

Summer 10-1-2013

# Catecholamine Interactions with the Cardiac Ryanodine Receptor

Robert Carl Klipp  
*Portland State University*

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<https://doi.org/10.15760/etd.1438>

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Catecholamine Interactions with the Cardiac Ryanodine Receptor

by

Robert Carl Klipp

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

Master of Science  
in  
Physics

Thesis Committee:  
Jonathan Abramson, Chair  
Drake Mitchell  
Robert Strongin

Portland State University  
2013

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## **Abstract**

The cardiac ryanodine receptor (RyR2) is a  $\text{Ca}^{2+}$  ion channel found in the sarcoplasmic reticulum (SR), an intracellular membranous  $\text{Ca}^{2+}$  storage system. It is well known that a destabilization of RyR2 can lead to a  $\text{Ca}^{2+}$  flux out of the SR, which results in an overload of intracellular  $\text{Ca}^{2+}$ ; this can also lead to arrhythmias and heart failure. The catecholamines play a large role in the regulation of RyR2; stimulation of the  $\beta$ -adrenergic receptor on the cell membrane can lead to a hyperphosphorylation of RyR2, making it more leaky to  $\text{Ca}^{2+}$ . We have previously shown that strong electron donors will inhibit RyR2. It is hypothesized that the catecholamines, sharing a similar structure with other proven inhibitors of RyR2, will also inhibit RyR2. Here we confirm this hypothesis and show for the first time that the catecholamines, isoproterenol and epinephrine, act as strong electron donors and inhibit RyR2 activity at the single channel level. This data suggests that the catecholamines can influence RyR2 activity at two levels. This offers promising insight into the potential development of a new class of drugs to treat heart failure and arrhythmia; ones that can both prevent the hyperphosphorylation of RyR2 by blocking the  $\beta$ -adrenergic receptor, but can also directly inhibit the release of  $\text{Ca}^{2+}$  from RyR2.

## **Dedication**

This thesis is dedicated to my family, who have loved and supported me through my life in both the good and the bad. If it wasn't for them I would not be where I am today.

## **Acknowledgments**

I would first like to thank my adviser, Dr. Jonathan Abramson, who has always been willing and patient in helping me in my work. I would also like thank the Department of Physics for the financial assistance which has made this possible.

My special thanks to my lab mates, Tom, Laura, and Yanping, who were always there to teach and to guide me in the lab. I thank my thesis committee, Dr. Drake Mitchell and Dr. Robert Strongin, for taking their time to look into my research. And finally I would like to thank everyone else who has provided help to me, both inside and outside of school these past few years; without all of you, this would not have been possible.

Table of Contents	
Abstract.....	i
Dedication.....	ii
Acknowledgments.....	iii
List of Figures .....	vi
Chapter I Introduction to Cardiac Muscle .....	1
1.1 Muscle.....	1
1.1.1 Cardiac Muscle .....	2
1.2 The Calcium Release Channel .....	4
1.2.1 The Sarcoplasmic Reticulum .....	4
1.2.2 The Calcium Release Channel .....	5
1.2.3 Excitation-Contraction Coupling.....	10
1.2.4 Contraction of Muscle.....	11
1.2.5 Heart Failure and Arrhythmias Associated with RyR2 .....	12
Chapter II Catecholamines.....	15
2.1 Introduction to Catecholamines .....	15
2.2 Biosynthesis of Catecholamines .....	15
2.3 Catecholamine Function in the Body.....	18
2.4 Stimulation of the $\beta$ 1-Adrenergic Receptor.....	18
2.5 Studied Compounds .....	19
2.5.1 Epinephrine .....	19
2.5.2 Isoproterenol .....	20
Chapter III Methods and Materials.....	22
3.1 Materials .....	22
3.1.1 Cardiac SR Preparation.....	22
3.1.2 Determination of Protein Concentration.....	23
3.2 Planar Lipid Bilayer.....	23
3.2.1 Method .....	24
3.2.2 Analysis.....	27

3.3 Electron Donor/Acceptor Assay .....	29
3.4 Ryanodine Binding Assays .....	30
3.4.1 Initial Rate of Ryanodine Binding .....	31
3.4.2 Ca <sup>2+</sup> -Dependence Ryanodine Binding Assay .....	32
Chapter IV Results .....	33
4.1 Electron Donor Properties of Studied Catecholamines .....	33
4.1.1 Isoproterenol Displays Strong Electron Donor Properties.....	33
4.1.2. Epinephrine Displays Strong Electron Donor Properties .....	35
4.1.3. Comparison of Electron Donor Properties.....	37
4.2 RyR2 Inhibition by Isoproterenol .....	39
4.3 RyR2 Inhibition by Epinephrine .....	43
4.4 Dwell Times.....	47
4.4.1 Isoproterenol Increases the Closed Time of RyR2 .....	47
4.4.2 Epinephrine Increases the Closed Time of the Channel .....	51
4.5 Ryanodine Binding is Unaffected by Isoproterenol and Epinephrine .....	54
Chapter V Conclusions and Discussion .....	60
References: .....	65



## List of Figures

Figure 1 Space filling model showing F-actin interacting with the thick filament's myosin head .....	2
Figure 2 Illustration showing the structure of muscle fibers <sup>1</sup> .....	4
Figure 3 Cryo-EM 3D reconstruction of RyR1 in both the open and closed states.....	7
Figure 4 Biosynthesis of Catecholamines.....	17
Figure 5 Chemical structure of epinephrine.....	19
Figure 6 Chemical Structure of isoproterenol.....	20
Figure 7 Bilayer Setup .....	25
Figure 8 Schematic showing the process of measuring electron donor/acceptor properties .....	30
Figure 9 Electron donor properties of isoproterenol.....	34
Figure 10 Electron donor properties of epinephrine .....	36
Figure 11 Comparison of the electron donor properties of epinephrine and isoproterenol .....	38
Figure 12 Representative single channel recordings of isoproterenol .....	40
Figure 13 RyR2 inhibition by Isoproterenol .....	42
Figure 14 Representative single channel recordings of epinephrine .....	44
Figure 15 RyR2 inhibition by epinephrine .....	46
Figure 16 Open times of RyR2 with isoproterenol.....	48
Figure 17 Closed times of RyR2 with isoproterenol .....	50
Figure 18 Open times of RyR2 with epinephrine .....	52
Figure 19 Closed times of RyR2 with epinephrine.....	53
Figure 20 Isoproterenol does not affect the rate of ryanodine binding.....	55
Figure 21 Epinephrine does not affect the rate of ryanodine binding.....	57
Figure 22 Isoproterenol Ca <sup>2+</sup> dependent ryanodine binding .....	59

## **Chapter I Introduction to Cardiac Muscle**

### **1.1 Muscle**

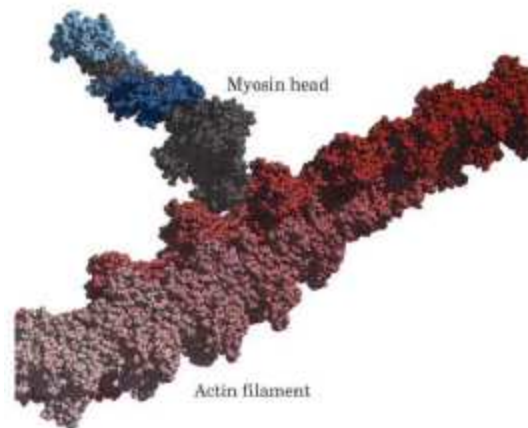
Muscles are the means by which animals move, maintain homeostasis, digest food, breathe, and accomplish various other tasks essential to maintain life. Contraction of muscle takes chemical energy and turns it into a force capable of doing mechanical work. Muscle comes in both the voluntary and involuntary varieties, which together make up a significant portion of an animal's mass. There are 3 classifications of muscle: skeletal, cardiac, and smooth.

Both cardiac and skeletal muscles are known as striated muscle, due to the appearance of striations in their structure. These striations are the contractile elements of the muscle tissue, which appear as alternating dark and light bands when viewed under a microscope. Skeletal is the only example of a voluntary muscle; animals control the contraction via their nervous system. Cardiac and smooth are involuntary muscles.

Overlapping contractile proteins in striated muscle allow for a greater force and distance of contraction; these are essential for physical movement of an animal and also for generating enough force to pump blood through the body. Although skeletal and cardiac muscles have similarities in their structure, they function through slightly different mechanisms. Smooth muscle lacks the alternating pattern of light and dark contractile elements; the seemingly random alignment of contractile proteins in smooth muscle allows it to maintain its contraction for longer periods of time.

### 1.1.1 Cardiac Muscle

Cardiac muscle is involved in the contraction of the heart. The cells that make up the muscle of the heart are referred to as cardiomyocytes. The main proteins making up the cardiac muscle are actin and myosin; the interaction between these 2 proteins is what leads to contraction in muscle (See Section 1.2.4). Thick filaments are made up of many myosin molecules, forming a rod-like structure, with many myosin heads coming off the filament. These myosin heads bind to the thin filaments made up from F-actin, troponin and tropomyosin. The thin and thick filaments are overlapping in the muscle cell; underneath a microscope, regions containing overlap between the thin and thick filaments appear denser (A bands) and regions where just the thin filaments exist are lighter (I bands). Z disks serve as anchors in the I bands, where all of the thin filaments are attached.

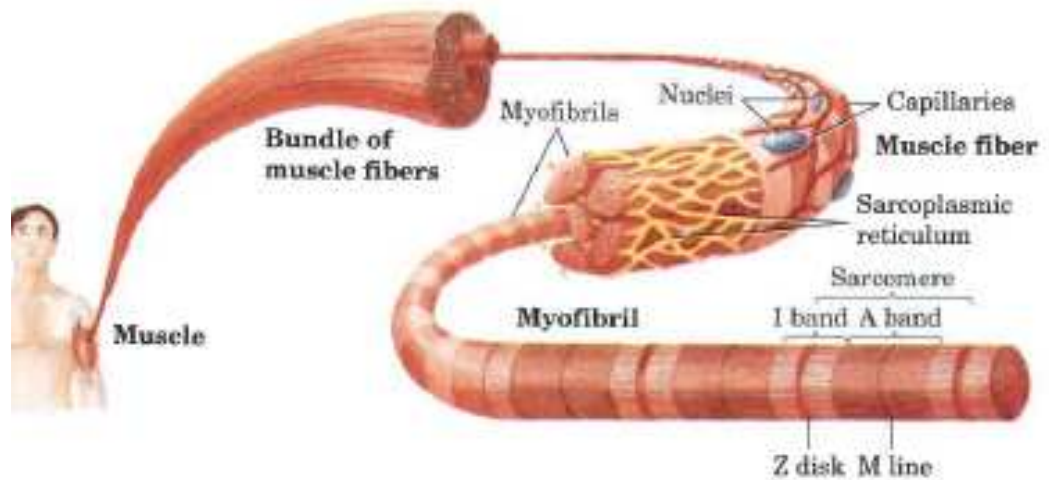


**Figure 1 Space filling model showing F-actin interacting with the thick filament's myosin head<sup>1</sup>**

The thin and thick elements together (sarcomere) give the muscle its striated appearance. Long chains of sarcomeres are referred to as myofibrils; many of these myofibrils together are what is known as a muscle fiber.

The sarcolemma, or cell membrane, encompasses the muscle fiber. The sarcolemma is responsible for maintaining electrochemical gradients between the cytosol and the extracellular fluid. It is these electrochemical differences that are the driving force for the action potential and muscle contraction.

The transverse tubules (T-tubules) are invaginations in the sarcolemma; they are typically perpendicular to the muscle fibers. The primary purpose of the T-tubule is to allow the action potential to propagate into the inner regions of the muscle fiber, and to activate L-type  $\text{Ca}^{2+}$  channels. The T-tubules in cardiac muscle tend to be larger and fewer in number than those found in skeletal muscle. In skeletal muscle the T-tubules are spaced at about  $1.2\mu\text{m}$  intervals, whereas in cardiac they are at  $2.5\mu\text{m}$  intervals<sup>2</sup>.



**Figure 2 Illustration showing the structure of muscle fibers<sup>1</sup>**

## **1.2 The Calcium Release Channel**

### **1.2.1 The Sarcoplasmic Reticulum**

Sarcoplasmic Reticulum (SR) is a subcellular organelle whose primary role is to control the levels of calcium in muscle cells. The SR runs along each myofibril and wraps around it. There is a region of enlargement in the SR where it meets the T-tubule, known as the terminal cisternae. A difference between skeletal and cardiac muscle is that in skeletal muscle, the T-tubules overlap 2 terminal cisternae, creating a triadic junction, whereas with cardiac muscle, the T-tubule is opposed to one terminal cisternae, creating a dyadic junction.

Two different types of SR can be classified: the longitudinal SR (LSR), which runs along the myofibril and junctional SR (JSR), the SR that forms the terminal cisternae and makes a dyad or triad with the T-tubule system. The mixture of the 2 types of SR we refer to as crude SR (CSR).

There are various proteins found both in the lumen of the SR, as well as membrane of the SR. The 2 main proteins responsible for calcium entering and exiting the SR lumen are the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) pump, and the calcium release channel. Ninety percent of the calcium pump is found in the longitudinal SR, whereas the highest density of release channel is found in the junctional SR. There is approximately one hundred times as much  $\text{Ca}^{2+}$  pump found in the SR than there is  $\text{Ca}^{2+}$  release channel.

### **1.2.2 The Calcium Release Channel**

The calcium release channel is commonly known as the ryanodine receptor (RyR), because of its high affinity for the plant alkaloid ryanodine when the channel is in its open state<sup>3</sup>. It is the largest known ion channel, with around 5,000 amino acids and a molecular weight around 2.3 MDa. It is comprised of 4 identical monomers, each of molecular weight 565 kDa, which together form a homotetramer.

There are three different isoforms of RyR which are found in many places in the body; however, each isoform is typically correlated to one of the three types of muscle. RyR1 is

primarily associated with skeletal muscle, RyR2 with cardiac muscle, and RyR3 with smooth muscle. The three isoforms share approximately a 70% sequence homology, leading to near identical three-dimensional structures.

The ryanodine receptor's three-dimensional structure was first determined by cryo-electron microscopy methods in the mid-1990s<sup>4</sup>. Reconstructions of the 3 isomers show similar structure overall. There are 2 main pieces associated with the channel: A large cytoplasmic assembly, which consists of 4 identical monomers, each of which has been hypothesized to have at least fourteen distinct domains<sup>5</sup>. The cytoplasmic assembly is 290x290Å looking from the top down, and 130Å thick. The second piece consists of a smaller, transmembrane assembly (TA). The TA is imbedded in the SR and connects the cytoplasm to the lumen inside of the SR; it measures 120x120x70Å.

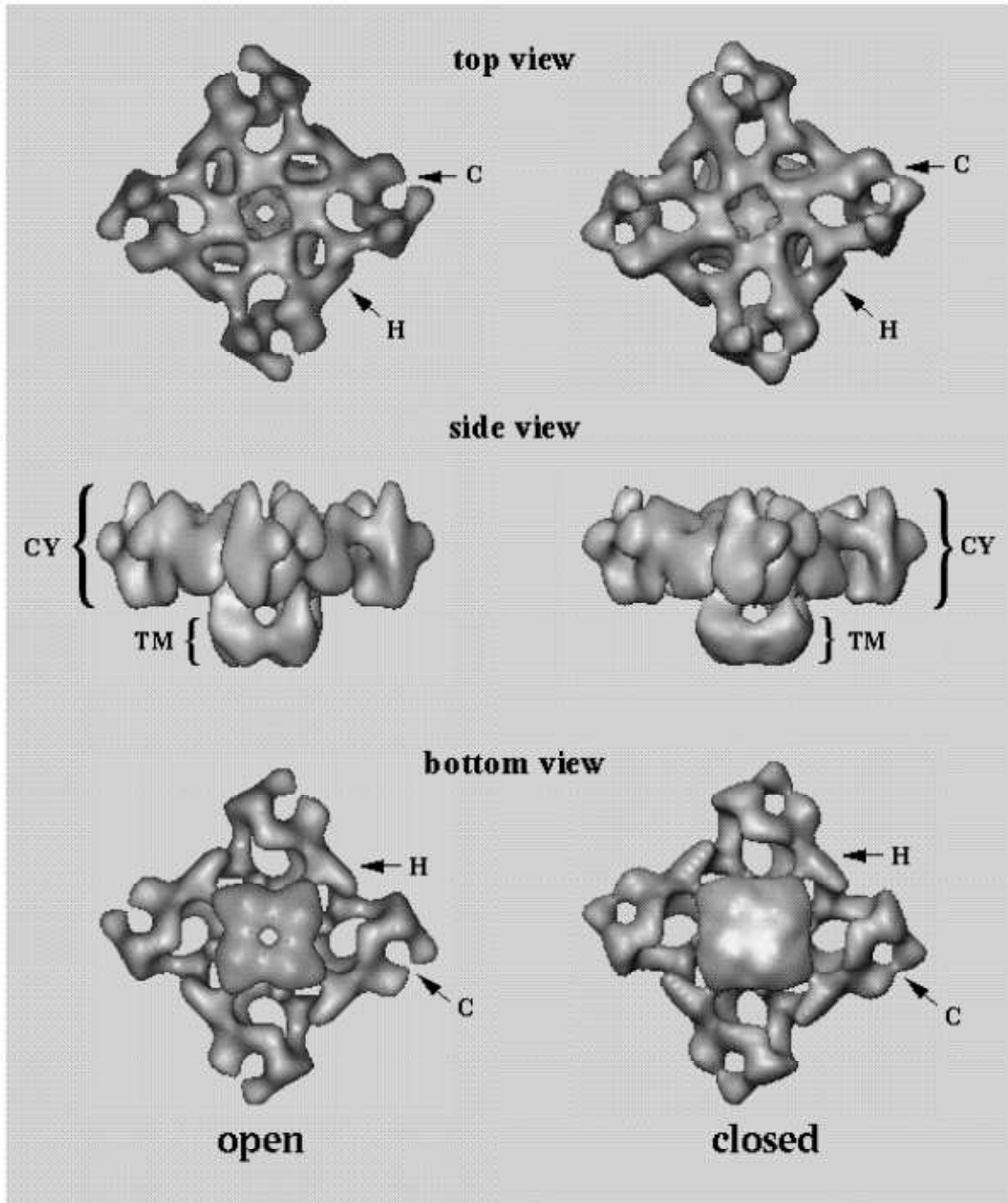


Figure 3 Cryo-EM 3D reconstruction of RyR1 in both the open and closed states<sup>6</sup>



The calcium release channel is imbedded in the SR membrane and is responsible for release of calcium ions from the lumen of the SR. Cryo-electron microscopy has shown large domain changes between the open and closed states of the channel as seen in figure 3.; it has also been a useful tool in determining binding locations for many of the molecules and proteins which interact with the channel.

### *1.2.2.1 Associated Proteins with the Ryanodine Receptor*

**FKBP 12:** Primarily associated with RyR1, but also found in RyR2. It has a molecular mass of 12kDa and is found at concentrations 10x that of FKBP12.6. FKBP12 stabilizes the closed conformation of RyR<sup>7</sup>. Removal of the FKBP has been shown to increase subconductance states in bilayer studies in both RyR1 and RyR2<sup>8</sup>.

**FKBP12.6:** Primarily associated with RyR2. It has a molecular mass of 12.6 kDa. Although FKBP12.6 is found at much lower concentrations than FKBP12, RyR2 has approximately a 500 fold greater affinity for it.

**Calmodulin (CaM):** A 16.8 kDa Ca<sup>2+</sup>-binding protein, capable of binding four Ca<sup>2+</sup> ions, two in each of its domains. In RyR1 CaM will stimulate the channel at low Ca<sup>2+</sup> concentrations and inhibit at higher Ca<sup>2+</sup> concentrations. In RyR2 CaM has only shown channel inhibition<sup>9</sup>. Recent studies have shown that mutant variations of CaM, that can exist in its Apo-CaM state (without Ca<sup>2+</sup>) even at high levels of Ca<sup>2+</sup>, binds in different domains than that of Ca<sup>2+</sup>-bound CaM<sup>10</sup>. This may account for the discrepancies in function between the two isoforms of RyR.

**Protein Kinase A (PKA):** A 96 kDa protein anchored to RyR2. It plays a role in the phosphorylation of the channel, leading to a more open or closed state.

**Junctin:** A 26 kDa protein found in the junctional SR in both skeletal and cardiac muscle. Junctin can bind to the RyR and couple to calsequestrin.

**Triadin:** A 35 kDa protein found in the junctional SR. Triadin plays a similar role to junctin in coupling to calsequestrin. It has also been shown that both triadin and junctin bind calsequestrin more strongly in the presence of low  $\text{Ca}^{2+}$ .

**Calsequestrin (CSQ):** A 45 kDa  $\text{Ca}^{2+}$ -binding protein. It is found in the lumen of the SR and each molecule of CSQ binds up to 50  $\text{Ca}^{2+}$  ions with low affinity ( $K_d \sim 1\text{mM}$ )<sup>12</sup>. By binding large amounts of  $\text{Ca}^{2+}$ , the SR is made a much better storage system for  $\text{Ca}^{2+}$  than would otherwise be possible.

**The Dihydropyridine Receptor (DHPR):** A 100 kDa L-type voltage-gated  $\text{Ca}^{2+}$  channel. It is found embedded in the T-tubule membrane and plays a key role in excitation-contraction coupling (See section 1.2.3). It makes direct contact with RyR1 and is essential to its function. The association between the DHPR and RyR2 is not as well defined.

**PP1 and PP2A:** Phosphatases associated with RyR2.

**Selenoprotein N:** Appears to be responsible for sensing the cellular redox environment and controlling the gating of RyR1<sup>13</sup>.

### ***1.2.2.2 Other Modulators of the Ryanodine Receptor***

**Calcium ( $\text{Ca}^{2+}$ ):** The most crucial ion associated with the receptor. Calcium has a biphasic effect upon the RyR. At low  $\text{Ca}^{2+}$  concentrations ( $<1\mu\text{M}$ ),  $\text{Ca}^{2+}$  stimulates the release of  $\text{Ca}^{2+}$  from RyR, a process referred to as calcium-induced calcium release (CICR). At higher concentrations of  $\text{Ca}^{2+}$  ( $>100\mu\text{M}$ ) the RyR is inhibited<sup>8</sup>. This biphasic effect is attributed to there being several binding locations for  $\text{Ca}^{2+}$  on the receptor; one which stimulates the channel and has a higher affinity for  $\text{Ca}^{2+}$ , this is the primary binding site at low  $\text{Ca}^{2+}$ . A second binding site, which inhibits when bound with  $\text{Ca}^{2+}$ , has a much lower affinity so it is only populated at higher concentrations of  $\text{Ca}^{2+}$ .

**Magnesium ( $\text{Mg}^{2+}$ ):** An inhibitor of RyR, believed to compete with high affinity  $\text{Ca}^{2+}$  binding sites<sup>14</sup>.

**Glutathione (GSH):** Plays a role in maintaining the redox status of cells. When an excess of reactive oxygen species is present, GSH can donate electrons to hydroperoxide resulting in a glutathione disulfide (GSSG) being formed. The ratio between GSH and GSSG play a role in the state of RyR<sup>15</sup>.

**Adenosine-5'-triphosphate (ATP):** ATP along with other adenine nucleotides (cAMP, ADP, and AMP) are stimulators of RyR<sup>16</sup>.

### **1.2.3 Excitation-Contraction Coupling**

All cells in the animal kingdom, including muscle cells, are electrically polarized. The electrical potential generated by the imbalance of charge on ions between the inside and outside of the cell is referred to as the membrane potential. In some cells, when a set

membrane potential is reached (threshold potential), the cell will rapidly depolarize; in muscle cells this leads to contraction. The process of an electrical signal being converted into a mechanical response is referred to as excitation-contraction coupling (ECC).

The mechanism of EC coupling is slightly different in skeletal and cardiac muscle. The difference arises from skeletal RyRs having a direct physical interaction with the DHPR. Here only cardiac EC coupling will be discussed.

In the heart, pacemaker cells first initiate an action potential. The action potential travels down the t-tubules which allow a threshold potential to be reached. This threshold potential depolarizes the cell allowing for an influx of  $\text{Ca}^{2+}$  through the DHP receptors. This influx of  $\text{Ca}^{2+}$ , along with the close proximity of DHPRs to RyR2 initiates CICR. This generates a positive feedback system allowing for more and more  $\text{Ca}^{2+}$  to be released from the SR.

#### **1.2.4 Contraction of Muscle**

Once  $\text{Ca}^{2+}$  floods the cell upon release from the SR, troponin C, a  $\text{Ca}^{2+}$ -binding protein attached to the F-actin thin filaments, binds  $\text{Ca}^{2+}$ . Upon binding of  $\text{Ca}^{2+}$  a conformational change occurs on troponin C, this allows the myosin head to attach itself to the actin filament.

ATP binds to the myosin head and the myosin dissociates itself from the actin filament. ATP hydrolysis occurs, generating ADP and inorganic phosphate ( $P_i$ ), as well as a conformational change in the myosin head. The myosin head shifts its position further down the actin filament and rebinds. Upon the binding of the myosin head to the actin filament,  $P_i$  is released. Finally, the myosin head returns back to its resting state and releases the ADP. This final step shifts the actin and myosin filaments relative to one another causing muscle contraction. This can be visualized under the microscope; upon muscle contraction, the Z disks will shift closer to one another. Skeletal muscle contraction occurs on the order of 2-5ms, while cardiac muscle contracts between 20-50ms.

### **1.2.5 Heart Failure and Arrhythmias Associated with RyR2**

Heart failure (HF) is the heart's inability to pump sufficient blood through the body to supply the organs with enough oxygen. It was reported in 2012 that approximately 5.7 million people living in the United States are living with heart failure<sup>17</sup>. Of these, half of them die within 5 years of the diagnosis. With such a high population of people being affected by failure, it is important to understand the mechanisms under which heart failure occurs, in order to both prevent and treat the disease.

It is known that electrical instabilities in cardiac muscle is what leads to the arrhythmias, but how and why this occurs is still not completely understood. It has been shown that there is a strong correlation between the improper handling of intracellular  $Ca^{2+}$  and HF.

Improper cardiac contraction via a malfunctioning RyR2 leads to a leak in  $\text{Ca}^{2+}$  during diastole. The leak causes a change in the normal polarization of the cardiomyocyte, altering the action potential in what is referred to as a delayed afterdepolarization (DAD). DADs lead to arrhythmias and in turn sudden cardiac death (SCD).

Reduced cardiac output during HF is compensated for in the body by an elevation in the body's catecholamine levels<sup>18</sup>. Elevated catecholamine levels, while increasing cardiac output, can worsen HF<sup>19</sup>. The catecholamines act indirectly on intracellular  $\text{Ca}^{2+}$  handling, triggering a receptor outside the cell, which in turn leads to a hyperphosphorylation of the channel (See Chapter 2).

One hypothesis on how this hyperphosphorylation leads to a malfunctioning RyR2 has to do with the FK binding proteins associated with RyR2. Phosphorylation of the channel causes the FKBP12.6 to dissociate (although this remains controversial<sup>20</sup>), which destabilizes RyR2<sup>21</sup>. This is likely due to an increased  $\text{Ca}^{2+}$ -dependent activation<sup>22</sup>.

Increased intracellular  $\text{Ca}^{2+}$  is not only due to the leak of  $\text{Ca}^{2+}$  from RyR2, but the SERCA pump found in cardiac SR also has reduced function. During HF, phospholamban (PLB), a membrane protein associated with RyR2, becomes hypo-phosphorylated. When PLB becomes hypo-phosphorylated it inhibits the pump's function. This along with the RyR2 leak leads to an increase in intracellular  $\text{Ca}^{2+}$  levels, and affects contractility<sup>23,24</sup>.

### ***1.2.5.1 Catecholaminergic Polymorphic Ventricular Tachycardia***

Catecholamines not only play a role in individuals with HF, but they play an especially important role in individuals with catecholaminergic polymorphic ventricular tachycardia (CPVT). During the “Fight-or-Flight Response,” elevated catecholamines increase heart rate, the strength contraction of the heart, and increased airflow to the lungs, in the presence of perceived danger. Under normal conditions this response is an evolutionary advantage; however, these elevated catecholamine levels in individuals (CPVT), a genetic disease involving a mutation on either RyR2 (CPVT1) or the associated protein calsequestrin (CPVT2), can lead to arrhythmias and heart failure. CPVT is typically treated with antagonists of the  $\beta$ -adrenergic Receptor ( $\beta$ -Blockers), such as carvedilol; these compounds bind to the  $\beta$ -adrenergic receptor, preventing agonists of the receptor from binding.

## **Chapter II Catecholamines**

### **2.1 Introduction to Catecholamines**

Catecholamines are an organic compound made up of 2 main parts; a benzene ring with 2 hydroxyl side groups referred to as a catechol, as well as an amine side chain. There are both synthetic and naturally occurring catecholamines, all sharing a similar structure. In the human body, the most abundant of the catecholamines include: epinephrine, dopamine, and norepinephrine. This work will focus on the catecholamine epinephrine as well as a non-natural catecholamine, isoproterenol, which shares a similar structure with epinephrine.

### **2.2 Biosynthesis of Catecholamines**

In the body, the first compound in the biosynthetic pathway is typically the amino acid L-tyrosine. In the liver, the process is started with phenylalanine being converted into tyrosine. Tyrosine hydroxylase, an enzyme, uses molecular oxygen and tyrosine substrates along with biopterin, a coenzyme, to add a hydroxyl group to L-tyrosine to produce 3,4-dihydroxy-l-phenylalanine (l-DOPA). It is thought that tyrosine hydroxylase provides the rate limiting step in the synthesis of catecholamines<sup>25</sup>.

The next step in the pathway is DOPA decarboxylase, often called aromatic L-amino acid decarboxylase, converting L-DOPA into dopamine and CO<sub>2</sub>. Dopamine is the first of the bodies catecholamines synthesized. For locations in the body such as the substantia nigra and ventral tegmental area of the brainstem, the process stops here, so that dopamine can be utilized.



Further along the pathway molecular oxygen and dopamine  $\beta$ -hydroxylase are used to convert dopamine into norepinephrine. Regions of the body containing the dopamine  $\beta$ -hydroxylase to make this conversion are referred to as, “noradrenergic.” Finally, norepinephrine gains a methyl group from S-adenosylmethionine, being catalyzed by phenylethanolamine N-methyltransferase, yielding epinephrine and homocysteine. The phenylethanolamine N-methyltransferase is found in several locations of the body including neurons in the brainstem and in adrenal cells.

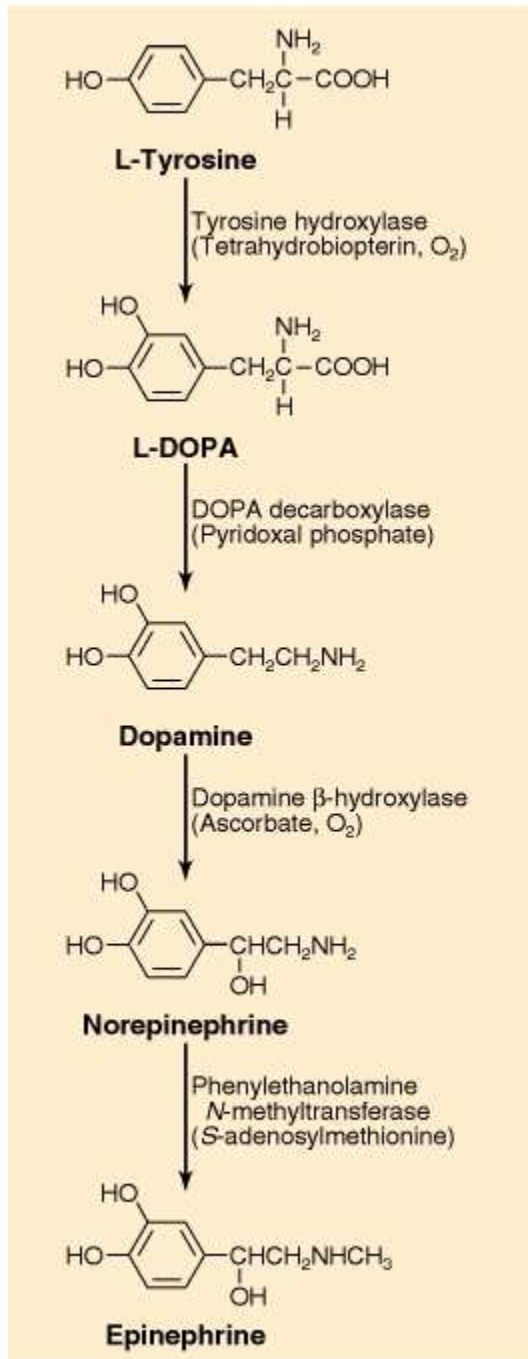


Figure 4 Biosynthesis of Catecholamines<sup>26</sup>

### **2.3 Catecholamine Function in the Body**

The catecholamines are often referred to as beta-adrenergic agonists due to their activation of the beta-adrenergic receptor, a G protein-coupled receptor located on the cellular membrane. There are three types of Beta-adrenergic receptors:  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ .  $\beta_1$  receptors are found primarily in cardiac muscle, but are also found in kidney and stomach tissue. When the  $\beta_1$ -adrenergic receptor is activated it activates adenylyl cyclase activity within the cell, which in turn stimulates calcium release from the ryanodine receptor thereby causing cardiac muscle contraction. The  $\beta_2$ -adrenergic receptor is found primarily in smooth muscle. When activated,  $\beta_2$ -receptors also stimulate adenylyl cyclase activity; however, in smooth muscle this lowers the intracellular calcium levels, thereby causing the dilation of the muscle. The  $\beta_3$ -adrenergic receptor is found primarily in adipose tissue and plays a role in the regulation of thermogenesis, as well as lipolysis.

This thesis focuses on the role of catecholamines in cardiac tissue, therefore only the  $\beta_1$ -adrenergic receptor will be discussed in detail.

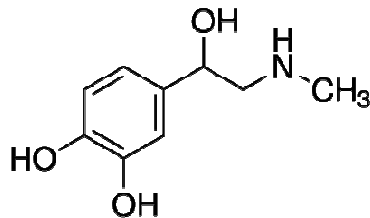
### **2.4 Stimulation of the $\beta_1$ -Adrenergic Receptor**

The  $\beta_1$ -adrenergic receptor is coupled to a G protein which spans the cellular membrane of cardiac myocytes. When an agonist of this receptor, such as epinephrine, binds to the receptor, it causes one of the G proteins subunits within the cell to activate adenylyl cyclase. The adenylyl cyclase, once activated, catalyzes the conversion of ATP into 3',5'-

cyclic AMP (cAMP). The elevated cAMP levels within the cell activate protein kinase A, an intracellular enzyme whose activity depends upon the level of cAMP within the cell. Protein kinase A, once activated, adds phosphate groups to other proteins in a process known as phosphorylation; leading to hyperphosphorylation of RyR2. Hyperphosphorylation of RyR2 leads to a more open state of the channel.

## 2.5 Studied Compounds

### 2.5.1 Epinephrine



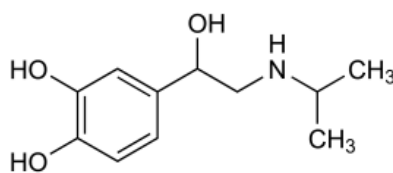
**Figure 5 Chemical structure of epinephrine**

Epinephrine (adrenaline outside the U.S.) was first discovered in 1895 in extracts from the adrenal gland by Polish physiologist Napoleon Cybulski. It can be synthesized in a number of locations in the body including: the adrenal medulla, chromaffin cells, and neurons in the central nervous system. The ability of epinephrine to be produced in one part of the body and be utilized in another classifies it as a hormone.

Epinephrine is naturally occurring, with a molecular weight of 183.2g/mol and existing at typical concentrations of a few nanomolar<sup>27</sup>. The half-life of epinephrine in blood plasma is on the order of 2 minutes, but is constantly being produced. In the early 1900s epinephrine was used as a bronchodilator in asthma patients. It is widely used in medical fields to treat a number of conditions including cardiac arrest and anaphylaxis. This compound increases the strength of contractions (positive inotrope) inside of the heart. It also increases the heart rate (positive chronotrope).

Epinephrine's effect on both the airways and heart makes it the key hormone in the "fight-or-flight response." This physiological reaction prepares one's body to either flee, or stay and fight in the presence of danger.

### 2.5.2 Isoproterenol



**Figure 6 Chemical Structure of isoproterenol**

Isoproterenol (Isoprenaline outside the U.S.) was developed in 1941 and is not found naturally in the body. It has a molecular weight of 211.3g/mol. Like epinephrine, isoproterenol is an agonist of the  $\beta$ -adrenergic receptor; however, unlike epinephrine it

does not stimulate the  $\alpha$ -adrenergic receptors, which lead to vasoconstriction of the veins and arteries; this made it an ideal drug for the treatment of asthma for a time before the release of albuterol, which is more selective for the  $\beta_2$ -adrenergic receptor, making it a better treatment of the airways.

Isoproterenol has a biphasic half-life when administered to patients, with a first phase lasting only a few minutes and a second phase lasting up to 5 hours. It can either be administered intravenously or by aerosol to the body.

Isoproterenol is an activator of all three types of  $\beta$ -adrenergic receptors, the affinity of  $\beta_1$  and  $\beta_2$  for Isoproterenol is greater than that of epinephrine,  $\beta_3$  receptors share about an equal affinity for the two catecholamines. Isoproterenol's primary use in the medical field is in its activation of the  $\beta_1$ -adrenergic receptor, which aids in the treatment of bradycardia (a heart rate below 60BPM). Isoproterenol acts as both a positive chronotrope and positive inotrope in cardiac muscle.

## **Chapter III Methods and Materials**

### **3.1 Materials**

#### **3.1.1 Cardiac SR Preparation**

Sheep heart SR isolation follows a modified protocol from Williams 1990<sup>28</sup>. Sheep hearts were removed and rinsed of excess blood from anesthetized sheep. The sheep heart was stored in an ice cold homogenization buffer containing: 300mM Sucrose, 10mM PIPES, 0.5mM PMSF, 1 $\mu$ g/mL Leupeptin at PH 7.4. Upon arrival to the laboratory the heart was dissected in a cold room, the atria along with extra fatty tissue were discarded.

Half (approximately 40g) of the ventricles of the heart were cut into cm<sup>3</sup> pieces and placed into 500mL of the homogenization buffer. The pieces were homogenized in a blender for 20 seconds at top speed, then for another 20 seconds after a 30 second wait period. The rest of the heart was homogenized following the same process.

The homogenates were combined and poured into 6 Type-19 rotor tubes and were centrifuged for 20 minutes at 9,000g in a Beckman Type-19 Rotor. The supernatant was filtered through 2 layers of cheesecloth and poured into 6 Type-19 tubes and centrifuged at 44,000g for 75 minutes in a Beckman Type-19 rotor. Supernatant was discarded and the pellet was placed into a resuspension buffer containing: 400mM KCl, 300mM Sucrose, 0.5mM MgCl<sub>2</sub>, .5mM CaCl<sub>2</sub>, .5mM EGTA, .5mM PMSF, 1 $\mu$ g/mL Leupeptin, 25mM PIPES at pH 7.0. The solution was all poured into one Type-60 centrifuge tube

and centrifuged at 50,000 rpms for 30 minutes. The pellet was collected and added to the resuspension buffer. The solution was homogenized using a glass homogenizer and stored in 300 $\mu$ L aliquots in liquid nitrogen until use in experiments.

### 3.1.2 Determination of Protein Concentration

Cardiac SR protein concentration is measured using the method developed by Kalckar<sup>29</sup>. The protein backbone has a maximum absorbance peak at 230nm and the amino acids tyrosine and tryptophan peak at 280nm. Using an Agilent 8453 UV-Visible spectrophotometer, the absorbance at three wavelengths was measured. Using the following two equations along with the absorbance at 260nm (to correct for nucleic acids), protein concentration is determined:

$$[SR]_{mg/ml} = 1.45(Abs_{280}) - 0.74(Abs_{260}) \quad (\text{eqn 3.1})$$

$$[SR]_{mg/ml} = 0.185(Abs_{230}) - 0.075(Abs_{260}) \quad (\text{eqn 3.2})$$

### 3.2 Planar Lipid Bilayer

The majority of work in this thesis consists of work done incorporating SR vesicles into a planar lipid bilayer. Incorporation of ion channels into artificial planar lipid bilayers allows for the kinetics of single ion channels to be studied. It also provides an extremely useful tool in studying how various compounds and proteins affect the gating of the channel.

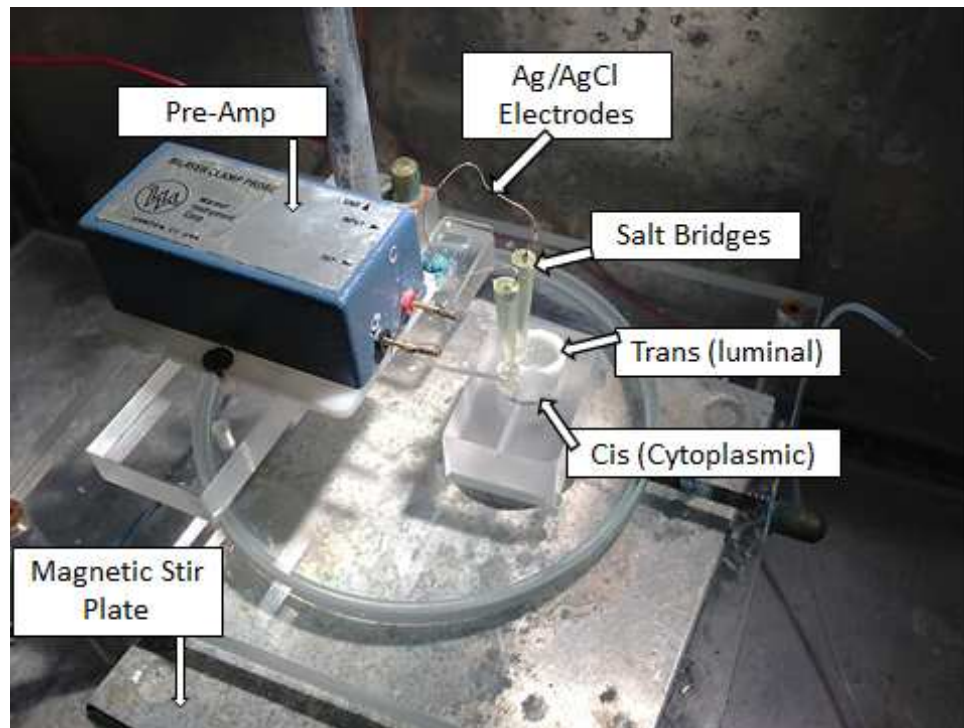


### 3.2.1 Method

Two chambers, comprised of either acetyl or delrin, are separated by a 100-200 $\mu$ m aperture. The aperture is created in one of three ways: by drilling with a 100-200 $\mu$ m drill bit, sparking through the delrin or acetyl barrier between the chambers, or a combination of drilling and sparking through the created aperture. The wall separating the two chambers is on the order of the size of the aperture itself. It has been reported that having a hole on the order of the wall size will help promote fusion of proteins into the planar lipid bilayer<sup>30</sup>.

The two chambers are filled with an aqueous solution of unequal concentration and volumes. The Trans (Luminal) chamber is filled with 650 $\mu$ L of buffer consisting of 25mM HEPES and 40mM CsMeSO<sub>4</sub> at pH 7.4. The CIS (Cytoplasmic) chamber is filled with 800 $\mu$ L of buffer consisting of 25mM HEPES, and 400mM CsMeSO<sub>4</sub> at pH 7.4. The unequal volumes create equal levels of fluid between the two chambers, reducing any hydrostatic pressure at the aperture between the two chambers.

Two electrically conducting salt bridges (Agarose, 4 M CsCl) connect the two chambers to two Ag/AgCl electrodes. The electrodes pass into a preamp which then runs into a Warner BC-525A amplifier. The amplifier passes its signal into an Axon Instruments Digidata 1322A digitizer and signals are recorded in Axon software (pClamp 9.0, ClampEx patch clamp chart recorder).



**Figure 7 Bilayer Setup**

Bilayers are formed between the two chambers using the film drainage method<sup>31</sup>. A lipid mixture consisting of a 5:3:2 mix of PE:PS:PC (PE: 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine; PS: 1,2-Dioleoyl-sn-Glycero-3[phosphor-L-Serine]; PC: 1,2-Dioleoyl-sn-Glycero-3-phosphocholine) (AVANTI) is made in decane at a concentration of 33.3mg/ml. A rounded glass capillary tube is then dipped into the lipid mix and dragged over the hole separating the two chambers. Two monolayers are formed separated by a layer of decane between them. The pressure from the salt bath causes the monolayers to form into a bilayer.

Formation and quality of the bilayer is determined by tracking the capacitance of the aperture. Square waves are indicative of good bilayers; a saw tooth capacitance is an indication of a leak across the bilayer. The bilayer capacitance is also a measure of the thickness of the bilayer; the capacitance is inversely proportional to the thickness of the bilayer.

Crude cardiac SR vesicles are homogenized in 300mM Sucrose, 400mM CsMeSO<sub>4</sub> and added to the CIS chamber (final protein concentration: 10-40μg/mL). A holding potential of +36mV is set, which promotes fusion of SR vesicles. Once fusion occurs, the Trans bath is brought up to 400mM CsMeSO<sub>4</sub> and the holding potential is set at -36mV.

Vesicle fusion occurs in such a way such that the cytoplasmic side of the RyR faces the Cis chamber<sup>32</sup>.

When the channel is in an open state, Cs<sup>+</sup> ions are able to pass through the channel; the conductance of the channel is monitored and recorded. Cs<sup>+</sup> is used as the current carrying ion through the channel for several reasons:

- The conductance of the RyR is higher for Cs<sup>+</sup> than Ca<sup>2+</sup><sup>33</sup>.
- Cs<sup>+</sup> does not modulate the channel in the same way that Ca<sup>2+</sup> does<sup>34</sup>.
- Finally, K<sup>+</sup> channels found in the SR are inhibited by high concentrations of Cs<sup>+</sup><sup>35</sup>; this makes K<sup>+</sup> channels distinguishable from RyR channels.

Subsequent recordings are made for no less than one minute each. Following each recording a measured concentration of compound being tested (made up fresh each day) is added and magnetic stir bars mix the solution for 30 seconds. Each recording of channel activity at the various compound concentrations is analyzed. The entire bilayer set-up sits in an aluminum cage to reduce outside noise.

### **3.2.2 Analysis**

#### ***3.2.2.1 Event Detection***

The Clampfit 9.0 software is mostly automated, the primary analysis tool used in Clampfit 9.0 is creating an events list using the half-amplitude threshold technique. A baseline is first set representing the channels closed state; a second level is then set representing the channel in its open state. In cases where multiple channels are present, 2<sup>nd</sup> and 3<sup>rd</sup> open levels may be set. The half-amplitude threshold technique distinguishes any event that is at least half of the open state level as an open event. Statistical analysis of the event list is then used to determine properties of the channel.

#### ***3.2.2.2 Open Probability***

Open probability ( $P_0$ ) of the channel represents the probability of the channel being in the conducting (open) state. A channel that is in a completely closed state will have a  $P_0$  of

zero, while a channel in a completely open state will have a  $P_0$  of 1.  $P_0$  is calculated as the ratio of the total time open to the total time of the recording.

$$P_0 = \frac{T_0}{T_0 + T_C} \quad (\text{eqn 3.3})$$

Where:  $T_0 = N\tau_0$ ,  $T_C = N\tau_C$

### ***3.2.2.3 Open and Closed Dwell Times***

A list of dwell times for each level set is generated in the events window. The dwell time is a representation of the time spent during each event, or the time spend at one level before changing to a different level. The channel's  $P_0$  depends upon three key factors; the open dwell time ( $\tau_0$ ), the closed dwell time ( $\tau_C$ ), and the frequency of events. It is for this reason that open and closed times must be analyzed to determine the kinetics of the gating.

Logarithmic histograms of the number of events vs. the dwell time are generated in the Clampfit 9.0 software. The histogram is fit to determine average  $\tau_0$  and  $\tau_C$  times.

### ***3.2.2.4 Student T-Test***

A t-test was applied to data for open and closed times between the control and varying concentrations of tested compound.

$$t = \frac{X_1 - X_2}{S_{X_1X_2} \cdot \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad (\text{eqn 3.4})$$

Where

$$S_{X_1X_2} = \sqrt{\frac{(n_1 - 1)S_{X_1}^2 + (n_2 - 1)S_{X_2}^2}{n_1 + n_2 - 2}} \quad (\text{eqn 3.5})$$

Where  $n_1, n_2, S_{X_1}, S_{X_2}, X_1,$  and  $X_2$  are the sample size, standard deviation, and mean value for the 2 concentrations of compound being compared.

From the t-test p-values between concentrations were calculated. The p-value is a measure of the statistical significance of the data. Here, a p-value equal or less than .05 is considered statistically significant.

### 3.3 Electron Donor/Acceptor Assay

Methylene blue (MB) is a dye that when illuminated with light will produce both dye anion and a dye cation radicals. Under normal circumstances, with no electron donor or acceptors present, the dye anion and cation will quickly recombine. If an electron donor is present, the electrons from the donor will pass to the dye cation, which will in turn increase the amount of dye anion radical present in the solution. When two dye anions combine, methylene blue bleaches. When dye anions pass electrons to  $O_2$ , superoxide is generated.

XTT is a compound whose spectrum changes increasing absorbance at 470nm by reacting with superoxide. The more superoxide that is present, the larger the increased absorbance at 470nm. With 50µM XTT, varying concentrations of electron donor, 10µM MB, oxygen, and light, electron donor properties of a given compound can be quantified by monitoring the spectrum change in XTT, as a function of time.

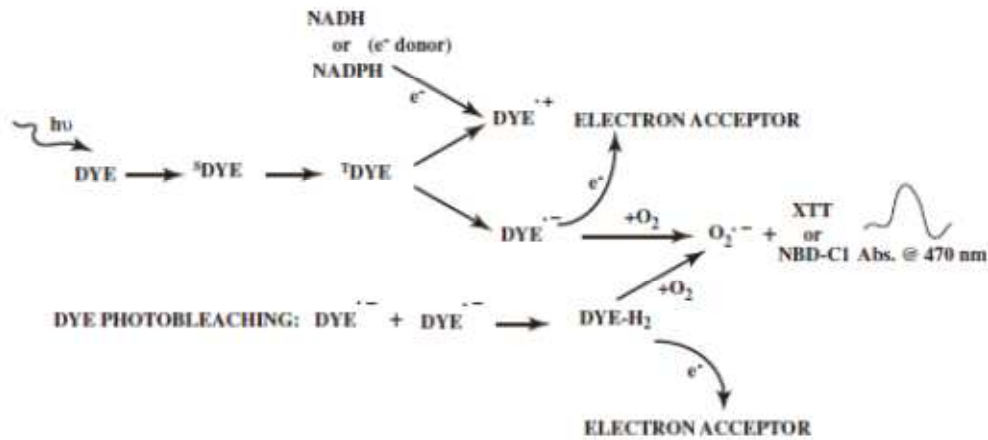


Figure 8 Schematic showing the process of measuring electron donor/acceptor properties<sup>36</sup>

### 3.4 Ryanodine Binding Assays

Ryanodine is an alkaloid found in the plant *Ryania Speciosa*. It binds with very high affinity to the ryanodine receptor, and at low micromolar concentrations, can lock the channel in a half open state.

Nanomolar levels of tritium labeled Ryanodine ( $[^3\text{H}]$ ryanodine) bind to the ryanodine receptor when it is in an open state. This gives it a power of being a strong molecular probe to assay the open state of the channel. By adding varying concentrations of inhibitors or activators of the channel, several different assays involving binding of  $[^3\text{H}]$ ryanodine can be performed.

### **3.4.1 Initial Rate of Ryanodine Binding**

Varying concentrations of either isoproterenol or epinephrine were combined in test tubes containing: 250mM KCl, 15mM NaCl, 20mM PIPES, 50uM  $\text{Ca}^{2+}$ , .5mg/mL crude cardiac SR, 13nM unlabeled ryanodine at pH 7.4. The test tubes were placed in a hot bath at 37°C and the reaction is initiated by the addition of 2nM  $[^3\text{H}]$ ryanodine to the test tubes at varying time points. For isoproterenol, the reaction was terminated at times 5, 10, 15, 20, 25, and 30 minutes. For epinephrine only four time points were used: 5, 10, 15, and 20 minutes.

The reaction was quenched by rapid filtration using a Brandel cell harvester and filtering through Whatman glass fiber filters. The filters were subsequently washed with a buffer containing: 250mM KCL, 20mM TRIS, 15mM NaCl, 100uM  $\text{CaCl}_2$  at pH 7.4. Filters were placed in an oven at 50°C for two hours, at which point 3mL of CytoScint scintillation fluid was added and the solution was agitated for at least 1 hour.

A Beckman LS 6000 scintillation counter was used to measure the levels of  $[^3\text{H}]$ ryanodine present in each sample. The total activity was measured by adding the same amount of  $[^3\text{H}]$ ryanodine used in the experiment directly to the scintillation fluid



and measuring activity. Nonspecific binding was also measured with 200nM unlabeled ryanodine and 4mM EGTA.

In the initial rate of ryanodine binding assay it is anticipated that inhibitors of the channel decrease the amount of binding that occurs over a given period of time. Channel stimulators have the opposite effect; the channel is more open, therefore allowing more ryanodine binding to occur.

### **3.4.2 Ca<sup>2+</sup>-Dependence Ryanodine Binding Assay**

Ca<sup>2+</sup> dependent ryanodine binding was measured with 10μM isoproterenol following a similar overall method to the initial rates binding assay. Here, drug concentration is kept constant and the Ca<sup>2+</sup> concentration is varied. A control is also performed with the same concentrations of Ca<sup>2+</sup> but with no drug present. The incubation at 37°C is carried out for a period of three hours and assaying of the activity is performed the same as the initial rate assay.

Rise in ryanodine binding can be seen in varying concentrations Ca<sup>2+</sup> because Ca<sup>2+</sup> is itself a modulator of the openness of the channel. A compound that is an inhibitor may shift the Ca<sup>2+</sup> dependence of RyR2 so that more Ca<sup>2+</sup> will be needed to reach maximal binding (this would be illustrated by a shift of the curve to the right). A channel activator may lower the Ca<sup>2+</sup> dependence, which can be seen by a shift in the curve to the left. In RyR2, the Ca<sup>2+</sup> dependence is very abrupt, binding goes from a minimum to a maximum over a very small range of Ca<sup>2+</sup> concentrations.

## Chapter IV Results

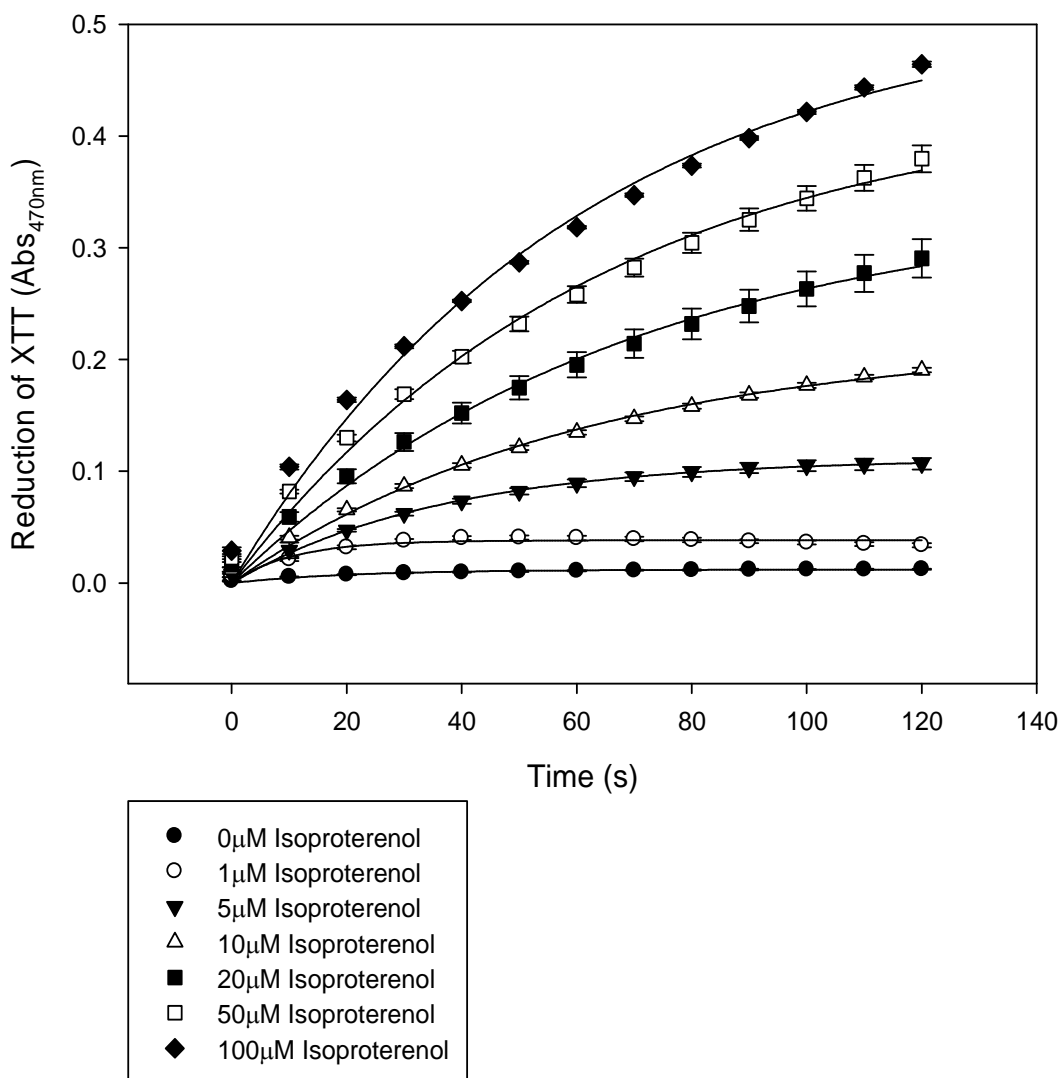
### 4.1 Electron Donor Properties of Studied Catecholamines

#### 4.1.1 Isoproterenol Displays Strong Electron Donor Properties

Isoproterenol was assayed for its ability to donate electrons as explained in the methods section. Concentrations ranging from 0-100 $\mu$ M isoproterenol were tested and the reduction of XTT vs. time for each concentration was plotted. Figure 9 shows data from a total of three experiments, averaged and fit to a 2-parameter exponential rise to maximum:

$$y = c(1 - e^{-dt}) \quad (\text{eqn 4.1})$$

The standard deviation was plotted for each data point from the 3 experiments. The experiment was performed at pH 7.4 to mimic the condition performed in the planar lipid bilayer.

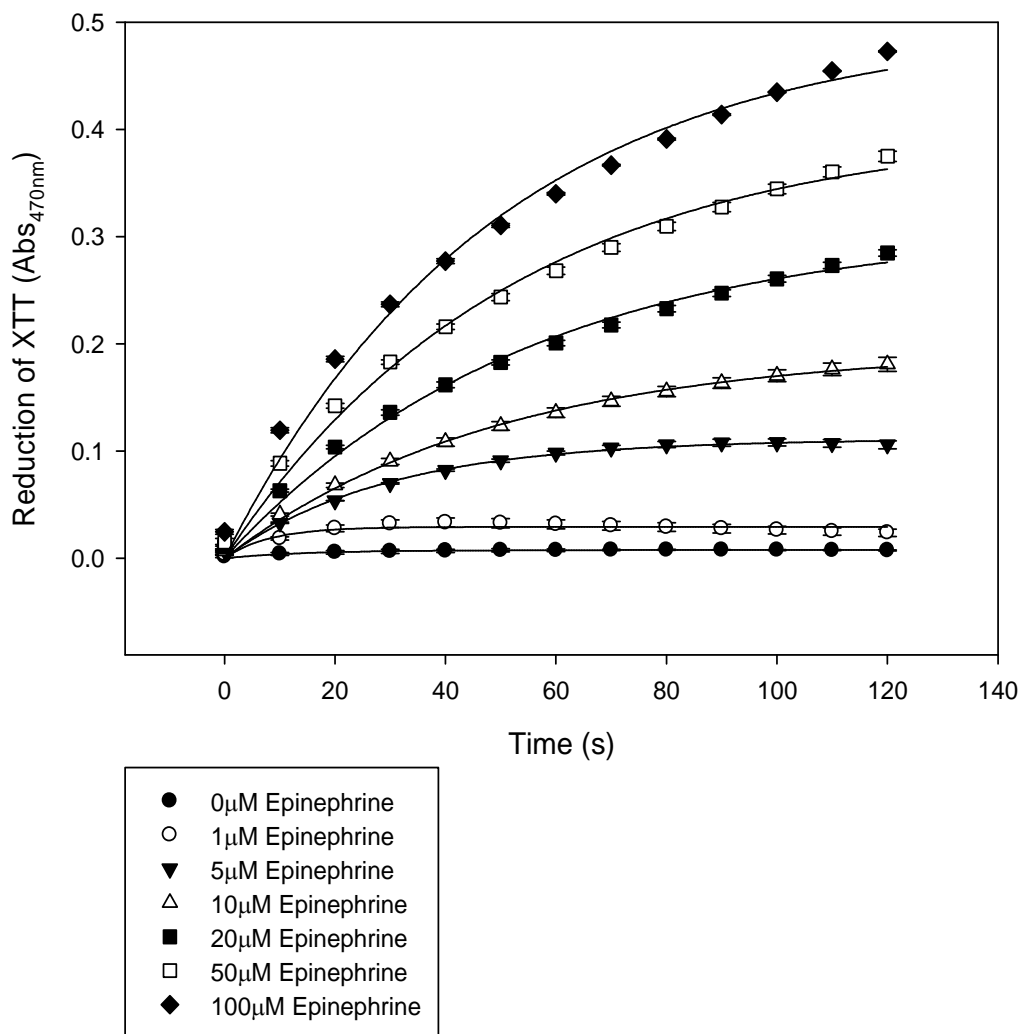


**Figure 9 Electron donor properties of isoproterenol**

Isoproterenol displays strong electron donor properties in 1mM Tris Buffer at pH 7.4, containing 10μM MB, and 50μM XTT. Reduction of XTT was measured as an increase in the absorbance at 470nm. Each data point is an average of 3 experiments  $\pm$  the standard deviation.

#### **4.1.2. Epinephrine Displays Strong Electron Donor Properties**

Epinephrine was assayed for its ability to donate electrons as explained in the methods section. Concentrations ranging from 0-100 $\mu$ M of epinephrine were tested and the reduction of XTT vs. time for each concentration was plotted. Figure 10 shows data from a total of three experiments, averaged and fit to a 2-parameter exponential rise to maximum. The standard deviation was plotted for each data point from the 3 experiments. The experiment was performed at pH 7.4 to mimic the condition performed in the planar lipid bilayer.



**Figure 10 Electron donor properties of epinephrine**

Epinephrine displays strong electron donor properties in 1mM Tris Buffer at pH 7.4, containing 10μM MB, and 50μM XTT. Reduction of XTT was measured as an increase in the absorbance at 470nm. Each data point is an average of 3 experiments ± the standard deviation.

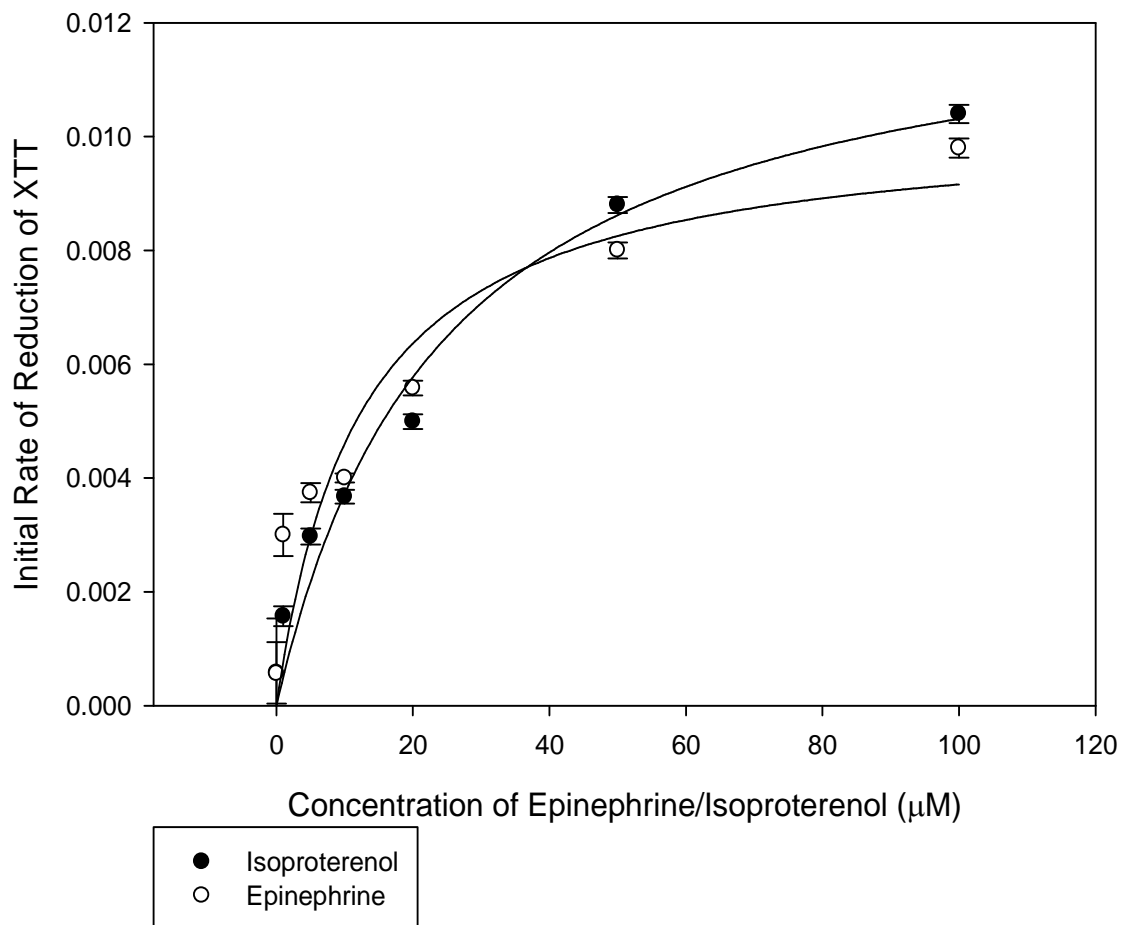
### 4.1.3. Comparison of Electron Donor Properties

The initial rates of XTT reduction were calculated using the 2-parameter exponential rise along with the values of c and d generated from eqn. 4.1 in SigmaPlot 9.0. The initial rates vs. concentration of concentrations for both isoproterenol and epinephrine were plotted in figure 11, and fitted to the Michaelis-Menten equation.

$$V = \frac{V_{\max} [S]}{K_d + [S]} \quad (\text{eqn 4.2})$$

Where: V=reaction rate, [S]=substrate concentration,  $V_{\max}$ =maximum rate, and  $K_d$ =the concentration at half maximal rate.

Standard error for the initial rate was calculated and plotted for each data point. The two donors show very similar strengths; half maximal donor activities were calculated from the fit for epinephrine and isoproterenol to be 17.5 $\mu$ M and 24.3 $\mu$ M respectively.



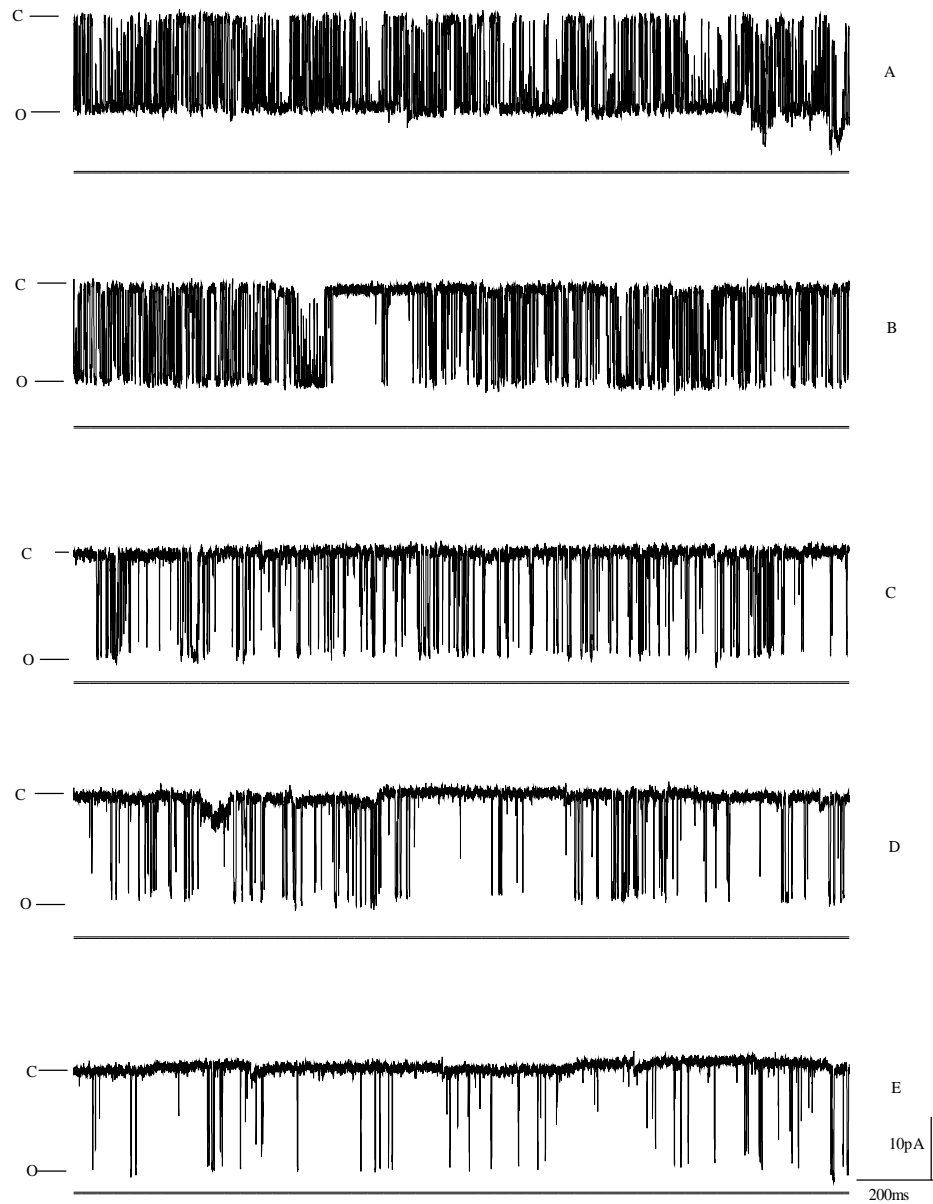
**Figure 11 Comparison of the electron donor properties of epinephrine and isoproterenol**

1mM Tris buffer, containing 10μM MB, 50μM XTT at pH 7.4. Initial rates of XTT reduction were calculated and plotted using a 4-parameter logistic fit. Each point represents 3 experiments averaged  $\pm$  the standard deviation.

## **4.2 RyR2 Inhibition by Isoproterenol**

Isoproterenol's effect on RyR2 was tested in the planar lipid bilayer. All experiments were done in the presence of 410nM  $\text{Ca}^{2+}$  (calculated from Bound and Determined, BAD). Isoproterenol was tested in a dose dependent manner ranging in concentrations of 100nM-7uM. A total of 8 experiments were performed under identical conditions.





**Figure 12 Representative single channel recordings of isoproterenol**

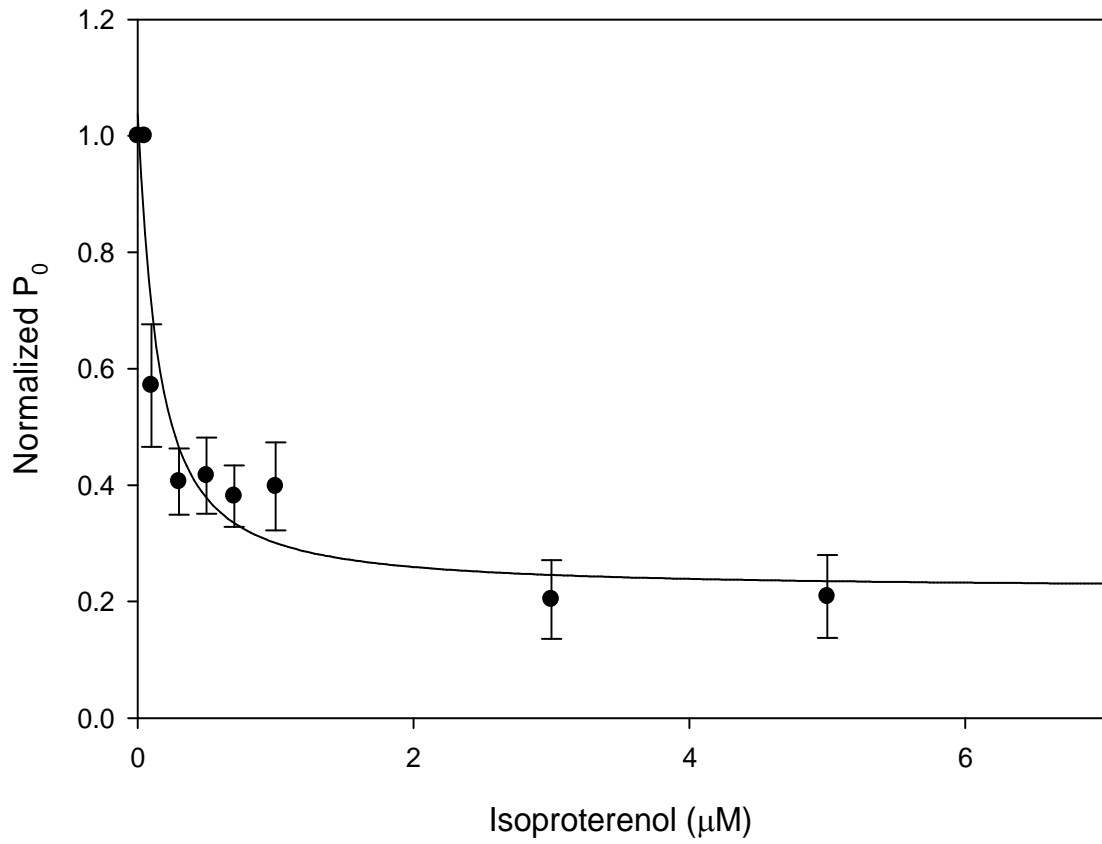
Two second representative traces of RyR2 inhibition by isoproterenol. A channel opening is indicated by a downward fluctuation. A) 410nM  $\text{Ca}^{2+}$  ( $P_0=0.50$ ) B) 100nM Isoproterenol ( $P_0=0.44$ ) C) 300nM Isoproterenol ( $P_0=0.26$ ) D) 500nM Isoproterenol ( $P_0=0.11$ ) E) 1µM Isoproterenol ( $P_0=0.06$ )

Figure 13 shows the concentration-dependent inhibition of single channel activity induced by isoproterenol. Normalized  $P_0$  was plotted vs. isoproterenol concentration. A 4-parameter logistic plot was used to fit the data:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b} \quad (\text{eqn 4.3})$$

The  $IC_{50}$  from the fit was determined to be 210nM. The  $IC_{50}$  is a measure of the compounds potency as a channel inhibitor; it represents the concentration at which the channel is half maximally inhibited. As can be seen from figure 13, the channel was never able to be fully closed by isoproterenol, making isoproterenol a partial antagonist of RyR2.

Ruthenium Red (R.R.), an inorganic dye that has been shown to completely block the ryanodine receptor when added to its cytoplasmic face, was added at the termination of each experiment when channel activity was still present. This is to ensure that the cytoplasmic face is facing the Cis chamber, as well as to ensure that the gating being observed is the ryanodine receptor and not some other ion channel present in the SR. In all of these experiments, 10 $\mu$ M R.R. decreases the  $P_0$  to zero.

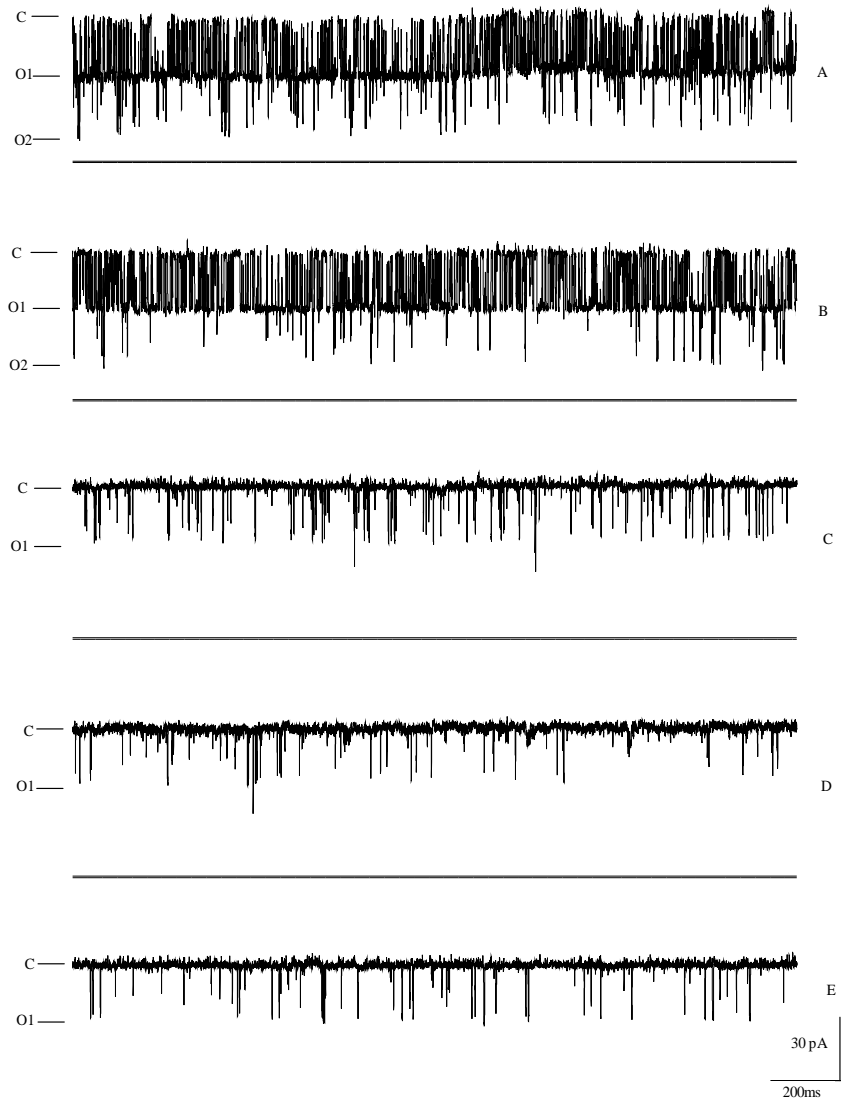


**Figure 13 RyR2 inhibition by Isoproterenol**

Control recordings at 410nM Ca<sup>2+</sup> were normalized to 1. Average P<sub>0</sub> with standard error before normalization was 0.50±0.06. Each point represents the average of 8 experiments ± standard error.

### **4.3 RyR2 Inhibition by Epinephrine**

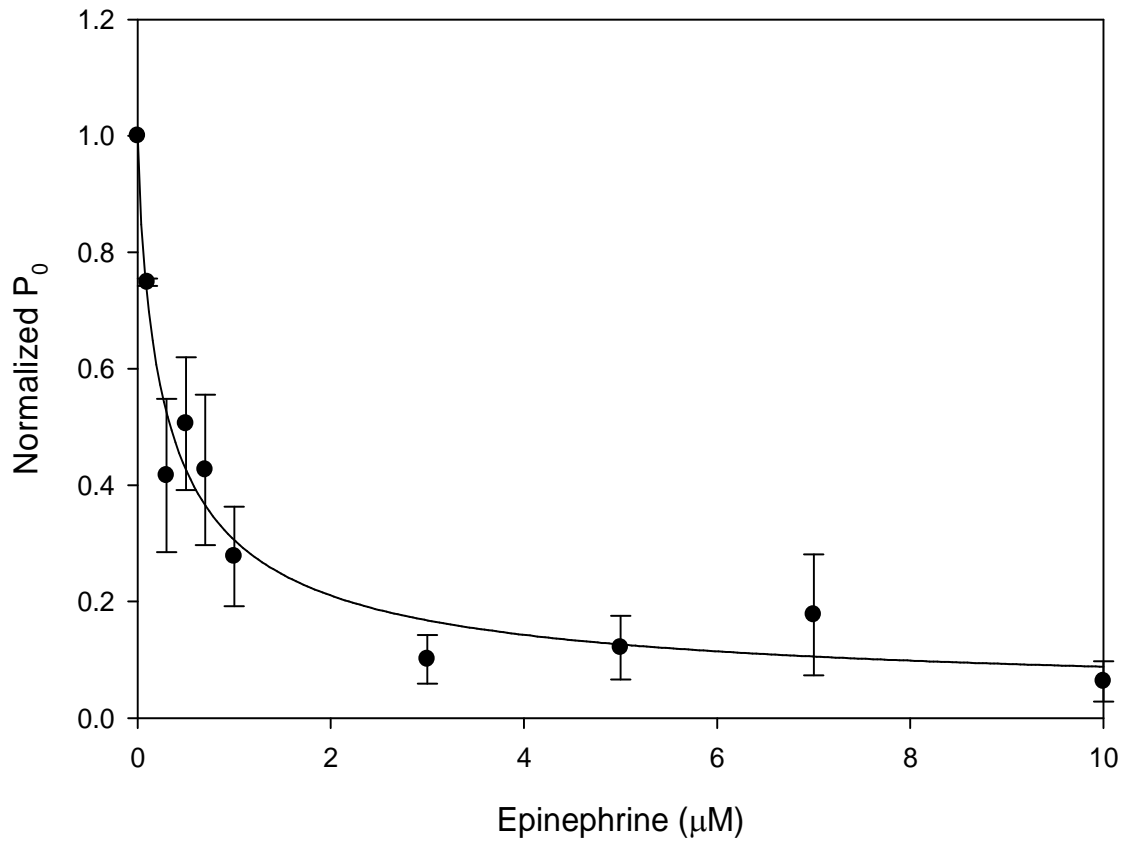
Planar lipid bilayer experiments were performed to test Epinephrine's potency on RyR2. 410nM free Ca<sup>2+</sup> was used (calculated using BAD). Epinephrine concentrations ranging from 100nM-30uM were tested in a total of 6 experiments. Standard error bars were generated and the IC<sub>50</sub> value was determined using the same method as used for isoproterenol.



**Figure 14 Representative single channel recordings of epinephrine**

Two second traces showing dose-dependent inhibition by epinephrine. Two channels can be distinctly identified in traces A and B. A channel opening is indicated by a downward fluctuation. A) 410nM  $\text{Ca}^{2+}$  ( $P_0=1.3$ ) B) 1 $\mu\text{M}$  Epinephrine ( $P_0=0.88$ ) C) 3 $\mu\text{M}$  Epinephrine ( $P_0=0.40$ ) D) 5 $\mu\text{M}$  Epinephrine ( $P_0=0.23$ ) E) 10 $\mu\text{M}$  Epinephrine ( $P_0=0.15$ )

Figure 15 shows the dose-dependent response of normalized RyR2 open probability vs. epinephrine concentration. Like isoproterenol, epinephrine displays strong inhibition of RyR2 characteristics. The  $IC_{50}$  was determined to be 310nM. Epinephrine at high concentrations (30 $\mu$ M) is more effective in inhibiting channel activity than is isoproterenol. However, neither compound completely closes down the channel. They are both partial antagonists of RyR2 activity. Once again, 10 $\mu$ M RR was added at the termination of each experiment.



**Figure 15 RyR2 inhibition by epinephrine**

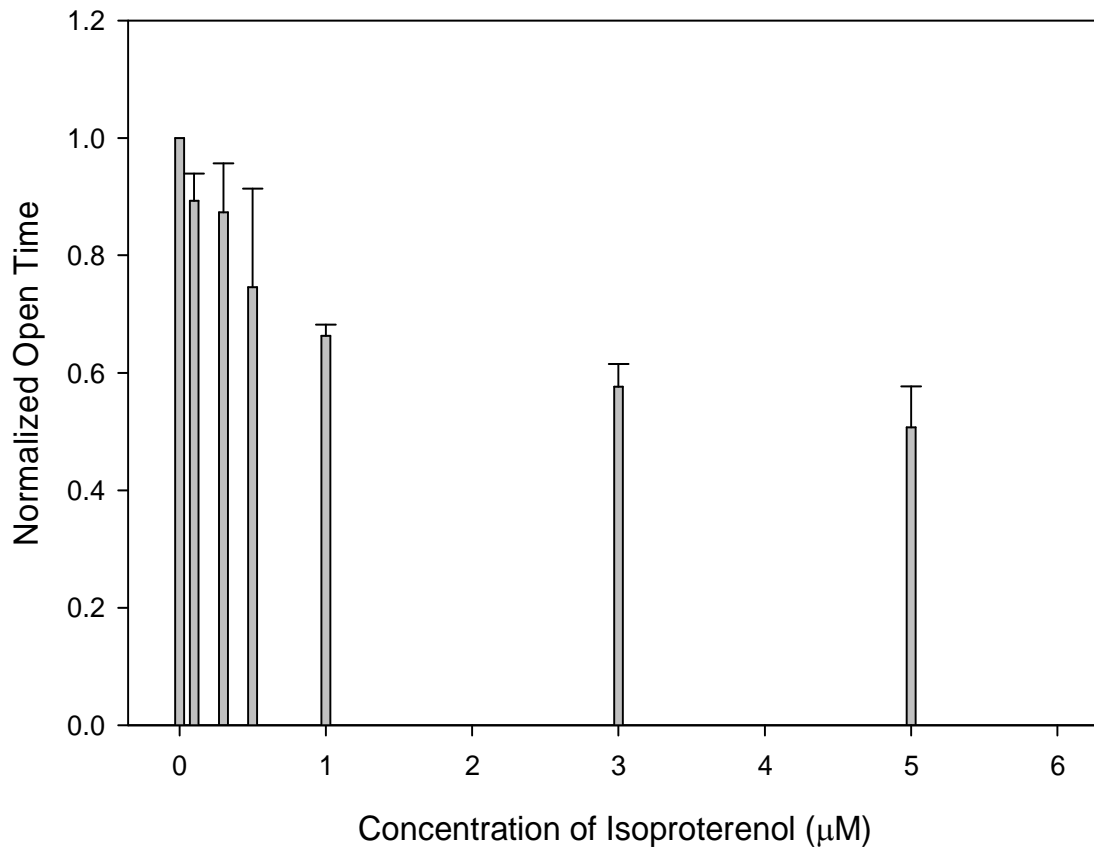
Control recordings at 410nM Ca<sup>2+</sup> were normalized to 1. Average P<sub>0</sub> with standard error before normalization was 0.56±0.09. Each point represents the average of 6 experiments ± standard error.

## **4.4 Dwell Times**

### **4.4.1 Isoproterenol Increases the Closed Time of RyR2**

Open and closed dwell times were calculated for isoproterenol as described in the methods section. Figure 16 shows normalized open times for varying concentrations of isoproterenol; open time decreases slightly. The p-values at 500nM and 5 $\mu$ M isoproterenol for the open time were determined to be 0.14 and 0.08 respectively, indicating a statistically insignificant result. However, fluctuations in the open time are hard to measure due to the already short open time of control recordings ( $\sim$ 1ms) and the resolution to which they can be measured; this leaves a level of uncertainty as to whether the measurements made in this experiment for the open times actually reflect the open time of the channel.

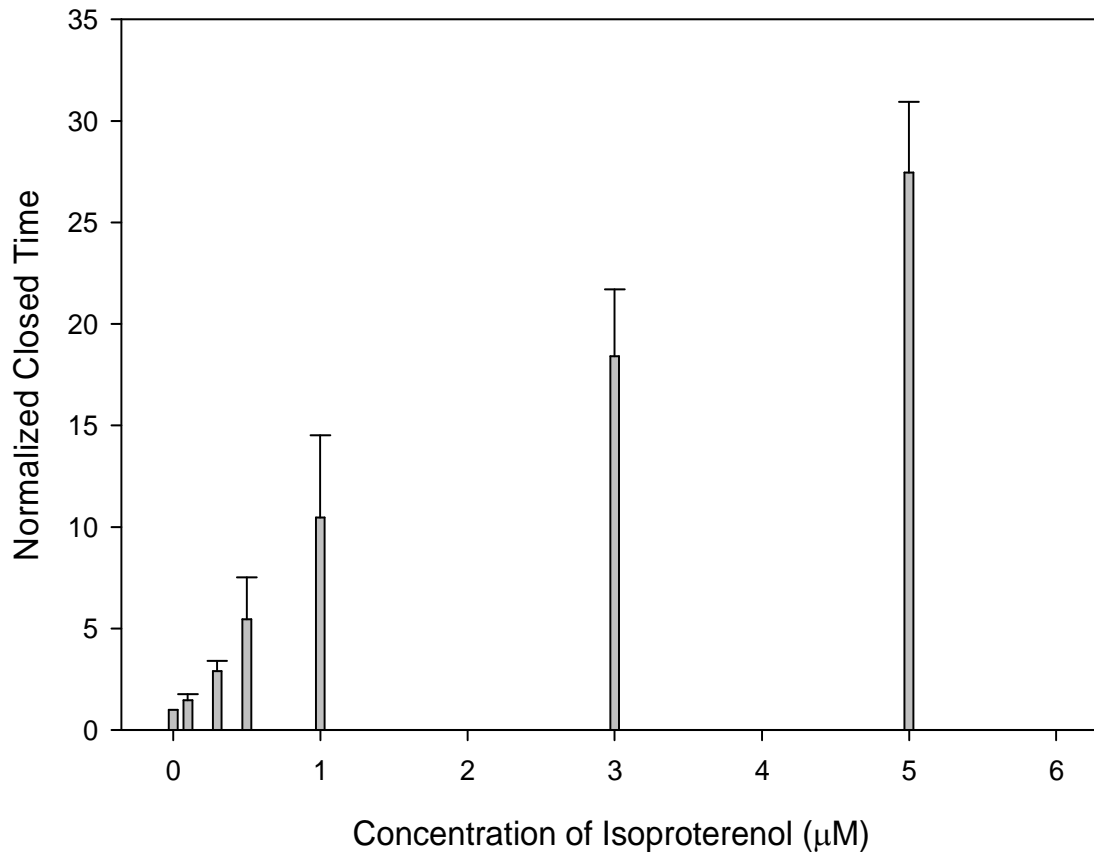




**Figure 16 Open times of RyR2 with isoproterenol**

Open time ( $\tau_0$ ) of single channel measurements at varying concentrations of isoproterenol. Isoproterenol slightly lowers the open time of the channel. Control recordings (410nM  $\text{Ca}^{2+}$ ) were normalized to one. Average  $\tau_0$  with standard error (n=7) prior to normalization was  $1.15 \pm 0.22$  ms. All data is the average of 7 experiments normalized as the percent of control  $\pm$  the standard error.

Figure 17 shows the normalized closed times as a function of isoproterenol. Closed time dramatically increases as increasing concentrations of isoproterenol is added. This means that isoproterenol's inhibition of RyR2 is mainly due to an increase in the closed time of the channel. The p-values at 500nM and 5 $\mu$ M isoproterenol were determined to be 0.02 and 0.01 respectively, making the epinephrine's effect of increasing the closed durations statistically significant.



**Figure 17 Closed times of RyR2 with isoproterenol**

Closed times of single channel measurements at varying concentrations of isoproterenol. Isoproterenol mainly decreases the open probability by increasing the closed time ( $\tau_C$ ). Control recordings (410nM  $\text{Ca}^{2+}$ ) were normalized to one. Average  $\tau_C$  with standard error (n=7) prior to normalization was  $2.35 \pm 0.47$  ms All data is the average of 7 experiments normalized as the percent of control  $\pm$  the standard error.

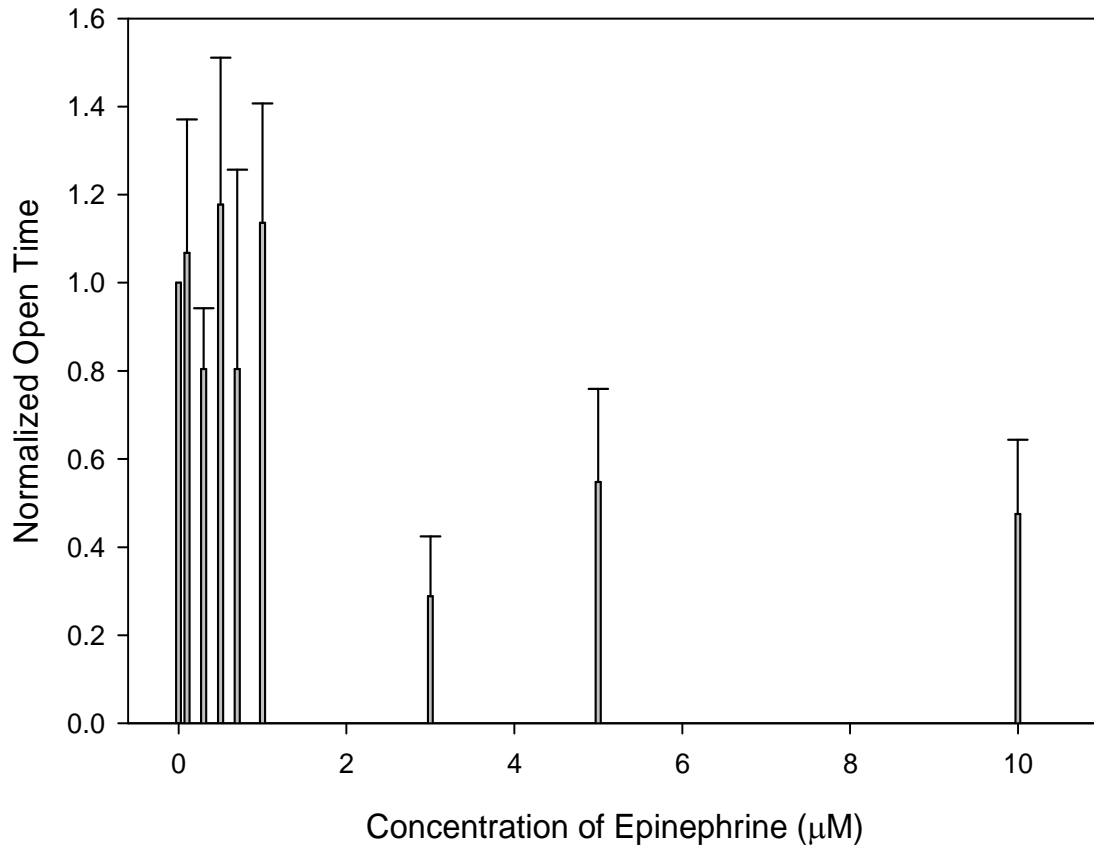
#### **4.4.2 Epinephrine Increases the Closed Time of the Channel**

Epinephrine open and closed times were calculated in the same way as isoproterenol.

Figure 18 shows a slight decrease in the open times as epinephrine concentration increases. The p-values at 500nM and 5 $\mu$ M epinephrine for the open time were determined to be 0.41 and 0.18 respectively, indicating a statistically insignificant result.

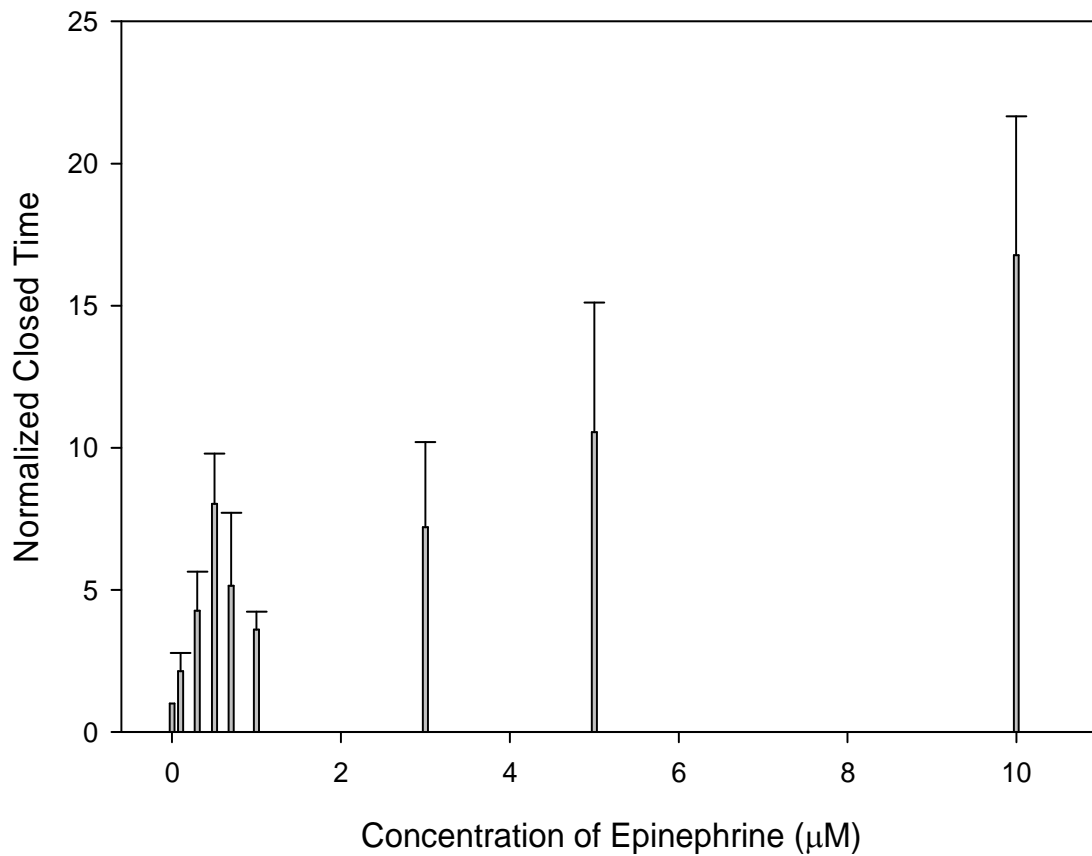
Once again, these measurements are at the time resolution limit of our setup, so it is unclear if this data reflects actual open times.

Figure 19 shows that like isopoteranol, epinephrine mainly decreases the open probability of the channel by increasing the amount of time the channel is in the closed state. The p-values at 500nM and 5 $\mu$ M epinephrine were determined to be 0.04 and 0.02 respectively, making the epinephrine's effect of increasing the closed durations statistically significant.



**Figure 18 Open times of RyR2 with epinephrine**

Open times of single channel measurements at varying concentrations of epinephrine. Epinephrine slightly lowers the open time ( $\tau_0$ ) of the channel. Control recordings (410nM  $\text{Ca}^{2+}$ ) were normalized to one. Average  $\tau_0$  with standard error (n=5) prior to normalization was  $0.97 \pm 0.13$  ms All data is the average of 5 experiments normalized as the percent of control  $\pm$  the standard error.



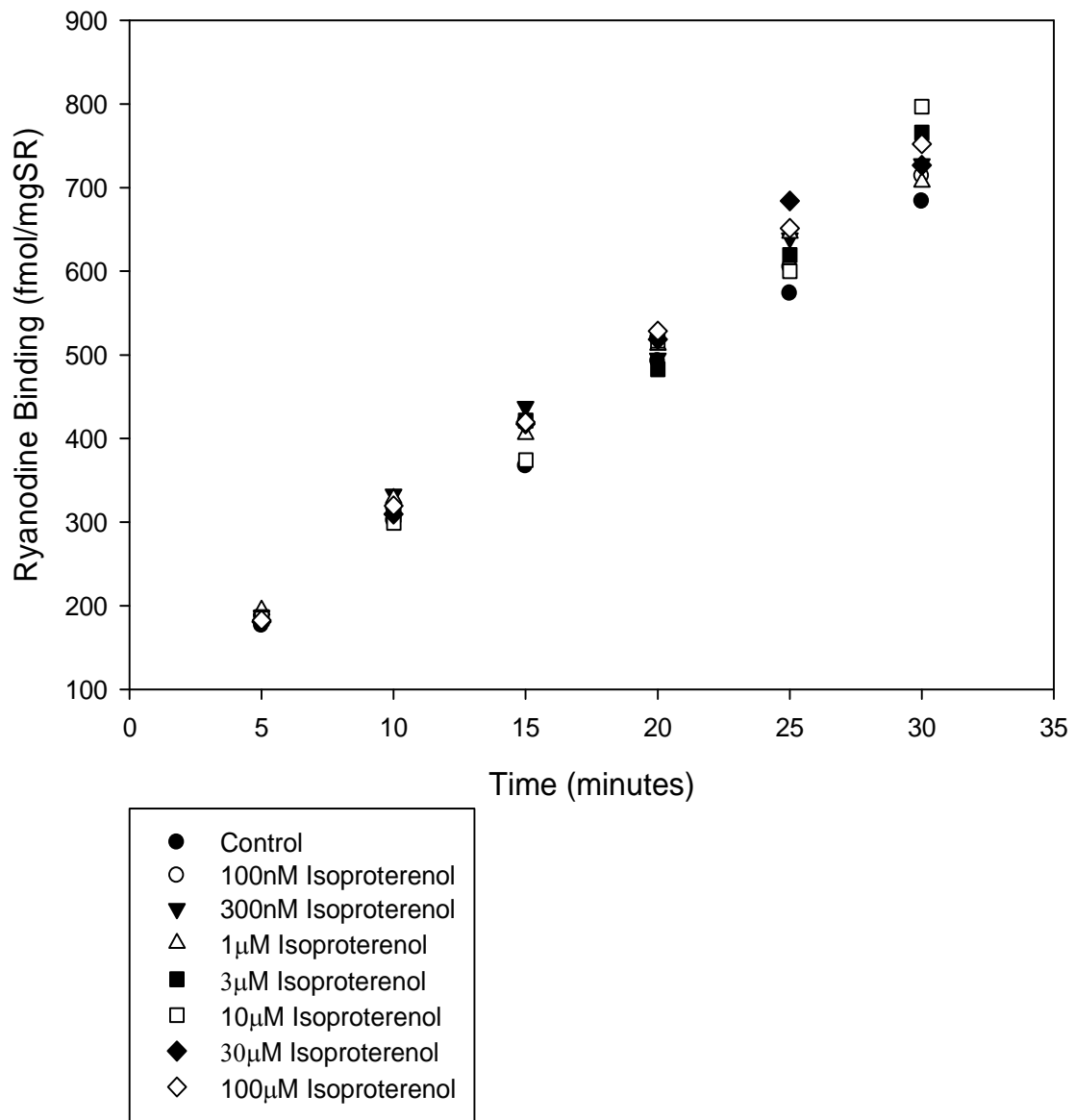
**Figure 19 Closed times of RyR2 with epinephrine**

Closed times ( $\tau_C$ ) of single channel measurements at varying concentrations of epinephrine. Epinephrine mainly decreases the open probability by increasing the closed time. Control recordings (410nM  $\text{Ca}^{2+}$ ) were normalized to one. Average  $\tau_C$  with standard error (n=5) prior to normalization was  $1.78 \pm 0.45$  ms All data is the average of 5 experiments normalized as the percent of control  $\pm$  the standard error.

#### **4.5 Ryanodine Binding is Unaffected by Isoproterenol and Epinephrine**

Initial rate experiments were performed for both isoproterenol and epinephrine as described in the methods section. Despite both compounds being strong inhibitors of RyR2 at the single channel level, they display no strong trends in the inhibition or stimulation in the initial rates of ryanodine binding.

As can be seen in figure 20, the rate of ryanodine binding is not sensitive to the isoproterenol concentration. All concentration tested start and end at roughly the same level as the control experiment ( $50\mu\text{M Ca}^{2+}$ , no drug). This indicates that the channels remained in roughly the same open percentage for all concentrations of isoproterenol as well as the control.

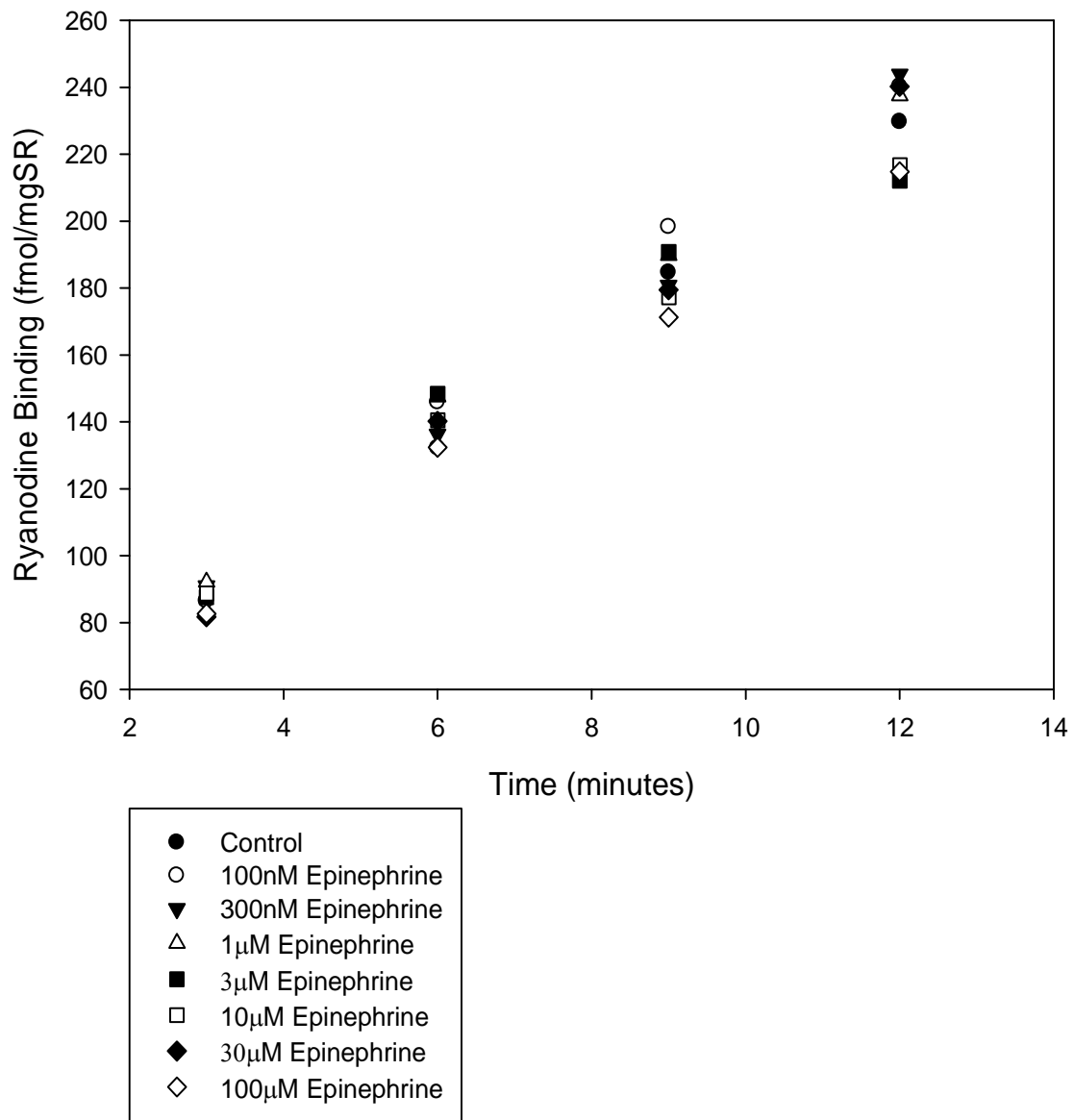


**Figure 20 Isoproterenol does not affect the rate of ryanodine binding**

Initial rate of ryanodine binding vs. isoproterenol concentration. No significant changes in the amount of ryanodine binding over time are observed as a function of [isoproterenol].



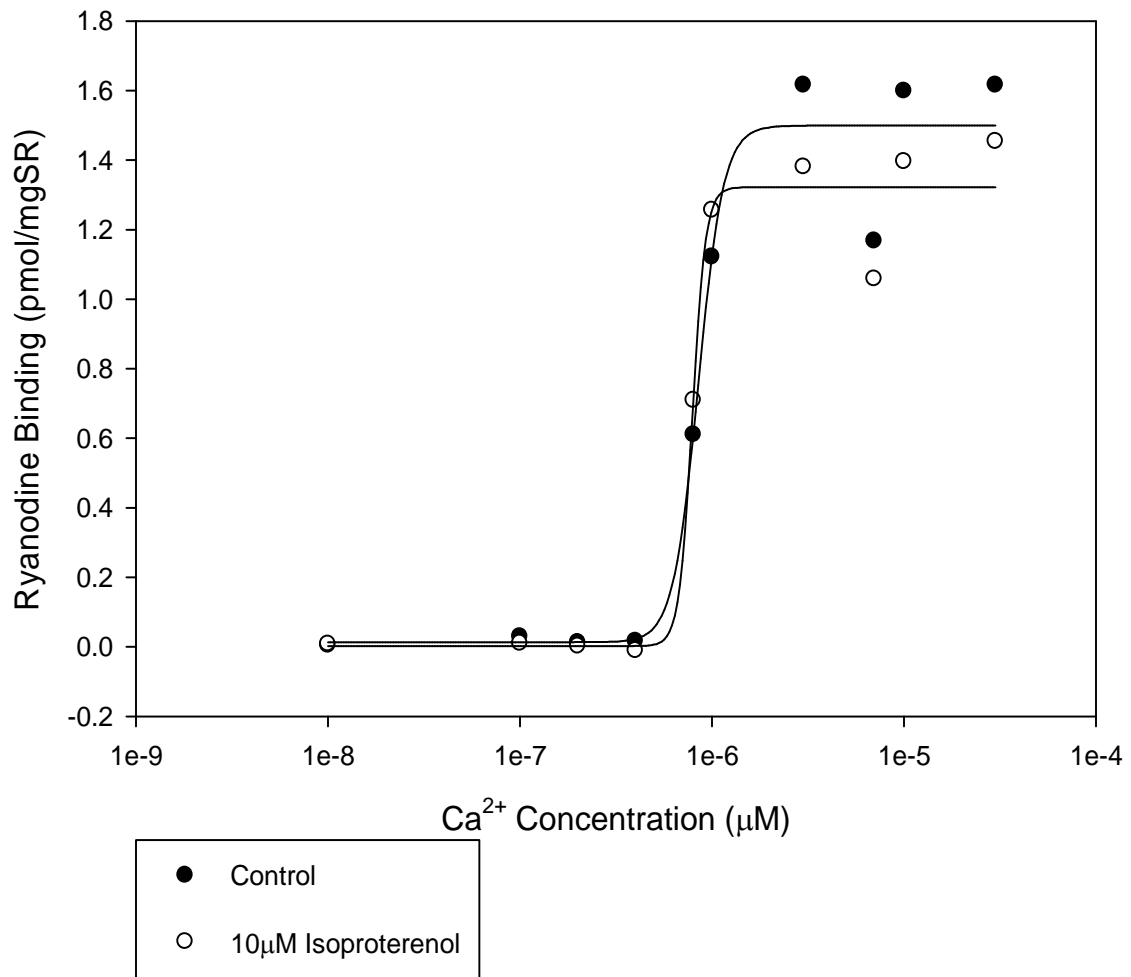
Figure 21 shows a similar trend for epinephrine compared to that seen with isoproterenol. The amount of binding increased in a linear manner over the 4 time points used. All traces roughly start and end at the same point as the control (50 $\mu$ M Ca<sup>2+</sup>, no drug). Once again, this is indicative of epinephrine of having no inhibitory effect on ryanodine binding.



**Figure 21 Epinephrine does not affect the rate of ryanodine binding**

Initial rate of ryanodine binding vs. epinephrine concentration. No significant changes in the amount of ryanodine binding over time are observed as a function of [epinephrine].

In addition to the initial rates experiments, a  $\text{Ca}^{2+}$  dependence experiment was carried out both with and without 10 $\mu\text{M}$  isoproterenol. Figure 22 shows a sharp rise of ryanodine binding over a small range of  $\text{Ca}^{2+}$  concentrations for both traces. There is no significant shift of the curve between the control and the 10 $\mu\text{M}$  isoproterenol. This is once again indicative of isoproterenol not having an effect on the openness of the channel in ryanodine binding type experiments.



**Figure 22 Isoproterenol Ca<sup>2+</sup> dependent ryanodine binding**

Ca<sup>2+</sup>-dependence equilibrium binding with and without 10µM isoproterenol. No significant shift in the Ca<sup>2+</sup>-dependence can be seen.

## Chapter V Conclusions and Discussion

The role of catecholamines has been studied since the early 1900s and their function in the body is thought to be well understood. In this thesis, the effect of two catecholamines, epinephrine and isoproterenol, acting directly upon cardiac ryanodine receptor has been examined for the first time.

We have shown that both isoproterenol and epinephrine act as strong electron donors. The concentrations at which epinephrine and isoproterenol half maximally act as electron donors are 17.5 $\mu$ M and 24.3 $\mu$ M respectively. We have also shown that these two compounds are potent inhibitors of single channels in the planar lipid bilayer. Bilayer studies performed produced IC<sub>50</sub> values for epinephrine and isoproterenol of 310nM and 210nM respectively. We have shown that the primary means of inhibition is an increase in the overall closed time of the channel. P-values calculated around and much higher than the IC<sub>50</sub>, yielded statistically significant results for the closed times. It is unclear whether these compounds also have an effect on the open time of the channel. P-values calculated for the open times yielded non-significant results, but measurements are being made at the time resolution our system.

It has previously been shown that inhibitors of the ryanodine receptor tend to act as electron donors, while activators of the channel tend to be electron acceptors<sup>37</sup>. Here, this hypothesis is confirmed for the two compounds studied. Both compounds share very similar electron donor properties as well as similar inhibition of RyR2 in the planar lipid bilayer.

These compounds, although strong inhibitors in the bilayer, had no effect on ryanodine binding experiments. Initial rates of binding indicated that the channels remained in roughly the same open percentage over all concentrations assayed. This is not the first time our lab has encountered this apparent dilemma; prior compounds tested, which have shown strong potency in the bilayer have had no effect on ryanodine binding.

One possible explanation to account for this is the means by which these catecholamines inhibit the channel. Ryanodine binds to the channel when it is in an open state. We have shown that these compounds do not significantly impact the duration of the open time of the channel. It is possible that without significantly altering the open time of the channel, the total amount of ryanodine binding will remain a constant. Inhibition of ryanodine binding may require a significant decrease in the open time of the channel.

In the body, catecholamines activate the ryanodine receptor via a non-direct interaction that is initiated outside of the cell. Epinephrine is hydrophilic, making it unlikely that it is able to cross the hydrophobic plasma membrane. Isoproterenol, which is not naturally produced, is also hydrophilic. This indicates that it is unlikely that epinephrine or isoproterenol have a direct interaction on RyR2 when administered, or produced in the body.

It is known that heart failure is associated with a RyR2 leak thought to be caused by a hyperphosphorylation of the channel<sup>37</sup>. This hyperphosphorylation hypothesis remains controversial; it has been shown that PKA-phosphorylation does not affect Ca<sup>2+</sup> sparks in

permeabilized myocytes<sup>38</sup>. Regardless,  $\beta$ -adrenergic receptor stimulation does seem to initiate a release of  $\text{Ca}^{2+}$  from RyR2, which in turn can lead to arrhythmia.

Two therapeutic ways to treat patients with arrhythmia can be imagined to alleviate a leaky RyR2; one where a direct interaction with a compound and the  $\beta$ -adrenergic receptor is utilized to prevent the catecholamines from binding; this is realized with the advent of the so called “ $\beta$ -blockers.” And a second method, where a direct interaction between a compound and RyR2 occurs, one that stabilizes the channel into a more closed state, thereby preventing  $\text{Ca}^{2+}$  leak.

$\beta$ -blockers are high affinity compounds which bind to the  $\beta$ -adrenergic receptor, but do not stimulate adenylyl-cyclase activity. When bound, they will prevent agonists of the  $\beta$ -adrenergic receptor, such as the catecholamines, from binding. It has been shown that treatment with  $\beta$ -blockers can restore cardiac function, reducing the death rate of patients with HF<sup>39</sup>. Some such  $\beta$ -blockers being used in the treatment of heart failure include: Carvedilol, propranolol, metoprolol, and atenolol.

The other method to decrease diastolic  $\text{Ca}^{2+}$  leak during arrhythmias is to directly inhibit the intracellular  $\text{Ca}^{2+}$  leak by inhibiting RyR2. It has previously been shown that compounds such as flecainide<sup>40</sup>, or benzothiazepine<sup>41</sup> will inhibit RyR2, suppressing arrhythmias in canine and Langendorff rat hearts.

Despite these two methods' ability to alleviate arrhythmia, it is unclear how these mechanisms are beneficial to heart failure; in normal hearts, inhibition of RyR2 and the blockade of the  $\beta$ -adrenergic receptor both lead to a decrease in cardiac output. It has

been proposed that the  $\beta$ -blocker carvedilol functions not only by binding to the  $\beta$ -adrenergic receptor, thereby down-regulating PKA activity, but it also directly inhibits RyR2. Specifically, carvedilol has been shown to suppress spontaneous calcium waves, also known as stored overload-induced  $\text{Ca}^{2+}$  release (SOICR), at concentrations<sup>42</sup> similar to what we show the catecholamines inhibit RyR2 activity.

Both the catecholamines and carvedilol are interesting compounds in that they can influence RyR2 activity, both on the cell membrane and on RyR2 itself. Carvedilol is known to cross the cell membrane<sup>43</sup>, but it is unlikely that it accumulates to levels within the cell that can inhibit RyR2 activity. The catecholamines neither cross the cell membrane, nor are present in high enough concentrations in the body to directly influence RyR2 activity.

Despite the problems with these 2 sets of compounds, this research does offer a promising insight into the potential development of a new class of compounds. The alteration of drugs like the catecholamines or carvedilol may lead to the production of a more lipophilic compound, one that can both inhibit  $\beta$ -adrenergic receptor activity, and exist in intracellular concentrations high enough to influence RyR2 activity. Our data suggests that making these compounds into better electron donors can alleviate the necessity of increasing the amount of compound needed to inhibit RyR2.

Whether it is possible to modify these compounds without altering the desired effects on the system remains to be unseen. Regardless, the development of compounds that can



both inhibit RyR2 activity inside the cell, and act as  $\beta$ -blockers could create a new frontier in drug development in the prevention of arrhythmia and HF.

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