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Calcium and Redox Control of the Calcium Release Mechanism of Skeletal and
Cardiac Muscle Sarcoplasmic Reticulum

by

Laura Jean Owen

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Physics

Thesis Committee:
Jon Abramson, Chair
Robert Strongin
Drake Mitchell

Portland State University
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Abstract

The sarcoplasmic reticulum is an internal membrane system that controls the Ca^{2+} concentration inside muscle cells, and hence the contractile state of both skeletal and cardiac muscle. A key protein that regulates the Ca^{2+} concentration in this membrane is known as the calcium release channel (CRC). The effects on Ca^{2+} dependent activation is of major importance in the study of CRC since other channel modifiers cannot effect the channel in the absence of Ca^{2+} , or they require Ca^{2+} for maximum results. In this study of the high-affinity Ca^{2+} binding site, expected increases in total binding and shifts in the sensitivity of the channel to Ca^{2+} were observed when the pH increased or the solution redox status became more oxidative. Ranolazine, a drug used for treating Angina Pectoris (chest pain), desensitized the cardiac CRC activation but had no effect on the skeletal CRC. This selective desensitization may be the cause of Ranolazine's beneficial therapeutic effects. Both Ranolazine, and homocystein thiolactone (HCTL), a naturally occurring derivative of homocysteine, alters Ca^{2+} dependent activation by calcium without changing the number of channels found in the open state. Surprisingly the effect of HCTL was observed only in a reduced redox potential which leads to speculation that the formation of an alpha-carbon radical by HCTL on the cardiac CRC only occurs if select thiols are in a reduced state.

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Chapter 1: Introduction

1.1 The contraction of a muscle cell

Vertebrate muscle provides mechanical force upon stimulation and can be separated into three types; skeletal, cardiac and smooth. In muscle cells, filaments called myofibrils are surrounded by the sarcoplasmic reticulum (SR), a net like membrane that is periodically penetrated by the transverse tubular system (T-tubules)¹ at intervals of approximately $1.2\mu\text{m}$ in skeletal muscle fibers and $2.5\mu\text{m}$ in cardiac ventricles. The initiation of contraction is provided by an electrical excitation called an action potential that travels along the surface membrane of the muscle cell and down the T-tubules allowing the action potential to travel across the surface of the entire muscle fiber rapidly. The time that it takes skeletal muscle to contract is approximately 2-5ms, where as cardiac muscle is slightly slower taking 20-50ms.

Figure 1: The sarcoplasmic reticulum and transverse tubule system of striated muscle cells².

T-tubules are separated from the terminal cisternae³ of the SR by approximately 10nm and the interaction between them is referred to as excitation-contraction coupling (ECC). In this process three things must happen; the detection of the propagation of an action potential along the T-tubules, the transmission of the information by the dihydropyridine receptor (DHPR) to the Calcium Release Channel (CRC), and finally the release of calcium from the SR. Figure 2 shows a schematic of the flow of calcium out of the SR through the CRC where it binds to troponin at micromolar concentrations, initiating contraction. After calcium is released from the SR, the Ca^{2+} - Mg^{2+} -ATPase (Ca^{2+} pump) a 110kDa protein, pumps the calcium back into the lumen of the SR causing the muscle to relax.

Figure 2: Excitation-contraction model⁴

One of the major differences between cardiac and skeletal muscle is that ECC in cardiac muscle requires a small amount of Ca^{2+} to cross the T-tubule, whereas in skeletal muscle ECC is independent of the extracellular calcium⁵. The activation of the CRC by calcium in cardiac muscle proceeds by a mechanism known as calcium induced calcium release.⁶

1.2 Impact of abnormal Intracellular Ca^{2+} cycling

Problems with intracellular Ca^{2+} cycling have been associated with heart failure, ischemic heart disease, and in several genetic forms of arrhythmias⁷. Heart failure is characterized by abnormal intracellular Ca^{2+} cycling that leads to progressive deterioration of cardiac function. Associated with heart failure is the inability of the SR to build up an adequate Ca^{2+} load required for generating contraction, due to an increased Ca^{2+} leak associated with the RyR2 channel. In cardiac muscle there are additional proteins not found in skeletal muscle. Since Ca^{2+} enters the cell through the DHPR to initiate cardiac muscle contraction there must be a mechanism of purging Ca^{2+} from the cell. The $\text{Na}^+/\text{Ca}^{2+}$ exchange protein (NCX) moves three Na^+ ions into the cell and one Ca^{2+} ion out of the cell. This is the primary mechanism for maintain low intracellular Ca^{2+} concentrations in spite of the fact that Ca^{2+} enters the cell on each beat of the heart. An abnormal Ca^{2+} leak from the SR during the relaxation phase of the heart, when the cytosolic Ca^{2+} concentrations should be low, have been shown to cause arrhythmias known as delayed afterdepolarizations (DADs)⁸. When intracellular Ca^{2+} levels rise the NCX assists in removing the Ca^{2+} from the cell, which

results in the influx of sodium and can lead to a depolarization of the extracellular membrane. This unsynchronized action potential can generate arrhythmias. CRCs that have a diastolic Ca^{2+} leak have consequently become a therapeutic target.

1.3 Calcium Release Channel

The calcium release channel is a cation selective trans membrane protein that is thought to be a homotetramer with each of its subunits having a molecular weight of about 565kDa. These subunits are arranged in a quatrefoil shape, 22 to 27nm on each side with a 2nm hole in the center⁹. The plant alkaloid ryanodine binds to this protein with high affinity and high selectivity when the channel is in its open state¹⁰. Because of this, the channel is commonly referred to as the ryanodine receptor (RyR). The RyR has three isoforms known as RyR1, RyR2 and RyR3. All three isoforms appear in brain tissue, however RyR1 is found predominately in skeletal muscle, while RyR2 is found primarily in cardiac muscle.

Figure 3: 3D reconstruction of the calcium release channel protein (commonly known as the ryanodine receptor).¹¹

1.4 Modification of Ca^{2+} Release

Ca^{2+} has a biphasic role in the regulation of Ca^{2+} release. Low micromolar concentrations stimulate the channel while high concentrations ($>1\text{mM}$) inhibit the channel. This bell shaped Ca^{2+} dependence curve has been hypothesized to result from two different binding sites, a high affinity site that opens the channel and a low affinity site that inhibits release.¹² While a rise in the Ca^{2+} concentration in the cytosol is the principal factor stimulating the CRC¹³ in cardiac muscle, in skeletal muscle, where ECC is independent of extracellular Ca^{2+} another mechanism of information transfer must be present. It is generally believed that there is some type of conformational coupling between the DHPR and the Ryanodine Receptor in skeletal muscle but the precise mechanism is unknown.

There is direct evidence, however, for the existence, location and function of hyperreactive thiols which have been reported in both skeletal¹⁴ and cardiac muscle¹⁵. Figure 4 shows a proposed model of the Ca^{2+} release channel that is regulated by several classes of sulfhydryl groups existing in close proximity and able to form mixed disulfides. Oxidation of these hyperreactive sulfhydryl groups to a disulfide linkage which opens the channel ($\text{S}_1\text{-S}_2$), is then followed by thiol-disulfide exchange ($\text{S}_2\text{-S}_3$) that closes the channel, and finally, reduction of the disulfide bond by the cellular oxidoreductive (redox) environment which resets the redox status. In support of this model, it has been shown that the CRC has a well defined reduction potential that is sensitive to the cellular environment.¹⁶ Channel activators lower the redox

potential, making the value more negative, which favors the oxidation of thiols and the opening of the channel, while channel inhibitors increase the redox potential to more positive values, which favors the reduction of disulfides and the closure of the channel. During oxidative stress or ischemia, these hyperreactive thiols could oxidize and alter the Ca^{2+} sensitivity of the CRC allowing small changes in the calcium concentrations to contribute to significant activation and release of Ca^{2+} .

Figure 4: Model of the Ca^{2+} release protein gated by SH oxidation and reduction of three endogenous SH groups.¹⁷

Glutathione (GSH) is part of an antioxidant system and plays an important part in maintaining the cellular redox status. It shares the job of scavenging peroxide, singlet oxygen and other free radicals, with antioxidant vitamins and antioxidant enzymes, and is the most abundant nonprotein thiol source in the muscle cell¹⁸. The eye lens has the highest GSH concentrations at ~10mM, whereas muscle has about 1-2mM¹⁹.

During excessive production of reactive oxygen species (ROS) GSH donates a pair of electrons to a hydroperoxide, resulting in 2 GSH being oxidized to 1 glutathione disulfide (GSSG). The GSSG can then be reduced back to GSH by NADPH, which is catalyzed by glutathione reductase (GR). When production of the ROS overwhelms the recycling capacity of the GR, GSSG levels rise, resulting in a decrease of the GSH/GSSG ratio and a more oxidized environment. While GSSG by itself is a activator of the channel, and GSH is a known inhibitor it has been reported that ratio of GSH/GSSG rather than the total GSH or total GSSG concentrations determine the response of the RyR²⁰. The typical mammalian cell ratio of [GSH]/[GSSG] in the cytosol is $\geq 30:1$ resulting in a reduction potential of approximately -230mV²¹. Trans-membrane redox potential differences are on the order of 50mV with the lumen of the SR being more oxidized than the cytosol³⁰.

1.5 Regulation of the calcium dependence of the CRC by $[H^+]$

During myocardial ischemia, when the flow of oxygenated blood to the myocardium is diminished, the cellular pH can fall to as low as pH5.8²². This condition, called acidosis, has been shown to reduce the open probability of the isolated CRC

incorporated into lipid bilayers²³ which produces an increase in SR Ca^{2+} content, and is also likely to decrease cytoplasmic Ca^{2+} buffering since Ca^{2+} and H^{+} ions share common binding sites within both the skeletal and cardiac cell²⁴. Therefore the pH dependent inhibition of the CRC likely influences the physiological mechanism of calcium induced calcium release.

1.6 Studied Compound - Ranolazine

The drug Ranolazine was approved for the treatment of Angina Pectoris (chest pain) and ischemic heart disease in 1996 by the FDA. While Ranolazine at micromolar concentrations is beneficial, it has been shown that it interacts with various ion channels and metabolic pathways leaving the exact mechanism of the useful effects in question. It is proposed that the beneficial effect of Ranolazine is due to a decrease in a spontaneous Ca^{2+} leak across the SR membrane, not the previously proposed shifting of myocardial energy metabolism from fatty acid to glucose oxidation²⁵.

Figure 5: (RS)-N-(2,6-dimethylphenyl)-2-[4-[2-hydroxy-3-(2-methoxyphenoxy)propyl]piperazin-1-yl]acetamide²⁶

1.7 Studied Compound - Homocysteine Thiolactone

It has been established that increases in homocysteine levels is a risk factor for cardiovascular disease and stroke in humans²⁷. The thioester Homocysteine Thiolactone (HCTL) is one of many metabolites of Homocysteine. Preliminary results

indicate it can activate the CRC at nanomolar concentrations. A possible mechanism by which the HCTL may react with the CRC is through lysine residues on the RyR which may form alpha-amino carbon-centered radicals²⁸ as shown in Figure 6.

Figure 6: HCTL, an activated carbonyl, conjugates readily to lysine residues of proteins.

It is widely accepted that a majority of protein backbone cleavage and related damage occurs via alpha-carbon radicals of amino acid residues. It has also been shown that cysteinyl residues and glutathione may aid the formation of alpha-carbon radicals on protein residues²⁹. Other possibilities include the α -amino group of HCTL reacting with aldehydes on the protein. It has been shown that the rate constant of HCTL reacting with streptomycin occurs with a half life of 10 minutes which is 400 times faster than the rate at which it reacts with lysine³⁰. Other possibilities of HCTL interaction are the oxidation of critical thiols or the homocysteinylation of the RyR protein.

Chapter 2: Materials and Methods

2.1 Materials

Tritium labeled ryanodine ($[^3\text{H}]$ -ryanodine) was purchased from PerkinElmer Life & Analytical Sciences, Boston Massachusetts and stored in the freezer at -18°C .

Unlabeled ryanodine was purchased from AgriSystems International, Wind Gap, Pennsylvania. A stock solution was prepared at 10mM in 50% Methanol/deionized distilled nanopure water. White New Zealand rabbits were purchased from Western Oregon Rabbit Company, Philomath, Oregon. Ketamine HCl and Acepromazine Maleate was purchased from the veterinary pharmacy at Oregon Health and Science University, Portland, Oregon. Sheep hearts were a gift from Dr. Kent Thornburg, Heart Research Center, Oregon Health and Science University, Portland, Oregon. Ethylene glycol tetraacetic acid (EGTA) was purchased from Research Organics, Cleveland, Ohio, and prepared in deionized distilled nanopure water and stored at room temperature. CytoScint scintillation fluid was purchased from MP Biomedicals, Costa Mesa, California. Homocysteine Thiolactone (HCTL) was a gift from Dr. Robert M. Strongin, Department of Chemistry, Portland State University, Portland, Oregon. HTLC stock solution was freshly prepared at less than $30\mu\text{M}$ in buffer containing 250mM KCl, 15mM NaCl, and 20mM Piperazine-1,4-bis(2-ethanesulfonic Acid (PIPES) set to pH 7.1 with KOH unless otherwise noted. Ranolazine was a gift from Dr. Guy Salama, Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania. A Ranolazine stock solution was freshly

prepared with 95% ethanol, which was purchased along with all other chemicals not listed above from Sigma Aldrich, St. Louis, Missouri. Glutathione in both its oxidized and reduced form was prepared on ice in ice cold deionized distilled nanopure water containing 10 μ M EGTA at a concentration of 0.5M. Once the pH was adjusted to pH 7.0, aliquots were frozen in liquid N₂.

2.2 Skeletal SR Preparation

Skeletal muscle SR vesicles were isolated from the fast twitch muscle of a 2-3 kg White New Zealand male rabbit based on the method of MacLennan.³¹ Euthanizing solution consisting of 58.8mg/ml Ketamine, 5.88mg/ml Xylazine, and 1.18mg/ml Acepromazine Maleate was subcutaneously injected at a dose of 0.5ml/kg. Rabbit's jugular vein was cut and muscle was removed from the back and hind legs. After removing the fat and connective tissue, muscles was minced and placed in a buffer containing 120mM NaCl, 10mM Imidazole, 100 μ M Dithiothreitol (DTT), 100 μ M EGTA, 0.1mM phenylmethanesulfonyl-fluoride (PMSF), 0.2 μ g/ml leupeptin, and 1 tablet per 500ml of EDTA free SigmaFAST protease Inhibitor Cocktail tablets (Skeletal Buffer A). Muscle was then blended for three cycles at a low speed for 15 seconds, high speed for 60 seconds, followed by a 30 second rest period. The resulting solution was centrifuged in a large Fiberlite rotor at 1600 x g for 10 minutes at 4°C to remove cell debris. The supernatant was then filtered through four layers of cheesecloth and adjusted to pH7.4 with dry Imidazole. The filtrate was then spun in a Type 19 rotor at 10,000 x g for 15 minutes at 4°C. The brown pellet composed of mitochondria was

discarded and the supernatant was then filtered through four layers of cheesecloth. The filtrate was then spun at 50,000 x g for 70 minutes at 4°C. The resultant pellet which consists of SR and myosin was then homogenized in 120mM NaCl, 10mM Imidazole, 100μM DTT, 50μM EGTA, 0.2μg/ml leupeptin, and 1 tablet per 500ml of EDTA free SigmaFAST protease Inhibitor Cocktail tablets (Skeletal Buffer B). The solution was then spun at 7,500 x g in a Ti-60 rotor for 10 minutes at 4°C. The resulting myosin pellet was discarded and the spin was repeated. The final supernatant, after removing the second myosin pellet was then spun at 123,000 x g for 30 minutes at 4°C which resulted in a SR pellet. That pellet was homogenized in a buffer containing 100mM KCl, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0 (KOH). Once the protein concentration was determined, and diluted, if needed, to approximately 20mg/ml, the crude skeletal SR aliquots were stored in liquid N₂ until needed.

2.3 Cardiac SR Preparation

Cardiac SR vesicles were isolated from Sheep hearts obtained from OHSU based on the method of Meissner and Henderson.³² Once the heart was removed from the sheep, it was immersed in ice cold buffer containing 300mM Sucrose, 10mM PIPES, 0.5mM PMSF, 1μg/ml Leupeptin at pH 7.4 with KOH (Cardiac Buffer A). The atria and other connective and fatty tissue were removed and the remaining ventricles were minced into small pieces to be blended at high speed in a waring blender for 20 seconds. After a 30 second rest period the solution was blended again on high for 20 seconds. The

resultant solution was centrifuged at 9,000 x g in the large fiberlite rotor at 4°C. The supernatant was then filtered through two layers of cheesecloth and spun at 44,000 x g for 75 minutes in a type 19 rotor at 4°C. The resultant pellet was homogenized in Cardiac Buffer A and spun at 25,000 x g for 30 minutes at 4°C. The resulting SR pellet was homogenized in a buffer containing 100 mM Sucrose, 5 mM HEPES at pH 7.2 (Tris-base). Once protein concentration was determined, and diluted if needed to approximately 20mg/ml, the crude cardiac SR aliquots were stored in liquid N₂.

2.4 Protein Determination

Protein concentrations of the SR were determined by the method of Kalckar³³. The absorbance peak at 280nm is mainly due to the presence of tyrosine and tryptophan and the peak at 230nm is mainly due to the peptide bond of the protein backbone. Using this information, absorption was measured on an Agilent 8453 UV-Visible photodiode array spectrophotometer at 230nm and 280nm. Data was also collected at 260nm in order to correct for nucleic acids using the following equations.

$$[\text{SR}]_{\text{mg/ml}} = 1.45 \times \text{Abs}_{280} - 0.74 \times \text{Abs}_{260} \quad (\text{Equation 1})$$

$$[\text{SR}]_{\text{mg/ml}} = 0.185 \times \text{Abs}_{230} - 0.748 \times \text{Abs}_{260} \quad (\text{Equation 2})$$

The process was repeated twice, and the average protein concentration was calculated.

2.5 Equilibrium Ryanodine Binding Assay

The molecule ryanodine binds with nanomolar affinity to a single class of proteins near the junctional region of the SR³⁴ under certain conditions. With a few exceptions, such as silver, CRC activators stimulate ryanodine binding, whereas compounds that inhibit the CRC, inhibit binding. Tritium labeled ryanodine ($[^3\text{H}]$ -ryanodine) binding assays were carried out at a $[^3\text{H}]$ ryanodine concentration of 1nM, and 14nM unlabeled ryanodine for skeletal SR, and a $[^3\text{H}]$ ryanodine concentration of 2nM, and 13nM unlabeled ryanodine for cardiac SR. SR vesicles (0.5mg/ml) were incubated in a buffer containing 250mM KCl, 15mM NaCl, and 20mM Piperazine-1,4-bis(2-ethanesulfonic Acid (PIPES), pH 6.8, 7.1 or 7.4 as dictated by each experiment, at 37° Celsius for 3 hours in order for binding to reach equilibrium. Samples were then quenched by rapid filtration through Whatman glass fiber filters using a Brandel cell harvester. Filters were washed twice with approximately 4ml of buffer containing 250mM KCl, 15mM NaCl, 20mM tris-(hydroxymethyl)- aminomethane (TRIS), and 100 μ M CaCl_2 at pH 7.1 (HCl). These filters were then dried in an oven at 50°C for two hours then incubated with agitation in 3ml of CytoScint scintillation fluid for one hour. Radioactivity was counted by a Beckman LS 6000 scintillation counter with an efficiency of approximately 55%. Nonspecific binding was measured in the presence of 200nM unlabeled ryanodine, and 4mM ethylene glycol tetra acetic acid (EGTA). Total specific activity was determined by adding an extra aliquot of experimental $[^3\text{H}]$ -ryanodine buffer solution to scintillation fluid and counting the sample.

2.6 Initial Rate Ryanodine Binding Assay

Binding kinetics were determined in a similar manner to equilibrium Ryanodine binding experiments described above with small modifications. When working with skeletal SR vesicles the addition of 1nM [³H]-ryanodine occurred over four time points, three minutes apart before being quenched with rapid filtration. When working with cardiac SR vesicles the addition of 4nM [³H]-ryanodine occurred over four time points, five minutes apart before being quenched with rapid filtration.

2.7 Hill Analysis

Calcium dependent ryanodine binding measurements were fit to the Hill equation.

$$B = B_{\max} \frac{[x]^n}{k_d + [x]^n} \quad (\text{Equation 3})$$

Where $B_{\max}$ is the maximum binding, $[x]$ is the free Ca^{2+} concentration determined with the computer program “Bound and Determined”, k_d is the dissociation constant of the binding site and n is the Hill coefficient. The graphing software SigmaPlot was used to determine the best fit parameters for the half maximal binding concentration (EC_{50}) which was calculated by equation 4.

$$\text{EC}_{50} = k_d^{\left(\frac{1}{n}\right)} \quad (\text{Equation 4})$$

2.8 Redox Potential

To study the effects of channel modulators in more reduced or oxidized environments it is possible to mimic the cellular redox status by using various concentrations of GSH and GSSG determined by the following equation.

$$E_{\text{solution}} = E^{\circ} + 2.3 \frac{RT}{F} (7.0 - pH_{\text{actual}}) + 2.3 \frac{RT}{nF} \log \frac{[GSSG]}{[GSH]^2} \quad (\text{Equation 5})$$

Where E_{solution} is the redox potential of the solution, E° is the standard potential of glutathione (-0.24V), pH_{actual} is the pH of the buffer, R is the gas constant ($8.31 \text{ deg}^{-1} \text{ mol}^{-1}$), T is the absolute temperature (K), n is the number of electrons transferred ($n = 2$ for glutathione) and F is the Faraday constant (96406 J/V).

2.9 Redox titrations

SR vesicles were incubated at various solution redox potentials and channel modifiers for 1 hr at 37°C before measurement of the initial rate of ryanodine binding assay was started with the addition [^3H]ryanodine. Values for the maximum initial rate of Ryanodine binding, B_{max} , were obtained from each individual redox titration at its most oxidized point, and the minimum initial rate of Ryanodine binding, B_{min} , was obtained from its most reduced point. With these values Equation 6 was used to plot E_{solution} as a function of the initial rate of Ryanodine binding, B , resulting in a curve in which the x intercept determines the redox potential of the RyR , E_{RyR} .

$$E_{\text{Solution}} = 2.3 \left(\frac{RT}{nF} \right) \log \left(\frac{B' - B'_{\text{min}}}{B'_{\text{max}} - B'} \right) + E_{\text{RyR}} \quad (\text{Equation 6})$$

Chapter 3: Results

3.1 Ca^{2+} dependence of Cardiac and Skeletal SR at various pH and redox potentials.

The Ryanodine Receptors found in cardiac muscle have different sensitivities to activators and inhibitors than those that are found in skeletal muscle. This variation in response is unlikely due to the difference in species as it has been reported that preparations from diverse species such as mouse, rabbit, and sheep exhibit similar trends, especially with respect to Ca^{2+} sensitivity³⁵. It has also been shown previously that Ryanodine Binding is affected by pH^{36,37,38} with optimal binding between pH 7.2 and pH 7.8³⁹. Figure 7 shows that ryanodine binding to rabbit skeletal muscle SR reaches a maximum between 30-100 μM free Ca^{2+} at pH 7.4 and between 3-10 μM free Ca^{2+} at pH 6.8 after a gradual increase in binding. Sheep cardiac muscle SR has a sharper response to Ca^{2+} than skeletal muscle SR showing a higher degree of cooperativity and reaches maximum binding between 300-700nM free Ca^{2+} at pH 7.4 and a maximum binding between 700nm-3 μM free Ca^{2+} at pH 6.8. There is also significantly higher binding of ryanodine to skeletal muscle SR at pH 7.4 than to cardiac muscle SR. At pH 6.8 there is little difference observed in maximal ryanodine binding to skeletal vs. cardiac muscle SR. Although there are differences in the Ca^{2+} dependence of ryanodine binding at pH 7.4 vs. pH 6.8, large changes in maximal binding between skeletal and cardiac SR are only observed at pH 7.4.

Ca^{2+} dependence of ryanodine binding in skeletal and cardiac muscle at pH 6.8 and pH 7.4

Figure 7: Ca^{2+} dependent ryanodine binding as a function of pH in skeletal and cardiac muscle SR. Skeletal muscle SR at pH 7.4 (●), pH 6.8 (■). Cardiac muscle SR at pH 7.4 (◆), pH 6.8 (▼).

The oxidation state of the cysteine residues on the cytoplasmic domain of the RyR have been shown to be important in determining the calcium sensitivity of the RyR⁴⁰. Shown in Figures 8 and 9, and summarized in Table 1, are the effects of two different redox potentials on Ca^{2+} dependent ryanodine binding to skeletal muscle SR.

Figure 8: Shift in Ca^{2+} dependent ryanodine binding due to solution redox potential at pH 7.4. Control Skeletal muscle SR at pH 7.4 (●); -90mV solution redox potential (■); -200mV solution redox potential (▼).

Figure 9: Shift in Ca^{2+} dependent ryanodine binding due to solution redox potential at pH 6.8. Control Skeletal SR at pH 6.8 (●); -90mV solution redox potential (■); -200mV solution redox potential (▼).

	Skeletal Muscle SR Control	Skeletal Muscle SR -200mV	Skeletal Muscle SR -90mV	Cardiac Muscle SR Control
k_d at pH 6.8 (μM)	0.8 ± 0.1	2.5 ± 0.3	0.5 ± 0.1	0.9 ± 0.1
k_d at pH 7.4 (μM)	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
Maximal ryanodine binding at pH 6.8 (pmol/mg)	1.0	0.8	1.2	0.9
Maximal ryanodine binding at pH 7.4 (pmol/mg)	2.3	1.5	2.7	1.1

Table 1: Shift in k_d Ca^{2+} and maximum binding due to pH and solution redox potential.

As summarized in Table 1 at both pH 6.8 and pH 7.4, a more oxidizing solution redox potential increases maximal ryanodine binding. At pH 7.4 sensitivity of the RyR1 to activation by lower Ca^{2+} concentrations is increased.

3.2 Shift in Redox Potential of the RyR as a function of pH

It has previously been shown that the ryanodine receptor acts as a redox sensor³⁵.

Channel activators shift the redox potential of the receptor to a more negative or reduced value. This results in the oxidation of critical thiols to disulfides. In contrast channel inhibitors shift the redox potential of the receptor to a more positive, or more oxidized potential resulting in the reduction of disulfide bonds and a decrease in the

rate of ryanodine binding. Figure 10 & Figure 11 show redox titrations at three different pH values and demonstrates that at lower pH thiols require stronger oxidizing conditions to activate the channel. This shift in value is expected as the $-S^-$ form is more easily oxidized than the $-SH$ form. The E_{RyR} values for each titration are summarized in Table 2.

	pH 6.8	pH 7.1	pH 7.4
Skeletal Muscle SR	-88mV	-133mV	-155mV
Cardiac Muscle SR	-141mV	-217mV	-236mV

Table 2: E_{RyR} values for skeletal muscle SR and cardiac muscle SR redox titration as a function of pH.

Figure 10: Shift of redox potential titration as a function of pH in skeletal muscle SR. pH 6.8 (●), pH 7.1 (▼), pH 7.4 (■). All experiments are carried out with $50\mu\text{M}$ free Ca^{2+} .

Figure 11: Shift of redox potential titration as a function of pH in cardiac muscle SR. pH 6.8 (●), pH 7.1 (▼), pH 7.4 (■). All experiments are carried out with 50μM free Ca^{2+} .

3.4 Change in Ca^{2+} dependent ryanodine binding due to the drug Ranolazine

Conditions that are associated with increased channel open probabilities in single channel artificial lipid bilayer experiments are usually associated with increases in ^3H -Ryanodine binding measurements. Ranolazine is one of the exceptions. No effect on Ryanodine binding was seen in cardiac or skeletal muscle as a function of increasing concentration of Ranolazine, however at the single channel level an inhibition with a k_d of $10 \pm 3 \mu\text{M}$ in cardiac SR vesicles (Yanping Ye ~ unpublished data) was shown. As the therapeutic effects of Ranolazine are due to decreasing intracellular Ca^{2+} load the effects of 30μM Ranolazine in cardiac SR were studied and a shift in the Ca^{2+} dependence of Ryanodine binding was observed at both pH7.1, where the k_d shifted from $0.49\mu\text{M} \pm 0.02$ to $0.66\mu\text{M} \pm 0.03$ and at pH 7.4 where the k_d shifted from $0.03\mu\text{M} \pm 0.01$ to $0.04\mu\text{M} \pm 0.01$ as shown in Figure 12.

Figure 12: Ca^{2+} dependent ryanodine binding in cardiac muscle SR with and without $30\mu\text{M}$ Ranolazine. Control at pH 7.1 (black sphere ●); $+30\mu\text{M}$ Ranolazine at pH 7.1 (black triangle ▲); control at pH 7.4 (red sphere ●); $+30\mu\text{M}$ Ranolazine at pH 7.4 (red triangle ▲).

3.5 Ca^{2+} dependence of Homocysteine Thiolactone

Homocysteine thiolactone like ranolazine does not affect maximal ^3H -Ryanodine binding measurements over the range of concentrations that it is soluble. HCTL has shown to shift Ca^{2+} dependent ryanodine binding in cardiac muscle SR from a k_d of $73.5\text{nM} \pm 1.8$ to a k_d of $43.2\text{nM} \pm 2.3$ at 100nM when the solution potential has been set to -210mV and pH 7.4 as shown in Figure 13.

Figure 13: Shift in Ca^{2+} dependent ryanodine binding in cardiac muscle due to 100nM HCTL. Control cardiac muscle SR at pH 7.4 and -210mV (●); +100nM HCTL at -210mV (▼); Control cardiac SR at pH 7.4 without redox potential set (■) +100nM HCTL without redox potential set (◆)

At other concentrations of HCTL, 1nM, 10nM, 1 μ M the k_d of the Ca^{2+} dependence was within two standard deviations of the control as shown in Figure 14 and summarized in Table 3.

	Control	10nM HCTL	100nM HCTL	1 μ M HCTL
k_d (nM)	73.5 ± 1.8	69.6 ± 1.3	42.1 ± 2.4	70.8 ± 8.6

Table 3: Shift in Ca^{2+} dependent ryanodine binding k_d as a function of HCTL concentrations

Effects of HCTL on Ca^{2+} dependent ryanodine binding in cardiac muscle SR at pH 7.4

Figure 14: Shift in Ca^{2+} dependent ryanodine binding as a function of HCTL concentration in cardiac muscle SR. Control Cardiac SR at pH 7.4 (●) +10nM HCTL (▼) +100nM HCTL (■) +1μM HCTL (◆). All experiments aer carried out at -210mV solution redox potential.

Skeletal muscle does not exhibit the biphasic effect of HCTL, however it does seem to have a effect on cooperativity, as the hill number generated from the fit of the 1μM addition of HCTL on Ca^{2+} dependent ryanodine binding is much less. Shifts in Ca^{2+} dependence is seen in Figure 15 and summarized in Table 4 with the largest shift occurring at 1μM HCTL.

	Control	10nM HCTL	100nM HCTL	1μM HCTL
k_d (μM)	0.393 ± 0.077	0.289 ± 0.054	0.338 ± 0.067	0.872 ± 0.255

Table 4: Shift in Ca^{2+} dependence k_d in skeletal muscle SR due to various concentrations of HCTL

Effects of HCTL on Ca^{2+} dependent ryanodine binding in skeletal muscle SR at pH 7.4

Figure 15: Shift in Ca^{2+} dependent ryanodine binding in skeletal muscle SR due to HCTL. Control skeletal SR at pH 7.4 (●); +10nM HCTL (▼) +100nM HCTL (◆) +1 μM HCTL (▲)

Chapter 4: Conclusions

The effects of Ca^{2+} dependent activation is of major importance in the study of RyR since other channel modifiers cannot effect the channel in the absence of Ca^{2+} , or they require Ca^{2+} for maximum activation. In this study of the high-affinity Ca^{2+} binding site, both an increase in pH and a more oxidative solution redox status increased binding as well as changed the amount of Ca^{2+} required for channel activation in both skeletal and cardiac muscle SR. These observation are in agreement with the previously reported conclusions that during muscle fatigue and a decrease in pH, higher concentrations of Ca^{2+} are required to stimulate contraction.

Activation by an oxidative solution redox potential follow similar trends in both cardiac and skeletal muscle SR. At higher pH the E_{RyR} values shift to the left, or a more reduced potential, while with the addition of an inhibitor, the E_{RyR} value shifts to the right, or a more oxidizing potential. While the trends are similar the actual values of the E_{RyR2} values are significantly more negative than the values that are found from skeletal muscle SR which is a possible explanation for why the RyR2 is much more easily oxidized than the RyR1.

In skeletal muscle SR lower Ca^{2+} concentrations shift the redox potential to a more positive potential, as opposed to cardiac muscle SR where when the free Ca^{2+} concentrations drop below $30\mu\text{M}$ there ceases to be an activation even at very

oxidized redox solution potentials. Preliminary data shows that the cardiac receptor activity can be modulated by redox potential only when there is a free Ca^{2+} concentration between $30\mu\text{M}$ to 1mM . This is outside the range of physiological Ca^{2+} concentrations in the heart and raises questions about the role of redox potential as a modulator of RyR2. Although oxidative stress is a critical challenge to the SR's ability to control Ca^{2+} homeostasis in the heart at this point it is not clear what role, if any, the redox potential plays in altering function of RyR2.

Ranolazine desensitized the cardiac channel to activate by calcium which should lead to higher concentrations of Ca^{2+} being required to activate the channel under physiological conditions. During heart failure and arrhythmias it is known that the cellular Ca^{2+} concentration is elevated, ranolazine's ability to desensitize the receptor to activation by Ca^{2+} should be beneficial towards maintaining calcium homeostasis under these conditions. It should prevent or decrease premature release of Ca^{2+} from the SR (arrhythmias).

Both Ranolazine and HCTL altered the Ca^{2+} dependence of ryanodine binding without effecting the maximum amount of binding. Surprisingly, the effect of HCTL was observed only in a reduced solution potential which leads us to speculate that the RyR's response to HCTL is caused by an interaction with sulfhydryl groups on the protein being in a reduced state. The mechanism underlying this interaction is still under investigation.

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