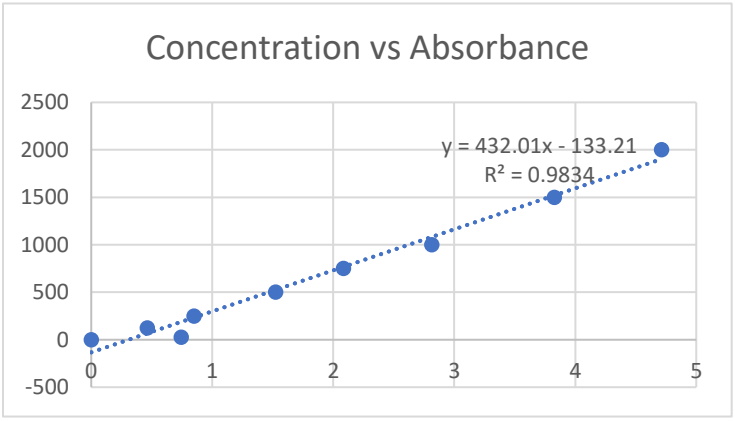

Figure 7, Concentration vs. Absorbance: These [] vs Abs results from the assay allow the rough calculation of the amount of protein present in the purified sample (this is for the WT proteins).

After collection of the microsome preps, multiple western blots of various sample aliquots were attempted to prove the presence of the RyR1 channels. A successful western blot procedure confirmed the presence of WT RyR1 in the sample using RyR1-specific antibodies [Figure 8]. The lab was not able to successfully confirm the presence of the double mutant channels [Figure 9]

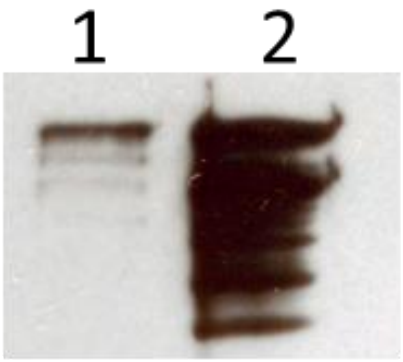

Figure 8, Western Blot WT: Western blot of wtRyR1 expressed in HEK293 cells (lane 1) and wtRyR1 derived pig skeletal muscle (lane 2). Note than lane 2 was over loaded resulting in multiple bands.

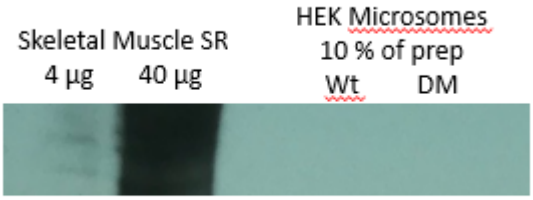

Figure 9, Western Blot WT/DM: The western blot shows 2 lanes of HSR prep as a control, 4µg in lane 1 and 40µg in lane 2. The blot also has wtRyR1 expressed in HEK293 cells (lane 3) and double mutant RyR1 expressed in HEK293 (lane 4), but these microsome lanes expressed no signal.

After confirmation of the presence of the wild type channels using the western blot technique, some attempts at the single channel electrophysiology tests were made. Some preliminary functional results of these tests are shown in Table 1 below. Attempts to record channel currents from microsomes containing RyR1 double mutant channels yielded a number of low conductance traces possible due to the presence of microsomal chloride channels, but no currents characteristic of RyR channels.

Table 1: Wild Type RyR Single Channel Electrophysiology

Class 1 = closed
Class 2 = open
Nevent = number events

Conditions

Cesium (mmol)	MOPS (mmol)	pH	EGTA (mmol)	Ca^{2+} (µmol)	mV
250	10	7	1	10	-50

Idealized Data Analysis

Class	Amp	Std	Occupancy	Lifetime (ms)	Nevent
1	-0.0515	1.1276	0.9289	14.7604	4589
2	-14.3922	11.4054	0.0711	1.1293	4588

Discussion

The goal of this project was to determine if adenosine binding in the hydrophobic cleft is required high affinity ATP binding. Site-directed mutagenesis was used to change ATP-interacting residues to alanine, which has the effect of removing the amino acid side chain. Altering residues of the hydrophobic cleft would reduce the affinity of the channel for adenosine nucleotides and have only minor effects on the maximal extent of channel activation by these compounds. The Phe4960Ala and Leu4985Ala mutations were chosen because of the large surface area contributed by their sidechains. Double mutant channel clones were produced through site-directed mutagenesis which allows control over the specific nucleotide changes in the overall receptor sequence and limits the randomness of mutations as seen in other protocols such as mutagenic PCR (Matsumura and Rowe, 2005). Through the work done in this experiment, WT channels have been expressed through HEK293 cells. There is a potential that F4960A/L4985A double mutant RyR1 channels were successfully expressed as well, but there is not sufficient evidence for this yet. The channels were transfected with the double mutant pCIneo_RyR1 plasmid, but the western blots of these microsome preps could not confirm the presence of the channel. In addition, single channel electrophysiology experiments failed to record RyR1 currents. There are a couple potential reasons for this. It is likely that channels were expressed only a small fraction of HEK293 cells due to low transfection efficiency of the plasmids. In addition, low microsome yield resulted in a low total channel yield in the microsome prep. The microsome preps from the wild type and double mutant transfected cells have been retained and aliquoted for further testing. Optimization of the HEK cell transfection and scaling up the cell culture may increase the likelihood of producing

sufficient protein for further channel characterization. Ultimately, the production of the double mutant clone allows for future production of these channels and further experimentation to be done to determine the role (if any) that these specific residues play in ATP binding of RyR1.

The impact of these mutations will be determined through single channel recording of wild type and mutant channels. If F4960 and L4985 are involved in the binding of ATP, there should be a significant increase in the ATP required to enhance channel activation. Because this affects the adenosine binding site, these mutations should have the same effect on all derivatives of adenosine (ATP, ADP, AMP, and adenosine).

RyR ATP-binding site mutants will also be useful in providing insight into the *in vivo* role of ATP in RyR-mediated Ca^{2+} release. Since ATP is critical for numerous cellular functions, it is not feasible to manipulate cellular ATP concentrations to examine the role of ATP in Ca^{2+} release. The alternative is to alter the ATP affinity of the RyR and measure Ca^{2+} release in HEK cells.

The expected results would provide evidence that the specific residues F4960 and L4985 within the binding pocket are involved in RyR activation, thus giving a fuller picture of the mechanisms required for full and sustained activation. This research will help elucidate how specific regulatory sites affect the function of the receptor to test the effects of ATP on *in vivo* Ca^{2+} release. This work can help determine if those sites can be targeted for pharmacological treatments. With this greater understanding of the hydrophobic cleft in the RyR1 adenosine binding site, the mechanisms by which sustained channel opening is achieved will be better understood. Further studies can then focus on determining the purpose of the phosphate tail binding site in conjunction with the adenosine binding site. This would provide a better understanding of the specific binding sites involved in sustained RyR1 activation. The long-term goal is to elucidate how specific regulatory sites affect the function of the receptor to determine if those sites can be targeted for pharmacological treatments of RyR diseases such as malignant hyperthermia (MH). Recent papers working with ATP binding sites in RyR1 have shown promising results to this effect. Melville et al. worked with Rycals in the ATP binding site showing an additional binding site where Rycals work cooperatively with ATP to alleviate receptor dysfunction (2022). Research on ATP and Ca^{2+} binding site mutants associated with RYR1-related myopathy showed functional differences in the mutant channels that inhibited channel opening. These successful projects provide evidence for continued with the ATP binding site mutants produced in this research. ATP concentration affects a multitude

of cellular processes, so RyR research will also lay the groundwork for understanding ATP's role in Ca^{2+} regulation.

References

Chan, W. *et al*. Structural factors that determine the ability of adenosine and related compounds to activate the cardiac ryanodine receptor. *British Journal of Pharmacology*. 130, 1618-1626 (2000).

Chan, W. *et al*. Structural Characteristics That Govern Binding to, and Modulation through, the Cardiac Ryanodine Receptor Nucleotide Binding Site. *Mol Pharmacol*. 63:174–182 (2003).

Chen, C. and Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol*. 7:2745-2752, 1987.

Dukta, T. L. & Lamb, G. D. Effect of low cytoplasmic [ATP] on excitation–contraction coupling in fast-twitch muscle fibres of the rat. *J Physiol*. l 560.2 pp 451–468 (2004).

Efremov, R. G. *et al*. Architecture and conformational switch mechanism of the ryanodine receptor. *Nature*. 517: 39-43 (2015).

Fill, M. & Copello, J. A. Ryanodine Receptor Calcium Release Channels. *Physiol rev*. 82: 893–922, 2002; 10.1152/physrev.00013 (2002).

des Georges, A. *et al*. Structural Basis for Gating and Activation of RyR1. *Cell*, 167:145–157 (2016).

Kampfer, A. J. & Balog, E. M. Electrical polarity-dependent gating and a unique subconductance of RyR2 induced by S-adenosyl methionine via the ATP binding site. *Journal of Biochemistry*. 170(6):739–752 (2021).

Kermode, H. *et al*. The Interactions of ATP, ADP, and Inorganic Phosphate with the Sheep Cardiac Ryanodine Receptor. *Biophysical Journal*. 74:1296-1304 (1998).

Litman, R. S., *et al*. Malignant Hyperthermia Susceptibility and Related Diseases. *Anesthesiology*, 128(1), 159-167 (2018).

Matsumura, I. & Rowe, L. A. Whole Plasmid Mutagenic PCR for Directed Protein Evolution. *Biomolecular Engineering*, vol. 22, no. 1, pp. 73–79., doi:10.1016/j.bioeng.2004.10.004 (2005).

Meissner, G. Ryanodine receptor/ca2 + release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol*. 56:485-508 (1994).

Melville, Z. *et al*. A drug and ATP binding site in type 1 ryanodine receptor. *Structure*. 30: 1025-1034. (2022).

Nakai, J. et al Two regions of the ryanodine receptor involved in coupling with L-type Ca^{2+} channels. *J Biol Chem.* 273:13403-13406, 1998.

Wei, Q.X. et al Rapid derivation of genetically related mutants from embryonic cells harboring a recombinase-specific Trp53 platform. *Cell Cycle.* 10:1261-1270, 2011.

Yuan, Q. *et al.* RyR1-related myopathy mutations in ATP and calcium binding sites impair channel regulation. *Acta Neuropathologica Communications.* 9:186. (2021).

Zalk, R. *et al*. Structure of a mammalian ryanodine receptor. *Nature*. 517: 44-48. (2015).