ARE RYANODINE RECEPTORS IMPORTANT FOR DIASTOLIC DEPOLARIZATION IN HEART?

A Dissertation submitted in partial fulfillment of the requirement for the Degree of Doctor of Medicine in Physiology (Branch – V) Of The Tamilnadu Dr. M.G.R Medical University, Chennai -600 032

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This is to certify that the thesis entitled "**Are ryanodine receptors important for diastolic depolarization in heart?"** is a bonafide, original work carried out by Dr.Teena Maria Jose , in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamilnadu Dr. M.G.R. Medical University, Chennai to be held in April- 2017.

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**Are ryanodine receptors important for diastolic depolarization in heart?**" were carried out by me during my term as a post graduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

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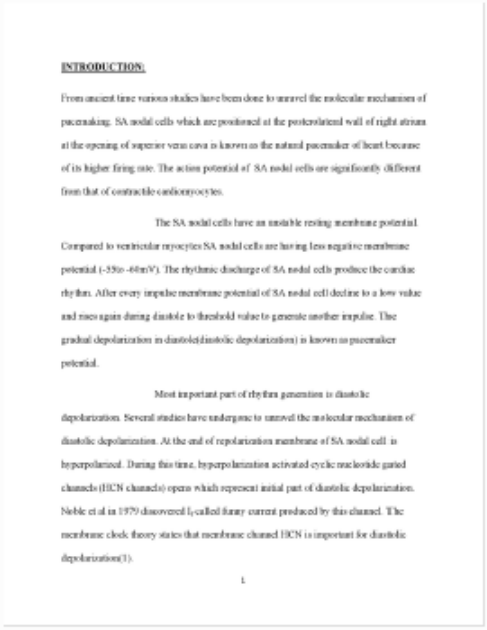

INTRODUCTION

From ancient time various studies have been done to unravel the molecular mechanism of pacemaking. SA nodal cells which are positioned at the posterolateral wall of right atrium at the opening of superior vena cava is known as the natural pacemaker of heart because of its higher firing rate. The action potential of SA nodal cells are significantly different from that of contractile cardiomyocytes.

The SA nodal cells have an unstable resting membrane potential. Compared to ventricular myocytes SA nodal cells are having less negative membrane potential (-55to -60mV). The rhythmic discharge of SA nodal cells produce the cardiac rhythm. After every impulse membrane potential of SA nodal cell decline to a low value and rises again during diastole to threshold value to generate another impulse. The gradual depolarization in diastole(diastolic depolarization) is known as pacemaker potential.

Most important part of rhythm generation is diastolic depolarization. Several studies have undergone to unravel the molecular mechanism of diastolic depolarization. At the end of repolarization membrane of SA nodal cell is hyperpolarized. During this time, hyperpolarization activated cyclic nucleotide gated channels (HCN channels) opens which represent initial part of diastolic depolarization. Noble et al in 1979 discovered I_f called funny current produced by this channel. The membrane clock theory states that membrane channel HCN is important for diastolic depolarization(1).

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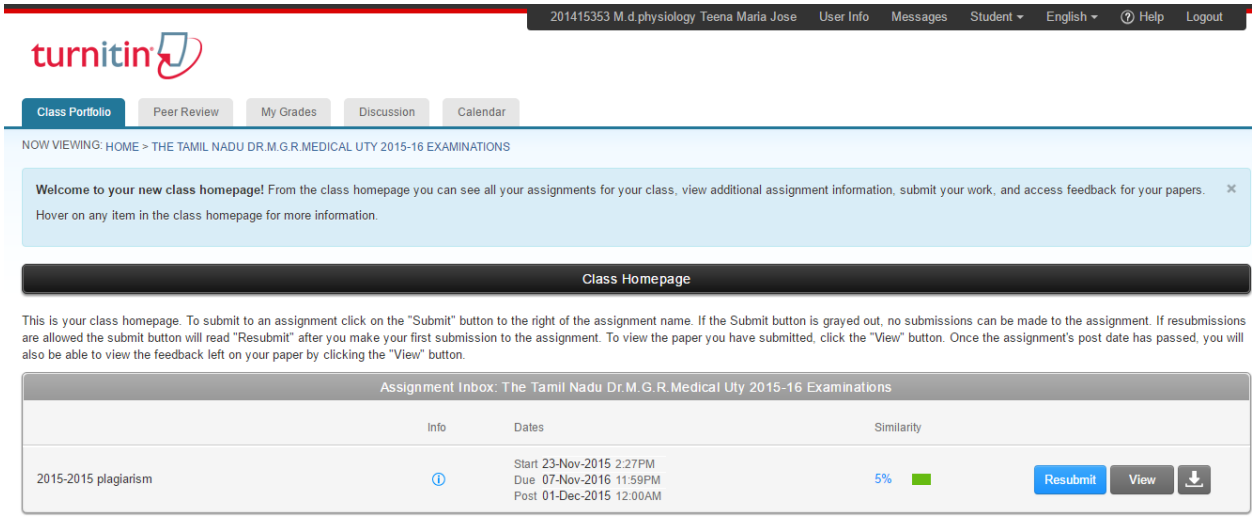
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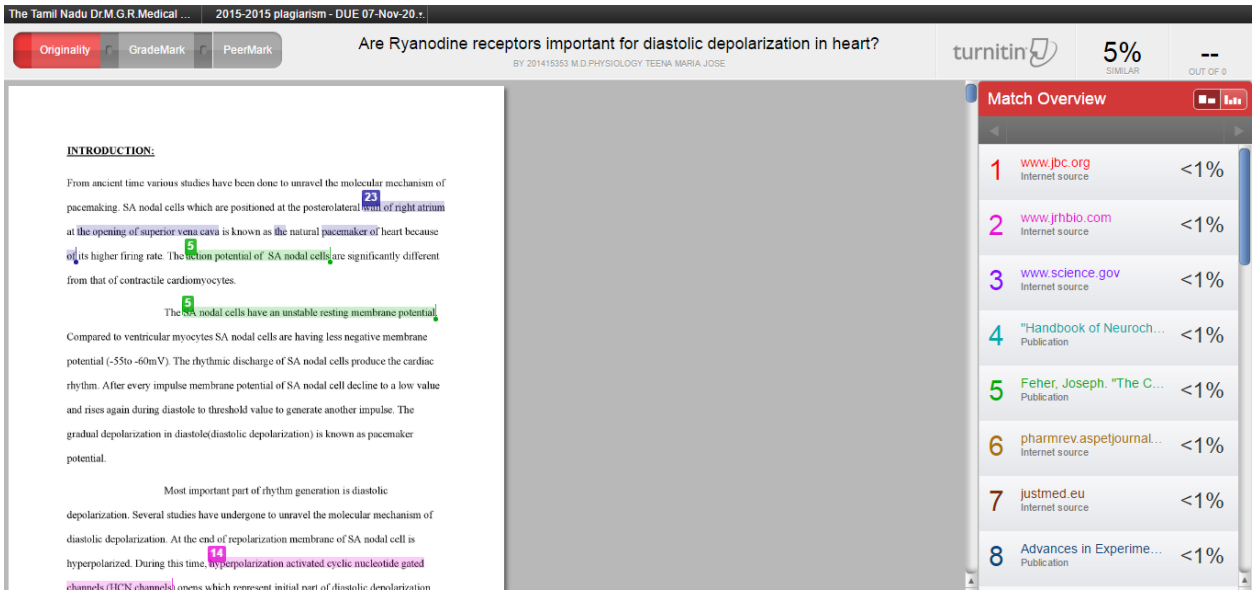
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TABLE OF CONTENTS

ABSTRACT:

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Introduction

It is known that during systole there is release of Ca^{2+} from SR (sarcoplasmic reticulum) via RyR (Ryanodine receptor) which is important for excitation contraction coupling. There is release of SR Ca^{2+} during diastole also. This released calcium will get extruded via sodium-calcium exchanger (NCX). Since the stoichiometry of NCX is $3Na^{+}$:$1Ca^{2+}$, calcium extrusion will result in an inward current. This current called I_{NCX} is important for late diastolic depolarization of SA (sinoatrial) nodal cells of heart.

Diastolic Ca^{2+} release from SR can occur through RyR(Ryanodine receptor) or IP_3R(Inositol 1,4,5 triphosphate receptor). A previous research work in the department has assessed the role of IP_3R in diastolic calcium release. The conclusion from this study is that IP_3R blockade decreased the heart rate –suggesting that IP_3R is involved in

calcium release during diastole. This study will assess the role of RyRs in pacemaker function.

Objective:

To study the changes in heart rate after perfusing isolated rat heart with normal ECF solution followed by addition of 100 µm Ryanodine.

Materials and methods:

Isolated hearts of male Wistar rats in Langendorff mode was used in this experiment (n=12). It was perfused with normal extracellular solution for first 15 minutes to record basal HR (heart rate). Then 0.25% DMSO (control) or 100µM Ryanodine in 0.25% DMSO was added to the normal extracellular solution and HR was recorded for 15 minutes. This was followed by wash with normal extracellular solution.

HR was calculated from surface ECG recorded with the help of surface electrodes using CMCdaq (computerised data acquisition system).

Results:

Control group (0.25% DMSO) shows that the heart rate does not change with time (p=0.075, with WSR (Wilcoxan signed rank test).With Ryanodine 100µM there is decrease in heart rate (p=0.028).Compared to control Ryanodine 100µM decreased the heart rate 55.56% (p=0.004, with MWU (Mann-Whitney U test).

Conclusion:

Both Ryanodine receptors and IP_3 receptors are important for pacemaking.

Key words:

Sarcoplasmic reticulum, Ryanodine receptor, Sodium calcium exchanger, Isolated rat heart, Pacemaking

INTRODUCTION:

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The SA nodal cells have an unstable resting membrane potential. Compared to ventricular myocytes SA nodal cells are having less negative membrane potential (-55to -60mV). The rhythmic discharge of SA nodal cells produces the cardiac rhythm. After every impulse membrane potential of SA nodal cell decline to a low value and rises again during diastole to threshold value to generate another impulse. The gradual depolarization in diastole (diastolic depolarization) is known as pacemaker potential.

Most important part of rhythm generation is diastolic depolarization. Several studies have undergone to unravel the molecular mechanism of diastolic depolarization. At the end of repolarization membrane of SA nodal cell is hyperpolarized. During this time, hyperpolarization activated cyclic nucleotide gated channels (HCN channels) opens which represent initial part of diastolic depolarization. Noble et al in 1979 discovered I_f called funny current produced by this channel. The membrane clock theory states that membrane channel HCN is important for diastolic depolarization(1).

There occurs rhythmic, regular and spontaneous calcium release from the sarcoplasmic reticulum during diastole(2). Various studies using confocal methods have shown this. There is calcium release from sarcoplasmic reticulum during systole as well as diastole(2). During systole calcium release is calcium induced calcium release from sarcoplasmic reticulum. There is calcium release during diastole also. Since the stochiometry of the sodium calcium exchanger is to move 3 Na^{2+} inwards and 2 Ca^{2+} outwards, the released calcium get extruded via NCX. This results in an inward current known as I_{NCX} which constitute next part of diastolic depolarization. This theory which states that intracellular calcium is important for diastolic depolarization is known as calcium clock theory(3).

Later these two theories were fused together to form coupled clock theory which states that both membrane clock and calcium clock are important for diastolic depolarization(4).

This diastolic calcium release is occurring from sarcoplasmic reticulum. The sarcoplasmic reticulum has got two types of calcium release channels: Ryanodine receptors and IP_3 receptors (inositol 1, 4, 5 triphosphate receptors). During diastole release of calcium can occur from both channels. It is not clear if diastolic release of calcium occurs from either or both channels. Hence this study has been done to substantiate the role of Ryanodine receptors in diastolic depolarization.

This study is done in isolated heart perfused in Langendorff mode with normal extracellular ringer with maintenance of 37 degree Celsius

temperature and continuous oxygenation. Heart rate is calculated from the surface electrogram obtained. To study the role of Ryanodine receptors blocker of the channel is added. Ryanodine is a chemical which is known to block the channel at micromolar concentration(5).

REVIEW OF LITERATURE:

Heart plays a vital role in our body by pumping blood to all parts of body. It helps to provide oxygen to the tissues, remove carbon dioxide, supply nutrients, and eliminate waste products. It is also required for circulation of hormones. Thus it helps in maintaining homeostasis. Even from ancient periods various studies have been done to understand heart and its functions. Aristotle a famous Greek philosopher acknowledged heart as a vital organ from his observations in chick embryo. In the fourth century BC he found heart as a three chambered organ located in the central part of body. Later a detail explanation about heart and its functioning was given by Leonardo da Vinci and Galen. In 1968 the publication named 'On the circulation of blood' written by William Harvey formed the modern basis of understanding about the working of heart. Harvey's ideas on circulation were accepted widely at the end of 17 th century.

Heart consists of four chambers made up of cardiac muscle. The properties of cardiac muscle is different from skeletal muscle in many ways. Cardiac muscle is a striated involuntary muscle which works as a syncitium. Cardiac muscle has got many special properties. It can generate its own impulses and conduct these impulses to various parts of heart via specialized conducting system.

Properties of cardiac muscle

The basic five properties of cardiac muscle includes

1. Chronotropy -ability to produce its own impulses

2. Dromotropy -propagation of impulses through gap junctions
3. Bathmotropy (excitability) - ability to respond to various stimuli
4. Inotropy (contractility) - ability to generate force
5. Lucitropy -relaxation

Heart consists of four chambers constituting two separate pumps right heart and left heart. Right heart pumps blood mainly to lungs and left heart to the peripheries. Each pump consists of one atria and one ventricle.

Heart is mainly made up of three types of muscle fibers. It includes atrial, ventricular and conducting or excitatory muscle fibers. Atrial and ventricular muscle fibers are similar to skeletal muscle fibers with longer duration of contraction whereas conducting and excitatory muscle fibers contain less contractile fibers. Cardiac muscle fibers act like syncytium because of presence of gap junctions(6)(7). They are concerned with rhythmic beating of heart because of production of action potential and its propagation.

<u>Cardiac action potential</u>

The simultaneous contraction of atria and ventricles occur because of generation of impulses known as cardiac action potential. It has got five phases(8).

1. Phase 4- ventricular muscles have stable resting membrane potential of about -90mV.

2. Phase 0- rapid depolarization occurs at this phase. After this phase membrane potential becomes more positive(due to rapid activation of sodium channels)
3. Phase 1- phase of rapid repolarization(sodium channel get inactivated)
4. Phase 2- this phase is the longest phase known as plateau phase (because of slowly opening Ca^{2+} channels)
5. Phase 3- brings back normal resting membrane potential by slow repolarization (opening of potassium channels)

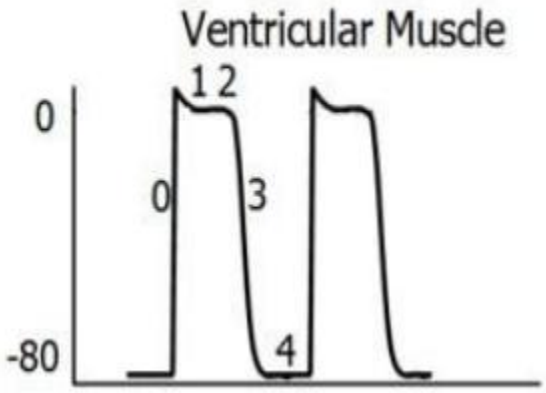


Figure 1: Action potential of ventricular muscle

SA nodal action potential differs significantly from ventricular muscle action potential. SA nodal cells do not have a stable resting membrane potential. It is more depolarized at phase 4 and undergoes slow diastolic depolarization

Excitatory and conductive system of heart

Excitatory and conductive fibers of heart control the contraction of heart.

The conductive system of heart is composed of

1. Sinoatrial node(SA node)

2. Internodal pathways

3. Atrioventricular node(AV node)

4. A-V bundle

5. Purkinje fibers

SA node

It is known as primary pacemaker of heart. It is located in the posterolateral wall of right atrium near opening of superior vena cava. It is a very small muscle strip which is ellipsoid in shape. Very few contractile fibers are seen in SA node. Impulses from SA node directly spread to various parts of atria because of direct connection. It has got the property of self excitation. The rate of beating of heart is controlled by SA node because of highest firing rate.

Resting membrane potential of SA node is about -55 to -60 mV which is less negative than that of ventricular muscles. The main types of channels seen in SA nodal cells include

1. Hyper polarization activated channel(HCN channel)
2. T type calcium channel
3. L type calcium channel

4. Potassium channels

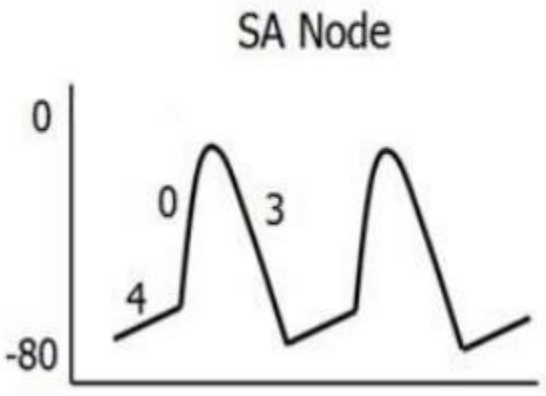


Figure 2: Action potential of SA nodal cells

SA nodal cells rhythmically discharge impulses that produce cardiac rhythm. After each impulse membrane potential of SA nodal cells decline to low value and rise again to reach a threshold to produce an impulse. This membrane potential is known as prepotential or pacemaker potential. It is also called diastolic depolarization of heart.

At the beginning of impulse the membrane is hyperpolarized due to potassium channels. After that a special type of channel which is permeable to both sodium and potassium opens. This channel opens only when the SA nodal membrane is hyperpolarized and hence this channel is called hyperpolarization activated channel (HCN channel) and current produced is called I_h. The characteristics of this channel seem to be funny so it is also called funny current. This current causes membrane potential to reach a less negative value constituting the first part of prepotential.

This membrane depolarization causes opening of T type Ca^{2+} channels. This causes release of calcium from sarcoplasmic reticulum via IP_3 and RyR receptors known as calcium induced calcium release. This released calcium is extruded via sodium calcium exchanger. It causes movement of 3 Na^{2+} inside and 2 Ca^{2+} outside since the stochiometry of NCX is to move $3Na^{+}$ inside and $2Ca^{2+}$ outside. The current produced is called I_{NCX} which constitute the next part of prepotential and membrane reach threshold value (-40mV) and opening of L type calcium channel occurs. This result in making up of next part of diastolic depolarization and result in production of impulse. Repolarization is produced by potassium channels which cause efflux of potassium ions simultaneous with closing of Ca^{2+} channels. After reaching resting membrane potential potassium channels remain open for some time causing membrane hyperpolarization(-60mV).Then potassium channels close and membrane potential reaches resting value(-55mV). This cycle continues till the lifetime of the patient. So in SA nodal action potential mainly Ca^{2+} channels are involved than Na^{2+} channels.

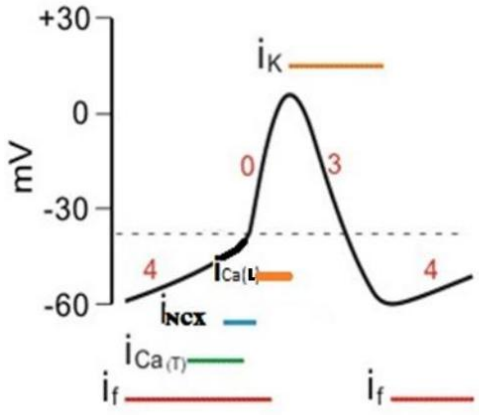


Figure 3: Various ion channels in production of diastolic depolarization

Chronotropy

Chronotropy is defined as the property to generate its own impulses. In the body some cells are provided with this property and such cells are called pacemaker cells. These pacemaker cells have got unstable resting membrane potential and they can undergo spontaneous depolarization resulting in production of action potentials. This can result in contraction of cells. Since neighboring cells act as syncytium this action potential is transmitted from pacemaker cells to others

Pacemaker cells seen in GIT

Pacemaker cells seen in GIT are called interstitial cells of Cajal. These cells are responsible for the phasic contractions produced by stomach, small intestine and large intestine by producing slow waves. These cells which are called primitive neurons were first described by Santiago Ramon y Cajal in 1911. The mechanism involved in pacemaking of these cells is still not clear.

Pacemaker cells in heart

Heart has got pacemaker cells. They can produce their own impulses without any neural and humoral stimulation. This was first reported by Galen when he found that heart continues to beat even after isolated from the body for some time. Most of the cells in the conducting system have got the property of pacemaking. Of all cells SA nodal cells have got the highest firing rate (60-100 per minute). So it is known as primary pacemaker of heart.

Theories of pacemaking

Many theories have been proposed to understand the ionic and molecular mechanisms involved in pacemaking. Recently more importance is given to the involvement of sodium calcium exchanger and intracellular calcium release in rhythm generation.

Membrane clock theory:

In 1979 Noble et al suggested the role of an inward current called I_f through HCN channels in producing diastolic depolarization in heart(1). These HCN channels are activated by hyperpolarization and permeable to sodium and potassium. The gene coding for this channel called HCN4 gene is found to be more expressed in SA node compared to surrounding atrial tissue(9). This funny current works like a clock to produce the rhythm. Hence this theory is called membrane clock theory.

Calcium clock theory:

Confocal imaging as well as immunofluorescence studies showed that intracellular calcium clock cause activation of many membrane ion channels to produce action potentials. This release of intracellular calcium occurs from the sarcoplasmic reticulum in a rhythmic fashion just before the next action potential. This calcium from intracellular stores gets extruded via sodium calcium exchanger producing an inward current. This inward current is important for depolarization(3). This theory which says that intracellular calcium release leads to action potential is known as calcium clock theory.

Coupled clock theory:

This theory states that calcium clock and membrane clock work together to produce the rhythm. Both can be regulated by membrane voltage, intracellular calcium, Ca^{2+}-calmodulin dependent protein phosphorylation and protein kinase A(4).

Cardiac rhythm and intracellular calcium

Initiation of cardiac rhythm is due to an array of currents produced by various ion channels present in the SA nodal pacemaker cells(10). Many of recent studies show inevitable role of intracellular calcium in generation of cardiac rhythm. Studies done by Rubenstein and Lipisus in cat pacemaker cells found that there occurs slowing of late diastolic depolarization in the presence of Ryanodine. Studies done on guinea pig SA nodal cells also showed decrease in heart rate with Ryanodine as well as cyclopiazonic acid(11). Both these show the importance of sarcoplasmic calcium in rhythm generation(12).

The last phase of diastolic depolarization was associated with a rise in intracellular calcium detected by fluo -3 fluorescence(12). This abrupt rise in calcium in the cytoplasm is called calcium sparks. Calcium sparks were first identified in isolated rat cardiac muscle cells in 1993 with the help of confocal microscope. This Ca^{2+} rise may be > 10 µm and frequency is 1.6 per second. Very localized increase in calcium sparks were seen beneath the sarcoplasmic membrane which can be suppressed by Ni^{2+} at

25-50µM. So from this we reach a conclusion that calcium release into the cell with the help of Ni^{2+} sensitive T type calcium channels act as a trigger for the calcium sparks in the cells. This calcium extrusion via NCX contribute to late part of diastolic depolarization(13)(14).

Calcium cycling in myocardial cells

Calcium plays important role in many physiological processes such as secretion, fertilization, signaling and muscle contraction. The role of calcium signaling in cardiac muscle fibers is studied extensively. Calcium is important not only for cardiac muscle contraction but also for rhythm generation. The increase in cytoplasmic calcium can occur by two methods. One is by influx of calcium from outside and other is by release of calcium from intracellular stores(15) (16).

Calcium transport can occur in response to various stimuli. That include

1. membrane depolarization which cause opening of various calcium channels on the membrane
2. certain neurotransmitters which act as second messengers to cause opening of certain channels eg.IP3 receptors located on sarcoplasmic reticulum
3. decline in intracellular calcium by store operated calcium channels
4. depolarization together with ligand eg NMDA receptors
5. sodium calcium exchange via NCX

In most cells sarcoplasmic caveolae function as intracellular calcium stores(17)(18).

Pathways that cause inward movement of calcium into the cell are

1. L type calcium channels

These channels can get inactivated by calcium as well as voltage. It is called L type calcium channels because of delayed inactivation. However when there is tremendous release of calcium from SR faster inactivation of L type calcium channel occurs and vice versa. In cardiac action potential plateau phase occurs because of opening of L type calcium channels. It is also required for the rhythm generation process in SA node by contributing to pacemaker potential along with action potential. L type calcium channels are important for calcium induced calcium release in cardiac muscle fibers. It is also involved in excitation-contraction coupling(19)(20).

2. T type calcium channels

It is called T type channel because of faster inactivation. It is shown to open at more negative membrane potentials compared to L type calcium channels. They do not contribute to cardiac action potential. But it contribute to pacemaker potential(21)(12).

3. Store operated calcium channels

When intracellular storage of calcium decreases calcium entry occurs by these channels(22).

4. Sodium calcium exchanger

It was first discovered by Carafoli's group in 1974. After contraction of cardiac muscle removal of calcium from the cytosol can occur through this channel. According to the stochiometry of the channel it causes movement of 3 sodium ions inside and 2 calcium ions outside in the forward direction. It can work in the reverse direction also. In the forward mode it causes production of a current called $I_{NCX.}$ NCX acts along with L type calcium channels in order to maintain plateau phase(23). Recently many research showed NCX's contribution in rhythm generation by acting as molecular partner with intracellular calcium release.

5. Ryanodine receptor

Ryanodine receptor is located on the sarcoplasmic reticulum. A chemical called Ryanodine act as a ligand for this receptor. Mainly three types of Ryanodine receptors are seen –

1.RyR 1-seen in skeletal muscle

2.RyR 2-seen in cardiac muscle

3.RyR 3-seen in brain

At nanomolar concentration Ryanodine causes the channel to be open in subconductance state and in millimolar concentration it closes the channel. RyR1 is mainly voltage dependent. Depolarization of sarcoplasmic reticulum is conducted to the receptor via t tubules. The voltage sensor in the t tubule is DHPR. There is some

mechanical connection between t tubule and RyR since both of them are situated proximally. This is called voltage induced calcium release.

RyR 2 does not respond to membrane depolarization but undergo calcium induced calcium release. Ltype calcium channels cause release of calcium into the cell. This acts as a triggering factor. Some studies have shown the importance of calcium release via T type Ca^{2+} channels which leads to calcium induced calcium release via RyR during diastole and is responsible for rhythm generation(12). The important regulators of RyRs include Ryanodine (plant insecticide) and ruthenium red(24). More about Ryanodine receptors are explained later.

6. IP3 receptor

It is a calcium release channel seen on the sarcoplasmic reticulum. The ligand for its activation is IP3 which is produced by phospholipase C. It is considered important in non excitable tissues, but its role in excitable tissues yet to be identified. Recent works show that it is important in excitable tissues also(25).

7. Calcium store in the mitochondria

Some of calcium ions secreted from sarcoplasmic reticulum is taken by mitochondria via a calcium uniporter seen in the inner mitochondrial membrane which get open at negative voltages(26).

Pathways causing calcium removal from the cytoplasm

Too much of calcium inside the cell can cause adverse effects on the cell. Excessive calcium should be removed from the cytoplasm. Three mechanism by which calcium is removed include

1. Calcium ATPase seen on the cellular membrane
2. Sodium calcium exchanger operating in the forward mode
3. Sarco endoplasmic reticulum calcium ATPase seen on the sarcoplasmic reticulum

Calcium ATPase

It helps in transport one calcium ion per ATP. It is very much similar to SERCA. Lanthanum can enhance its activity.

Sodium Calcium exchanger

Description is given earlier.

Sarco Endoplasmic Reticulum Calcium ATPase(SERCA)

It is seen on the surface of sarcoplasmic reticulum. Main trigger for its action is increased cytoplasmic calcium concentration. Main regulator of SERCA is a protein called phospholamban. It inhibits SERCA when unphosphorylated and the inhibition is removed on phosphorylation. Phospholamban in turn is regulated by a regulatory protein called FK-506 binding protein(26).

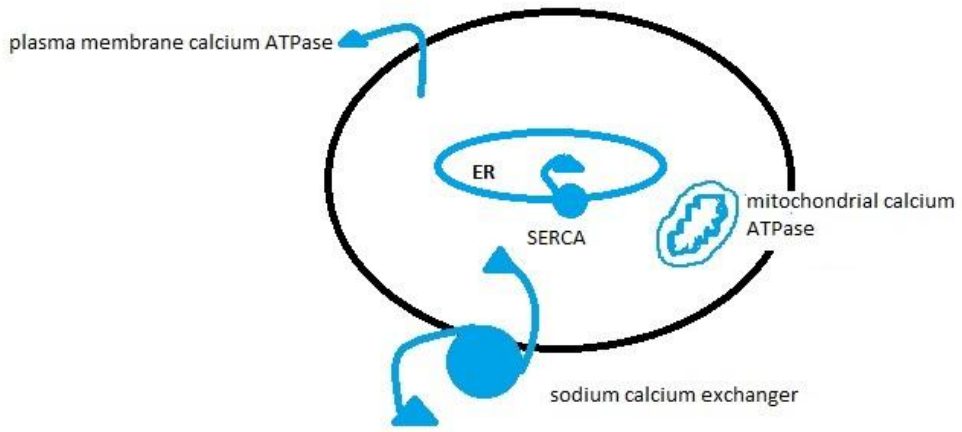


Figure 4:- shows calcium clearing mechanism

Ryanodine receptors-structure and function

The plant Ryania spesiosa which is seen in South America was found to be effective as insecticide many years before(27). Ryanodine, an active ingredient of this plant target Ryanodine receptors which is an eukaryotic membrane protein. But even before their isolation Ryanodine receptors were visualized in electron microscopic studies of ultrastructure of muscle. Using electron microscopic studies an electron dense region is seen to span the gap between sarcoplasmic reticulum and t tubular invagination of sarcolemma. These electron dense regions were named as feet structures. Later they purified it and confirmed to be Ryanodine receptors(28).

RyRs are the largest ion channels known till today. They form homo tetrameric assemblies. They are made up of proteins of molecular mass 2.2 MDa.

5000 amino acids are seen in each monomer. These channels are mainly involved in calcium release from endoplasmic reticulum and sarcoplasmic reticulum. Since they are involved in calcium release many cellular processes are affected by RyRs. Open state of channel is required for binding of Ryanodine with RyRs. It cause the channel to be locked in subconductance state at nanomolar concentration and blocks completely when concentration is above 100 micromolar concentration(29).

Various isoforms of RyRs and their gene expression

RyRs are seen in various mammalian cells like epithelial cells, neurons, exocrine cells, lymphocytes etc. The major function of the channel is to cause release of calcium during muscle contraction. In mammalians RyR1 is mainly seen in skeletal muscle and it was the first isoform to be isolated.RyR1 is studied in detail because of its higher expression rate and easiness in isolation. The location of RyR1 gene is on chromosome 19q13.2. RyR2 is found in heart and RyR3 is found in brain. The location of RyR2 is on chromosome1q43 and RyR3 on 15q13.3-14.They are 65% identical in structure and differs in three divergent regions D1,D2 and D3. The region D2 is required for mechanical coupling of RyR1 and $Ca_V1.1$.In lower animals only two isoforms are seen RyRα and RyRβ. In some lower animals like house flies and lobsters only one isoform is seen(30).

Expression of isoforms of RyRs

RyR1 mainly expressed in skeletal muscle. It is also seen in smooth muscle, cardiac muscle, thymus, cerebellum, kidney, stomach, adrenal glands, purkinje cells, testis and ovaries. RyR2 is the predominant cardiac isoform. A splice variant is also seen in heart which plays role in early apoptosis. It is also seen in higher amounts in cerebral cortex and purkinje cells of cerebellum and in lower amounts in kidney, stomach, adrenal glands, thymus, ovaries and lungs. RyR3 is seen in thalamus, hippocampus, corpus striatum, purkinje cells, smooth muscle, skeletal muscle (diaphragm), ileum, kidney, lungs, urinary bladder and ureter(30).

Gating of the channel

The main stimulator for RyR opening is calcium ion same ion which pass through the channel. Cardiac muscle fibers on depolarization cause opening of L type calcium channels and calcium influx occurs. This increased calcium in the cytoplasm is sensed by calcium sensors on RyRs and calcium release occurs from Sarcoplasmic reticulum. This phenomena is known as calcium induced calcium release.(31)(32) But when calcium level in the cytoplasm increases beyond certain level it cause inhibition of Ryanodine receptors. This implies RyRs have multiple binding sites with different affinities and binding kinetics. So a bell shaped curve is obtained while ploting open probability of the channel(33).The calcium concentration in the sarcoplasmic and endoplasmic reticulum is also sensed by RyR and they cause store overlaod induced calcium release(SOICR)(34).

Although calcium acts as a stimulating ligand it is not an absolute requirement. In RyR1 there exists physical interaction between voltage dependent Ca^{2+} channels and the receptor so that any change in the membrane potential can cause opening of RyR1.Here there is no requirement of calcium for opening of the channel. There occurs primary interaction between $Ca_v1.1$ and transmembrane loops II and III. The channels which are not nearby $Ca_v1.1$ get activated by Ca2+ released from other channels(35)(36)(37)(38).

Ryanodine receptor and striated muscle

Interaction between dihydropyridine receptors present on the t tubular membrane and Ryanodine receptors present on the sarcoplasmic reticulum is required for the excitation contraction coupling occurring in skeletal muscle. Dihydropyridine receptor is a voltage sensing L type calcium channel. Both the dihydropyridine receptor and Ryanodine receptors are arranged so closely leaving a narrow gap between them. So both proteins can interact structurally and functionally.

Physical interaction between these receptors in skeletal muscle is proved by studies done in individual myocytes. The micro regions that help in this interaction is already identified. Yet the actual mechanisms of interaction between these proteins are unknown. Whenever there is depolarization of sarcolemma it is sensed by dihydropyridine receptors which in turn cause release of calcium from Ryanodine receptors because of physical coupling between the two in skeletal muscle.

Few Ryanodine receptors which are away from dihydropyridine receptor get activated by calcium released from nearby channels(39).

In cardiac muscle opening of Ryanodine receptors occurs in response to calcium released from L type calcium channels.

Ryanodine receptors in rhythm generation

Recently it is found out that Ryanodine receptors are important for rhythm generation. Calcium release from sarcoplasm occurs not only during systole but also during diastole(2). Two main receptors that are present on the sarcoplasmic reticulum are Ryanodine receptors and IP3 receptors. During diastole release of calcium occurs from intracellular stores. This calcium will get extruded via sodium calcium exchanger and a current called I_{NCX} is thus produced. I_{NCX} is important for later phase of diastolic depolarization. Isolated cardiomyocytes already showed slowing of heart rate with addition of Ryanodine which is a blocker of the channel(3).

Ryanodine receptor and sodium calcium exchanger

Sodium calcium exchanger causes exchange of three sodium ions for one calcium ion. So intracellular calcium concentration as well as calcium transport depends on Na+ concentration inside the cell. Thus minute alteration in sodium concentration can affect the muscle contractility. Membrane potential also can affect the function of sodium calcium exchanger. The current generated due to inward movement of sodium ions (I_{NCX}) is involved in plateau phase of cardiac action potential

during systole. Abnormal release of calcium release called after depolarizations can lead to various arrhythmias. NCX plays a role in maintaining calcium concentration in sarcoplasmic reticulum constant(40).

Both Ryanodine receptor and NCX are known as molecular partners in rhythm generating mechanism.

In SA nodal cells it is found that calcium induced Ca^{2+} release occurs before firing of action potential. This release of calcium is believed to occur via Ryanodine receptors. In case of ventricular muscle fibers this Ca^{2+} release occurs via t tubular depolarization which causes activation of L-type calcium channels and calcium induced Ca^{2+} release causing muscle contraction. SA nodal cells lack this orderly t-tubular system and have very little contractile fibers. Studies have already proved the release of sub sarcolemmal calcium before SA nodal action potential(41). This released Ca^{2+} can modulate diastolic depolarization via NCX pump. Yet the characteristics of this localized Ca^{2+} release is not known fully(42)(3).

Imaging using electron microscopy

There is extensive study about the receptors which have been done in purified muscle fibers. Till today the whole crystallized structure of receptor is not available. The structure of Ryanodine receptors resembles mushroom with a cap and stalk. Cap is located in the cytoplasm forming 80% of mass. Stalk is passing through the membrane of

sarcoplasmic or endoplasmic membrane. Cytoplasmic area is bigger than transmembrane region.

The cytoplasmic area and transmembrane region are joined together by four thick columns. The cytoplasmic area is not rigid and it is made up of cavities filled with solvents and globular masses. Globular masses constitute subregions. The various parts of RyR receptors are described as clamps, handles and central rim. Central rim of RyR act as pore portion of the channel. Structure of all three isoforms of RyRs are similar with slight changes(43)(44).

There are found to be 6 or 8 transmembrane helices. But using electron cryo microscopy only 5 or 6 can be visualized. The inner helices form the pore region. The channel undergoes motion while opening and closing of the channel. During channel opening inner helices kink forming causing widening of pore region of the channel. It is evident from all studies that RyR act as an allosteric protein which can undergo rearrangement in the structure of cytoplasmic region during opening of the channel(45)(46). The columns transmit movements in transmembrane and cytoplasmic regions. Because of this coupling if any ligand bind to mobile part of cytoplasmic region, it causes opening of pore region of channel.

Opening of one channel can affect opening of other channels also. This property is known as coupled gating.

Regulators

A few transmembrane helices are required to form a calcium channel. But the protein is made up of > 2MDa subunits to accomplish this function. This implies the tight regulation of RyRs by proteins, small molecules and post translational modification. The large cytoplasmic region and solvent filled cavities increase the surface area of RyRs onto which various regulators can bind. These regulators can either activate or inhibit the channel.

One of the important regulators is FK506 binding protein. They can bind to all the three isoforms of the channel. They maintain the channel in closed state(47). Another regulator is calmodulin whose effect depends on Ca^{2+} concentration and isoforms of the channel. At elevated Ca^{2+} level inhibits RyR1 and RyR2. At low Ca^{2+} levels activates RyR1 and inhibits RyR2. One other modulator is Calsequestrin. It can cause either activation or inhibition of channel depending circumstances. More details about this is not available. Homer 1c is a protein which can turn on RyR1 and turn off RyR2(48).

RyRs can be altered by molecules like Mg^{2+} which cause inhibition, ATP which cause activation, toxin called natrin which cause inhibition, several ligands (ruthenium red, caffeine, 4-chloro-m-cresol, volatile anesthetics and dantrolene) which can affect the channel and imperatoxin which cause activation of the channel. RyRs are susceptible to redox conditions and NO can activate the channel(49).

Phosphorylation

Many type of kinases including PKA,PKG and calcium/calmodulin dependent protein kinase II and also several phosphatases can affect the channel. Of these PKA has got major role because it acts as an association between physiological stress and RyR mediated activation of β adrenergic receptors. Mainly two regions of the RyR can be affected by phosphorylation. Still now it is not evident which site phosphorylation lead to cardiac failure.

Studies have shown that in cardiac failure Ser 2808 in RyR2 is hyper phosphorylated leading to dissociation of FKBP12.6. This dissociation can lead to increased activity of RyRs and this can lead to heart failure. It is shown that different levels of oxidation can also affect the association of FKBP12.6 to the RyRs.

Cardiac arrhythmia and dysfunction can also occur in conditions of CAMKII induced open probability of the channel(50).

Disease mutations

It is now evident that different types of mutations of RyRs can cause mishandling of calcium ions and can lead to cardiac failure and other cardiac disorders. Mutations of RyR1 and RyR2 mutations can cause various genetic diseases because both receptors are involved in muscle contraction.

Ryanodine receptors and human diseases

Mutation of RyR1 and RyR2 are associated with many human diseases.

Diseases caused by mutation of RyR1 receptors include

1. Malignant hyperthermia
2. Central core disease
3. Exercise/heat induced exertional rhabdomyolysis
4. Atypical periodic paralysis
5. Multi mini core disease

Cardiac Ryanodine receptors and arrhythmias

The two main arrhythmias which can occur due to mutation of cardiac Ryanodine receptors include

1. catecholaminergic polymorphic ventricular tachycardia (CPVT)
2. arrythmogenic right ventricular dysplasia type 2(ARVD2)

Inheritance of ARVD2 is autosomal dominant and in this condition myocytes are replaced with fibrofatty tissues. It is manifested by the presence of various ventricular arrhythmias. RyR mutations causing ARVD2 is homologous to mutations occurring RyR1 receptor in malignant hyperthermia and central core disease.

CPVT is caused by abnormal phosphorylation by Ca2+/calmodulin dependent protein kinase 2 (CaMKII) and protein kinase A. This causes an increase in open probability of RyR2 and lead to stress induced rhythm abnormalities. These arrhythmias are particularly seen during β adrenergic stress and exercise. In autosomal recessive type of CPVT mutation of calsequestrin 2 also found(30).

Mutation of a closely related protein can cause abnormality in function of Ryanodine receptor 2 and can lead to long QT syndrome. Leaky RyR 2 channels can lead to overload of calcium in the mitochondria and can lead to cardiac dysfunction and cardiac failure(51).

Cardiac Ryanodine receptors and heart failure

The role of RyR2 in cardiac diseases has become a subject of interest recently. Numerous studies are undergoing in this field. Cardiac Ryanodine receptors are main regulators of calcium release from the sarcoplasmic reticulum. They play major role in excitation-contraction coupling. In cardiac failure increased adrenergic stress is prominent. This leads to hyperphosphorylation of RyR2 and cause leaking of calcium from sarcoplasmic reticulum. Delayed afterdepolarizations are produced due to leakiness of channel leading to various arrhythmias and this can lead to cardiac failure. Drugs which block β adrenergic receptors are effective in heart failure(52).

Cardiac Ryanodine receptors and cardiomyopathy

RyR2 is found to play a crucial role in anthracycline mediated cardiomyopathy. Anthra cyclins are known to cause cardiomyopathy and cardiac failure. Studies were done by administering daunorubicin in repeated doses and noting changes in the calcium regulatory protein expression. A tetrameric protein complex is seen near sarcoplasmic reticulum which is constituted by RyR, junctin, triadine and calsequestrin. Normally when luminal calcium is low CSQ will bind to TRN and junctin causing inhibition of RyR2 channels. When luminal calcium increases it relieves the inhibition of RyR2 by binding of calcium with CSQ. FK506 protein also stabilizes RyR2. SERCA is responsible for calcium uptake into sarcoplasmic reticulum. Daunorubicin by binding to SERCA and RyR2 receptors affect the calcium cycling and lead to cardiomyopathy and cardiac failure. Usually upregulation of RyR2 receptors occur in daunorubicin cardiomyopathy(53).

Pharmacology of Ryanodine

Ryanodine is a chemical isolated from a plant ryania speciosa which grows as a shrub in Central and South America. A closely related compound is ryanodol obtained from wood Persea indica. Even from ancient times it was known that this plant contains toxic compounds and was used as an insecticide. The toxin was isolated from stem and roots of plant Ryania speciosa. Crude extract of this plant was used to poison arrow head by local people.

Ryanodine is a perfect insecticide because of three reasons. It is effective as DDT or parathion against insects. Half life of this chemical is very low providing environmental safety. Its action is specific against crop damaging insects than beneficial insects. Most important historical significance of this chemical is that it lead to the discovery of intracellular calcium releasing channels called Ryanodine receptors.

The effects of Ryanodine on conductance and gating of RyR is very complex. The conclusions from studies done by Fairhurst and Hasselbach on cardiac muscles showed the increase in conductance of SR Ca^{2+} by Ryanodine. Also it is found that Ryanodine can decrease the calcium conductance(54) (5).

There are three observation showing effects of Ryanodine on SR Ryanodine receptors. The channel activity showed full conductance at sub micromolar concentrations. Also at sub micromolar concentration channel showed sub or partial conductance state. Channel is closed at micromolar and above concentration. There are two probabilities about how Ryanodine alter the conductance of RyRs. One reason is that it may be due to allosteric modulation and another one is physical interaction of Ryanodine with Ca^{2+} influx through the pore region. Most of the studies support allosteric mechanism(55).

Another finding that was found was Ryanodine binds with the receptor only in active state. In one experiment when Ryanodine was infused into a muscle, stimulated area of muscle showed contracture not the unstimulated parts of same muscle. Several other studies showed that tetrameric form of receptor is required for

binding with Ryanodine(56).This was demonstrated by using a detergent Zwittergent 3-14 which caused dissociation of subunits. After dissociation, Ryanodine failed to bind to its receptor even when the monomers were reassembled again(57).This can be explained by two ways. Binding site of Ryanodine is an associated domain contributed by all the four components. Or the binding site is formed only by the tetrameric conformation.

There are two types of binding process for Ryanodine with RyRs which include high affinity binding and low affinity binding. The stoichiometry of high affinity binding is 1:1 and that for low affinity binding is either 3:1 or 1:1.Because of this difference in stoichiometry there are two different models for binding. Interconvertable site model(ISM) is one in which there are four sites which can serve either as high affinity or as low affinity sites. Where as in distinct site model (DSM) there are two distinct binding sites for high affinity and low affinity binding. In ISM model initially all the binding sites equally show high affinity for binding to RyR with open status. But after one site is bound by Ryanodine rest sites undergo negative co-operativity that lowers its affinity. In one type of receptor binding with one site causes reduction in affinity of all the three remaining sites. After three sites are bound by Ryanodine it leads to channel closure. Binding of each molecule of Ryanodine to the receptor decreases the conductance of the channel. This results in stabilization of channel in closed state. In DSM out of the two different types of binding sites the one with high affinity having a stochiometry 1:1 occurs only in open state of RyR and shows some conductance. Binding of Ryanodine to low affinity sites prevents dissociation of

Ryanodine from high affinity sites. This results in trapping of Ryanodine in the high affinity sites(58).

By the use of Pharmacological approach it is found that other ryanoids also affect the conductance of Ca^{2+} ions from SR. The concentration required to control calcium permeability of SR by different ryanoids varies.

Nonryanoid chemicals which can alter RyR Channel function.

Many compounds which are structurally not related to Ryanodine can affect Ryanodine receptor functions.

A. Proteins

1. FK 506 binding proteins

FK 506 binding proteins can tightly bind to RyRs and affect its functions. It causes change in RyR receptor activity by cis/trans-peptidyl-prolyl isomerase activity. When FK 506 binding proteins associate with RyRs there is decreased probability for opening of the channel and this can lead to decreased conductance states. An anticancer drug FK506 can cause dissociation of FK binding protein from RyR receptors. A derivative from the plant lanthella basta called bastadins can increase Ryanodine binding and calcium permeability.

2. Calmodulin

Interaction of Ryanodine receptor with calmodulin is complex. It causes activation of the channel at low calcium concentration and cause inhibition of calcium channel at high calcium concentration.

3. Triadin

It is a sarcolemmal protein. It acts as intermediate between DHPR and RyR. It acts both with RyR as well as a sarcoplasmic reticulum luminal protein calsequestrin. It plays role in both calcium storage and calcium release from sarcoplasmic reticulum. Recent studies show that interaction between triadin and RyR is associated with redox state of sulfhydryl group of both. It is already shown that changes in oxidation influence SR calcium release. This can be partially explained by triadin-RyR interaction.

4. Sorcin

It is a protein seen on the SR membrane of cardiac muscle. Importance of this protein should be identified in the future.

B. Peptides

1. Myotoxin

It is a poly peptide isolated from snake venom and shown to activate RyR receptor channel.

2. Helothermine

It is also prepared from the venom of lizard which can inhibit both cardiac and skeletal muscle RyRs.

3. Ryanotoxin

It is prepared from scorpion venom shown to have action on RyR channel

4. Imperatoxin

It is also prepared from snake venom which specifically act on RyR 1 receptor not on RyR 2.

C. Cyclic Adenosine Diphosphate Ribose(cADPR)

It has been shown that this acts as a second messenger in release of calcium from cytoplasmic stores. Many studies have shown that it is due to its action on RyRs. It acts as a modulator of cardiac Ryanodine receptors (RyR2).

D. Local anesthetics- RyR channel activity is affected by various local anesthetics also.

E. Polyamines

Spermine, spermidine and putrescine are polyamines seen in many cells which at millimolar concentration cause rectification of RyR2. They act as blockers of RyRs(59).

F. Suramine

It is an antiparasitic agent derived from urea. It is very interesting that this can regulate the activities of RyRs. It was more effective against RyR2receptors.

G. Ortho substituted polychlorinated biphenyls

It is an aromatic hydrocarbon capable of increasing the release of calcium from SR(60).

Methodologies and tools to study Ryanodine receptors

- Negative staining

It is a simple way of getting more information about the structure of receptor. One disadvantage of this technique is that dehydration that occurs during the procedure can produce distortion of macromolecular structure. It is usually used in adjunction with other methods.

- Cryo Electron microscopy

In this technique sample remains in solution and thus prevent distortion of structure. This help in getting high resolution 3D images of proteins.

- Mapping of 3D structure

This is done by two methods. One is by identifying peptide regions in the protein using biochemical methods. Other way is by using antibodies, various modulators and green fluorescent proteins.

- Crystal structure of different terminals

In this smaller fragments are crystallized to study N terminal domain.

- Protein stabilization assays

It helps to study the various types of mutations in Ryanodine receptor structure.

- Calcium sparks

Functioning of Ryanodine receptors can be studied by visualizing Calcium sparks that occur from sarcoplasmic reticulum to the cytosol. Calcium sensitive fluorescent dyes made this possible. Calcium events that occur from a cluster of Ryanodine receptors are studied using confocal microscopy.

- Heterologous cell systems

In this mutant or wild type RyR is inserted into a cell that doesn't have a Ryanodine receptor. Then that portion which contains RyR is used to study biochemical, biophysical or molecular properties of receptors. It is used to study the functioning of the receptors.

- Subcellular assays

In this subcellular fractionation to get intracellular vescicles is done. It helps to observe calcium release from various cellular constituents. Sarcoplasmic reticulum vescicles are isolated from cells and release of calcium from that can be studied.

- Monitoring function of individual RyRs

For studying individual Ryanodine receptors first they are isolated from the cell and inserted into planar phosholipid bilayers.

- Pharmacological manipulation

It is most commonly used to study Ryanodine receptor functioning. In this various activators and inhibitors of the receptor is used. Chemical Ryanodine was shown to cause either increase or decrease in permeability of sarcoplasmic reticulum to calcium in a dose dependent manner. The local anesthetic tetracaine causes inhibition of calcium release from sarcoplasmic reticulum. Cafffeine even though not selective when compared to Ryanodine caused increase in open probability of Ryanodine receptor channels. Recently scorpion toxins have emerged that specifically target Ryanodine receptors(61) (62).

Rat experiments

Animal research played an essential role in most of the medical breakthrough during the last decade. Since 1901 most of the scientists who won Nobel Prize have used animal data for their work. Mouse genome and human genome share 95% similarities. So they

can serve as efficient model of human body. Functioning of various organ systems in animals and human beings are similar. Humans and animals share many diseases such as flu, Tb, asthma, cancer etc. Many medicines that we use for humans like pain killers and antibiotics are same for human beings and animals. Many modern drugs including insulin, penicillin and anesthetics are the results of animal research. Most of surgical and orthopedic tips were initially done in animals. Transgenic mice can carry same genes that cause human diseases. Thus it is very practical to use animals for research purpose. Most of the animal experiments are done in mouse and rats. Other animals used for animal experiments include primates, cats, dogs, fish, birds and invertebrates.

Rats are used most commonly for animal experiments because of they are inexpensive, easy to handle, easily available, small size and have quick reproduction rate. In case of testing various pharmacological agents rats are very good. Most of the rats which are used for animal studies are inbred, so they are genetically similar. This will help in getting uniform results.

Isolated heart studies

Isolated heart studies are mainly used for hemodynamic and metabolic studies in animals. This method plays important role in in vitro studies in Physiology and Pharmacology. The main advantage of this method is that using this method we can study various intrinsic properties of heart like rhythmicity, contractility and conductivity without neural and humoral involvement.

Langendorff setup

In 1985 Oscar Langendorff first demonstrated the technique of perfusion of isolated heart using Langendorff setup. From then this method was used for isolated heart studies with slight modification. This method is useful only in animals which are homeothermic or warm blooded with intact coronaries. These homeothermic animals can keep their internal temperature at a constant level despite changes in external environment. Animals like frog and fish have got numerous pores in the cardiac musculature. They get nutrition by diffusion through the pores. Since they have no coronaries this method cannot be done in them. Heart can be perfused with various solutions containing nutrients, drugs and oxygen by inserting a canula through aorta. The aortic valves get closed by the force of flow of the perfusing solution and coronary ostium will get opened. Thus the perfusate will reach the coronaries.Since the fluid is rich in nutrients it helps the heart to remain viable even after a long time after removing from the body. This method will help to study the various intrinsic properties and the effects of various pharmacological agents on heart. Another usefulness of this technique is that we can obtain individual cardiomyocytes with enzymatic digestion technique.

The aim of this study is to find out the importance of calcium release from sarcoplasmic reticulum via Ryanodine receptors in diastolic depolarization of heart. This study is done in isolated perfusion of heart of wistar rats in Langendorff method. ECG is recorded using surface electrodes and heart rate is

calculated from the ECG. Ryanodine receptor blocker Ryanodine is added to the heart to study its effect in heart rate.

AIMS AND OBJECTIVES

Aim:

To study the role of Ryanodine receptors in rhythm generation in isolated wistar rat hearts

Objective:

To study change in heart rate due to addition of Ryanodine, the Ryanodine receptor blocker in an isolated rat heart that is perfused with normal extracellular solution

MATERIALS AND METHODS

MATERIALS AND SOLUTIONS USED

1. EQUIBATH Circulating Water Bath
2. MasterFlex Peristaltic Pump
3. Langendorff perfusion setup
4. Oxygen pump
5. Perfusing Solutions – Normal mammalian ringer
6. CMCdaq Data Acquisition Device
7. Drugs- DMSO

    -Ryanodine

PREPARATION OF SOLUTION

Isolated heart is perfused in Langendorff setup. Perfusate used was very similar to mammalian extracellular fluid. The amount of electrolytes, glucose and pH were almost comparable to normal mammalian extracellular fluid.

Before preparing normal mammalian ringer solution amount of each salt to be added to a litre of distilled water was calculated. Salts were weighed with precision using an electronic balance (Precisa XB 320M). All the weighed salts were added to one litre of water. For each experiment solution was made freshly to avoid contamination.

A magnetic stirrer was used to mix the salt thoroughly in the distilled water. For checking pH an electronic pH meter was used (METTLER TOLEDO MP220). Before checking pH with the pH meter it was calibrated using solutions of known pH (pH4 and pH7). Using 1 molar NaOH the final pH of solution was adjusted to be at 7.4.

NORMAL EXTRACELLULAR SOLUTION COMPOSITION

SALTS	CONCENTRATION(mmol/L)
NaCl	135
KCl	5.4
NaH_2PO_4	0.4
$MgCl_2$	3
$CaCl_2$	1
Hepes	10
Glucose	10
pH	7.4
Osmolarity	307

PREPARATION OF STOCK SOLUTION

Before experiment stock solution for each drug was prepared. Stock solutions were made in millimolar concentration. It helps to make final concentration thousand times diluted than stock concentration.

Ryanodine was soluble in DMSO (29mg/ml). For control experiments DMSO is used at concentration of 250µl in 100 ml of normal ringer to give a final concentration 0.25%.

Ryanodine stock solution was prepared by dissolving 1 mg in 100µl DMSO to get a stock solution of 20mM. Molecular weight of Ryanodine is 498 mg. From the stock solution 50µl is added to 10ml to get a final concentration of 100µM.

Prepared stock solution is kept in fridge at -20 degree Celsius.

The various salts involved in making up of normal extracellular solution were obtained from the following companies:

1. Sodium chloride : Sigma Aldrich
2. Potassium chloride : Sigma Aldrich
3. Calcium chloride : Sigma Aldrich
4. Magnesium chloride : Sigma Aldrich
5. Glucose : Sigma Aldrich
6. Disodium hydrogen phosphate : Qualigens
7. Sodium hydroxide : Merck
8. HEPES : Lobachemi

Drugs:

1. DMSO : Sigma Aldrich
2. Ryanodine : Enzo life sciences

Storing of these salts and drugs were done as mentioned in the data sheet and all of them were utilized within the expiry dates of each.

LANGENDORFF'S SETUP:

In 1985 Oscar Langendorff first demonstrated the technique of perfusion of isolated heart using Langendorff setup. From then this method was used for isolated heart studies with slight modification. This method is useful only in animals which are homeothermic or warm blooded with intact coronaries. These homeothermic animals can keep their internal temperature at a constant level despite changes in external environment. Animals like frog and fish have numerous pores in the cardiac musculature. They get nutrition by diffusion through the pores. Since they have no coronaries this method cannot be done in them. By inserting a canula through the ascending aorta heart can be perfused with various solutions containing nutrients, drugs and oxygen. The aortic valves get closed by the force of flow of the perfusing solution and coronary ostium will get opened. Thus the perfusate will reach the coronaries.

This method will help to study the various intrinsic properties and the effects of various pharmacological agents on heart. Another usefulness of this technique is that we can obtain individual cardiomyocytes with enzymatic digestion technique.

The Langendorff setup consists of one bubble trapper to prevent entry of air into the blood vessels and two reservoirs which are graduated. In case of experiments using Ryanodine reperfusion setup is used. Here solution which comes after perfusing the heart is used to perfuse the heart again. Temperature of the whole setup is maintained at

37 degree Celsius by using water bath and continuous oxygenation is maintained using oxygen cylinder.

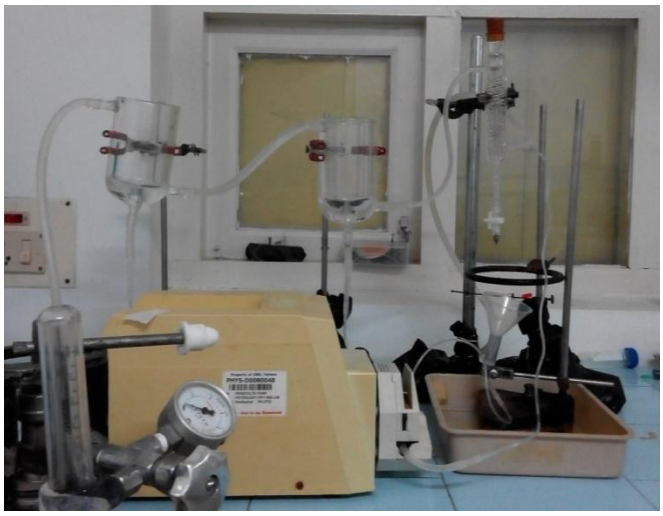


Figure 6: Langendorff perfusion setup

PERFUSION:

A Master Flex peristaltic pump is used to pump the solution at a constant rate of 10 ml in every minute. Perfusion pressure is kept at a constant level of 60mm of Hg to ensure the perfusion of coronaries.

OXYGENATION:

The reservoir was connected to an oxygen cylinder to obtain continuous 100% oxygenation of the perfusing solution. This high flow of oxygenation was maintained throughout the whole experiment.

Figure 7: Peristaltic pump

TEMPERATURE MAINTENANCE

With the help of a circulating water bath temperature of the circulating solution was kept at 37 degree Celsius. This was obtained by circulation of water at 37 degree Celsius through the outer compartment of the bubble trapper and the reservoirs throughout the experiment.

RECORDING OF HEART RATE:

Using Langendorff setup heart was perfused with normal extra cellular solution and time was given for stabilization of heart rate.

For recording surface electrogram three surface electrodes were placed on the surface of heart mounted. It was then connected to CMC daq ECG amplifier (designed by Bioengineering department of Christian Medical College Vellore). The recording was done in such a way that negative electrode was touching right atrium and positive electrode was touching left ventricle. In order to reduce the noise third

electrode was connected to ground. The arrangement of electrodes showed resemblance to lead II in ECG. The electrodes were coupled to the computer through CMC daq ECG amplifier. Recording of ECG is done using CMC daq software in the computer. Calculation of heart rate was done manually from tracings obtained with surface electrodes called surface electrogram.

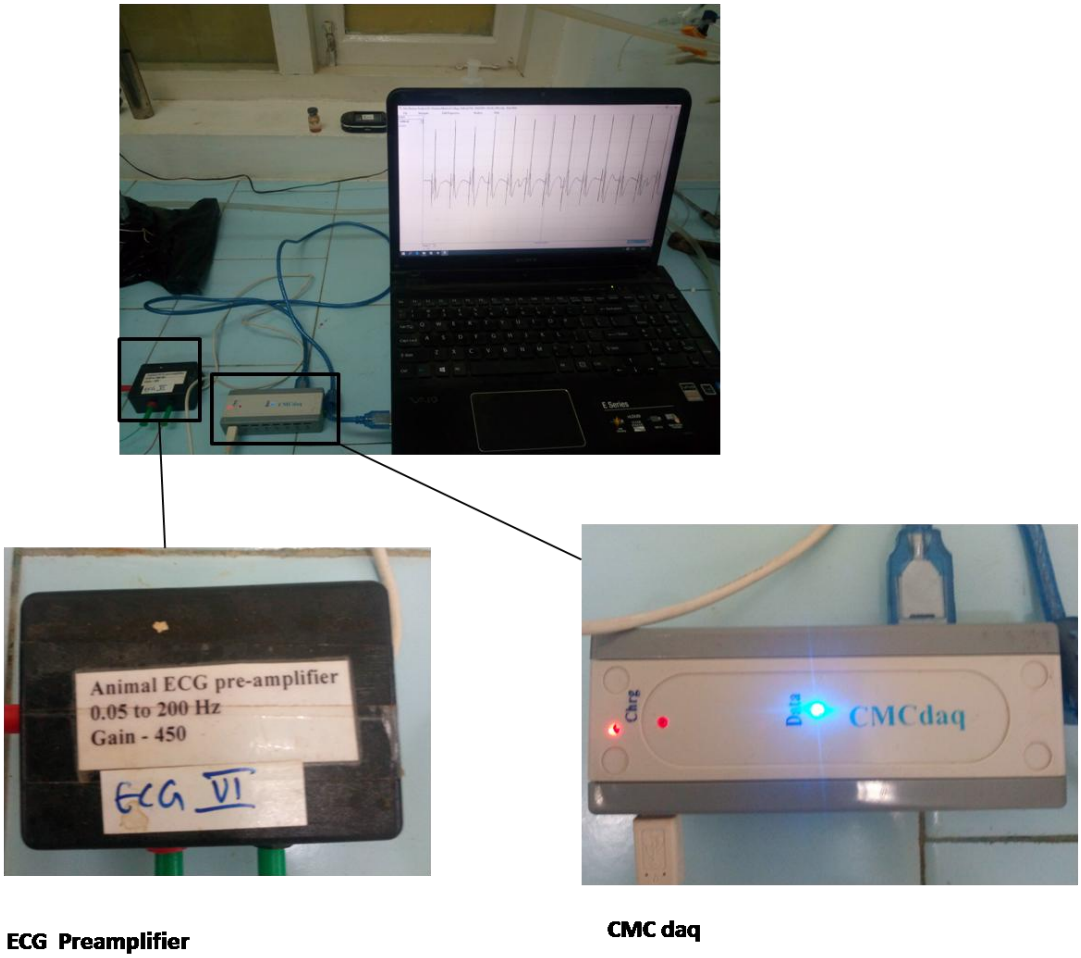


Figure 8: surface electrogram obtained using ECG amplifier and CMC daq

EXPERIMENT PROTOCOL:

The ethical clearance for the usage of animals was provided by Institutional animal ethics committee. (IAEC No:13/2014)

Both male and female Wistar rats weighing between 200 and 300 grams were used during the experiment. Anesthesia was given before all procedures. Till the date of experiment rats were maintained in the animal house in CMC Vellore. Out of the 16 rats used for experiment 4 rats were used for drug dose standardization.

Figure 9: Wistar Rat

ANAESTHESIA

Ketamine at a dose of 100mg per kg of body weight intraperitoneally was used for anaesthesia. After giving anaesthesia, the four limbs of the animal were attached to a plastic board using micropore. Completeness of anaesthesia was confirmed by pinching the soles before starting the experimental procedure.

PROCEDURE:

Before starting the procedure area of chest overlying heart is wiped using distilled water. The abdominal cavity is opened by putting an incision over the xiphi sternum and extending the incision through skin and muscle layer. The ribs were cut on both sides and heart was exposed.

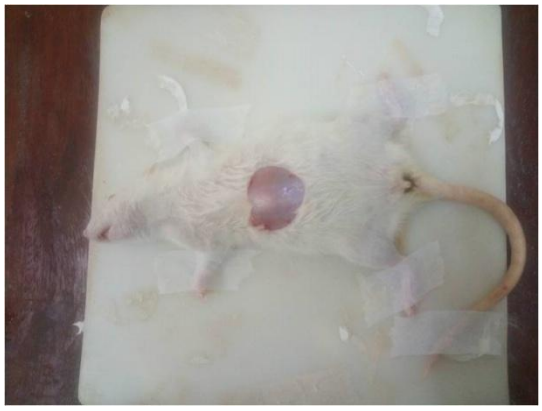


Figure 10: subcoastal incision given below xiphi sternum

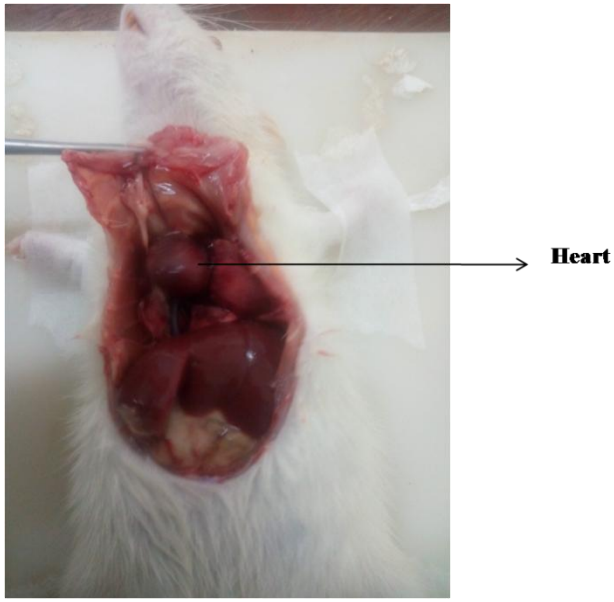


Figure 11: Exposure of heart

After identifying aorta a bull dog artery clamp is used to clamp the aorta. Then heart is removed by cutting with scissors above the aorta after lifting the clamp and aorta together. Care was taken to ensure that sufficient length of aorta is attached to the heart for mounting.

The heart, after removal from the chest cavity was put into a petridish containing ice cold extracellular solution. Slow massage is done to get rid of any blood in the heart. Non removal of blood can cause clotting of blood in blood vessels or in the chambers in the heart which can affect the perfusion as well as contraction of heart. Then heart was mounted on the Langendorff set up by cannulating aorta immediately. Perfusion of coronaries was done with normal extracellular solution at 37 degree Celsius at a rate of 10 ml/minute.

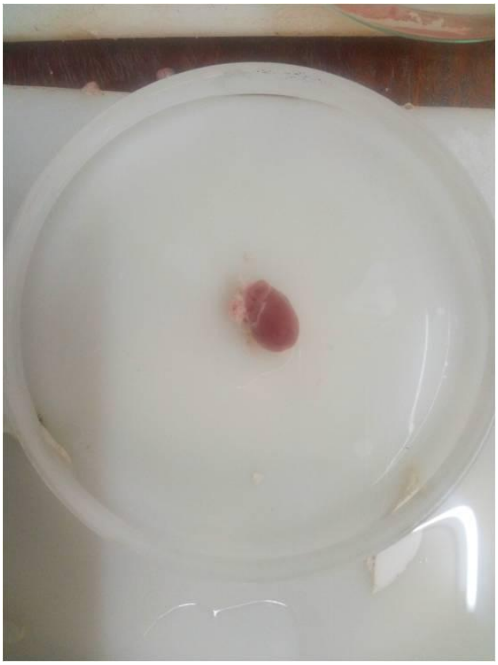


Figure 12: Heart is kept in a petri dish containing ice cold extracellular solution

The solutions which were used to perfuse the heart were allowed to pass through the bubble trap before reaching aorta to prevent the entry of air into aorta. The heart is made to beat at its own pace. Recording of heart rate is done using surface electrodes and CMC daq amplifier connected to computer. Digital data is obtained using CMC daq data acquisition system.

Figure 13: recording of heart rate using surface electrodes

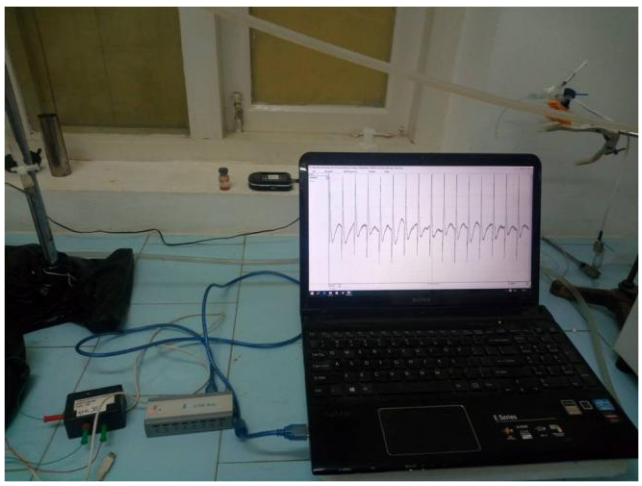


Figure 14: CMC daq data acquisition system

In case of perfusion with Ryanodine, reperfusion setup is used. Fluid coming after perfusing the heart is collected and again used to perfuse the heart while oxygenation and temperature is maintained.

Figure 15: reperfusion setup for Ryanodine perfusion

STANDERDIZATION OF DRUG DOSE:

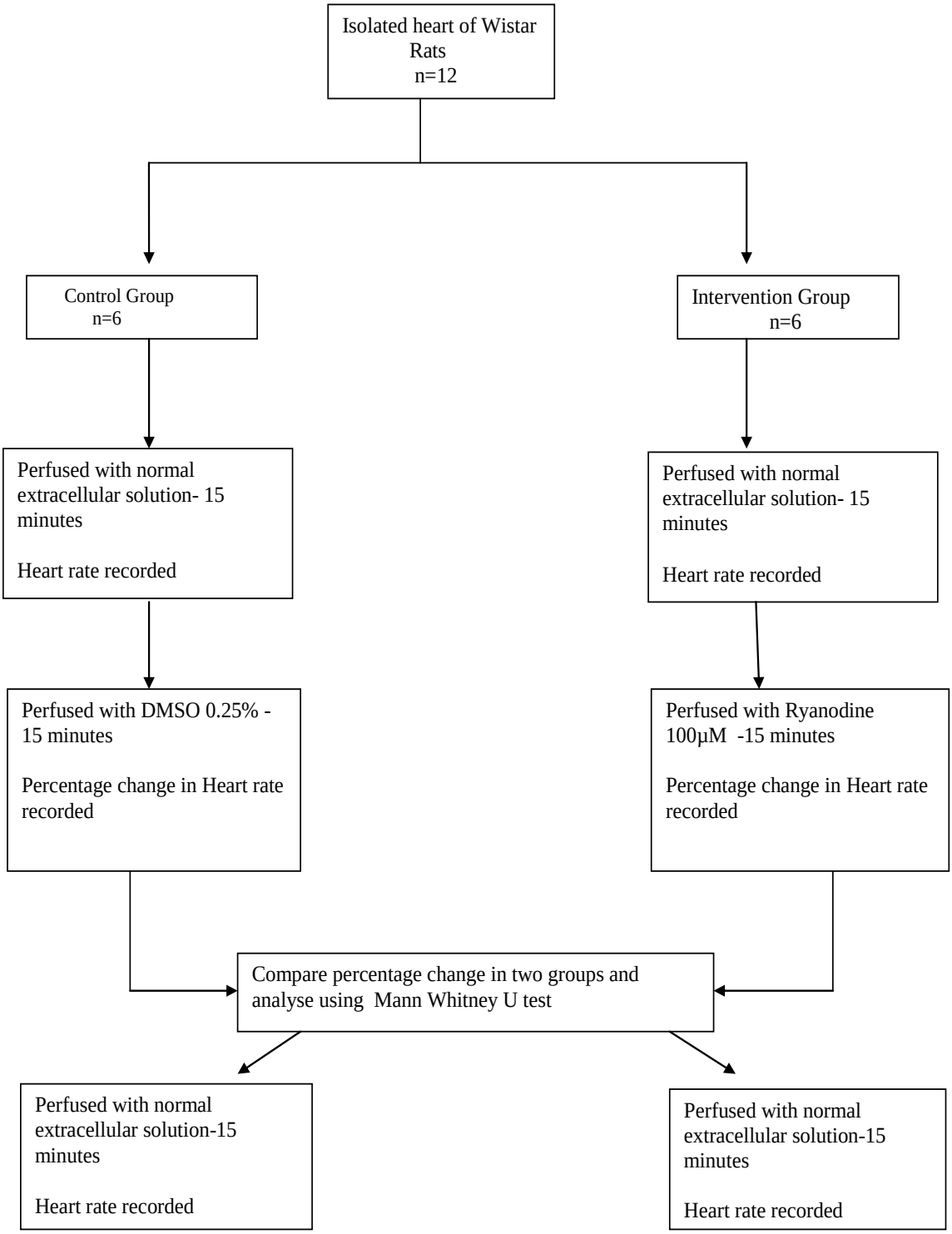
For standardization four rats were used. Various doses of Ryanodine (10µM, 40µM, 60µM and 100µM) were used in four different rats. Final dose used in the experiment was 100µM.

EXPERIMENTAL DESIGN:

Two groups of rats were used in the experiment (control group and intervention group) each containing 6 rats. Totally 12 rats were used in the experiment. Initially time was given for stabilization of heart rate. In the control group first 15 minutes perfusion is done with normal extracellular solution and next 15 minutes with DMSO 0.25% followed by a wash. Heart rate is recorded using surface electrodes and data is obtained using CMC data acquisition system.

In case of intervention group first 15 minutes perfusion is done with normal extracellular solution and next 15 minutes with Ryanodine 100µM dissolved in DMSO 0.25% followed by a wash. Heart is calculated from the ECG tracings obtained using surface electrogram.

FLOW CHART SHOWING EXPERIMENTAL DESIGN



ANALYSIS:

The continuous recording of ECG is done using surface electrodes which are connected to CMC daq ECG amplifier and CMC daq software. From the recording obtained manual calculation of heart rate is done. By counting the peaks(R wave) heart rate at each minute is calculated and tabulated. Calculation of change in heart rate is also done and expressed as percentage change.

STATISCAL ANALYSIS

Within a group, heart rate before and after the intervention is assessed.Since it is a non-parametric paired data it was analyzed using Wilcoxan Signed Rank test.

Comparison was also done between two groups. They were analyzed using Mann Whitney U test.

Analysis was done using SPSS software (version 17).

A p value less than 0.05 was considered significant.

IGOR Pro software (version 5.4) was used for analysis and plotting of graphs.

RESULTS

Sample of tracings of ECG obtained

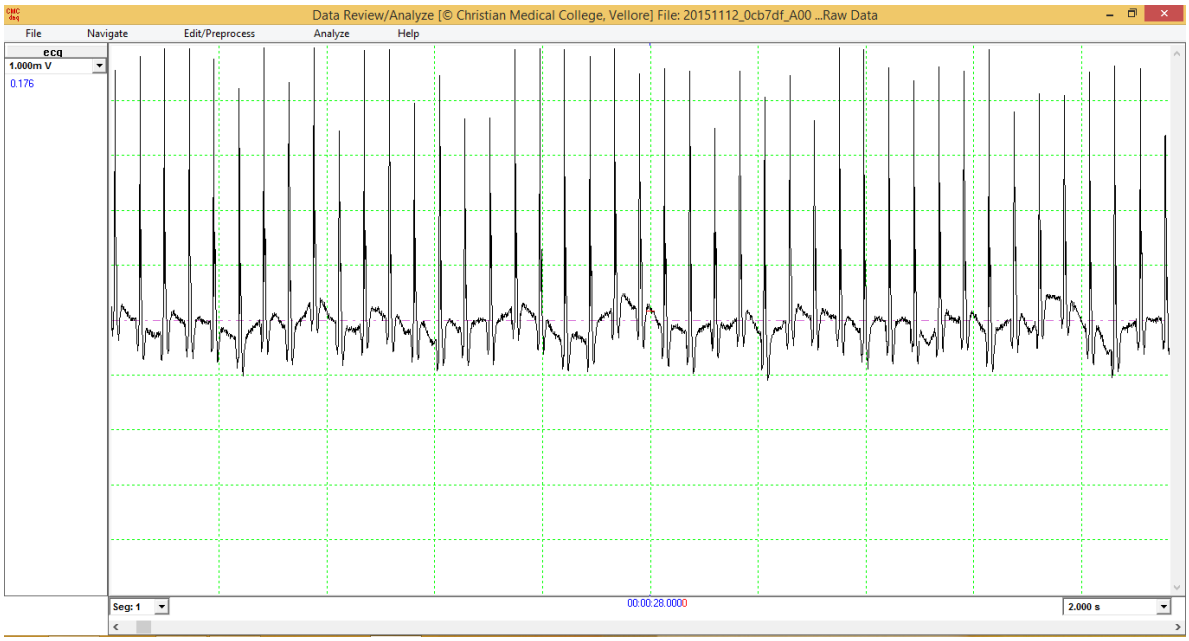


Figure 16: Sample of tracing after perfusing with normal ringer

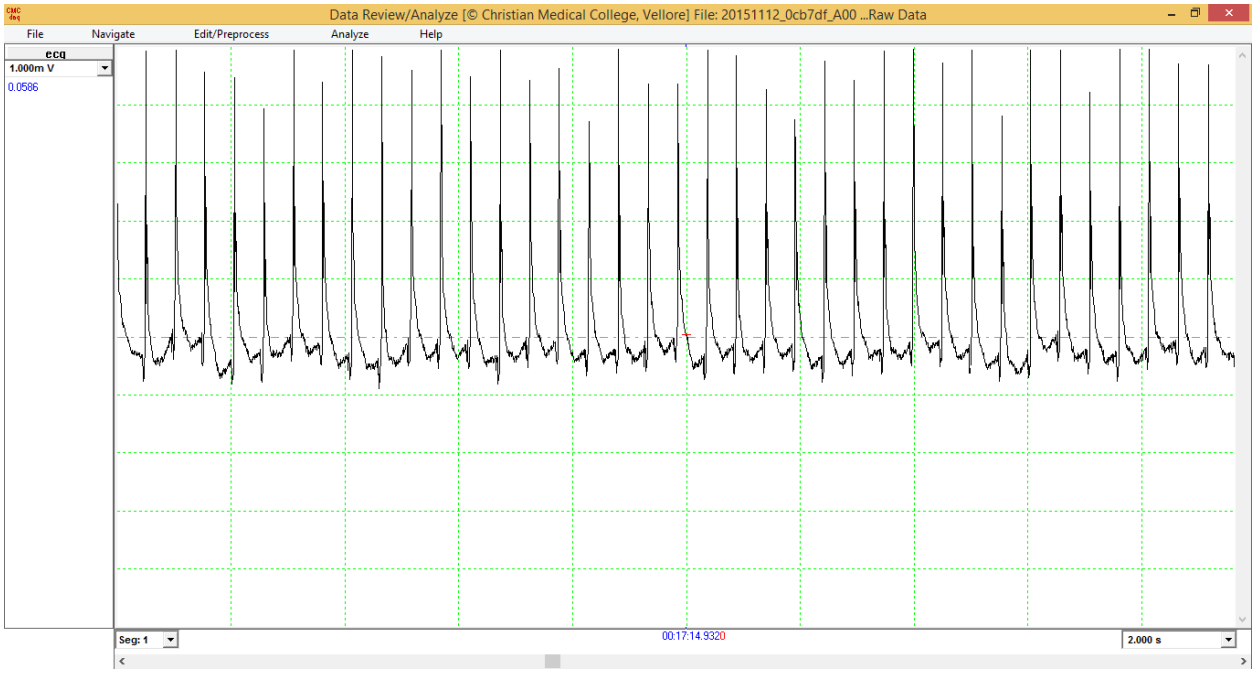


Figure 17: Sample of tracing after perfusing with 0.25% of DMSO

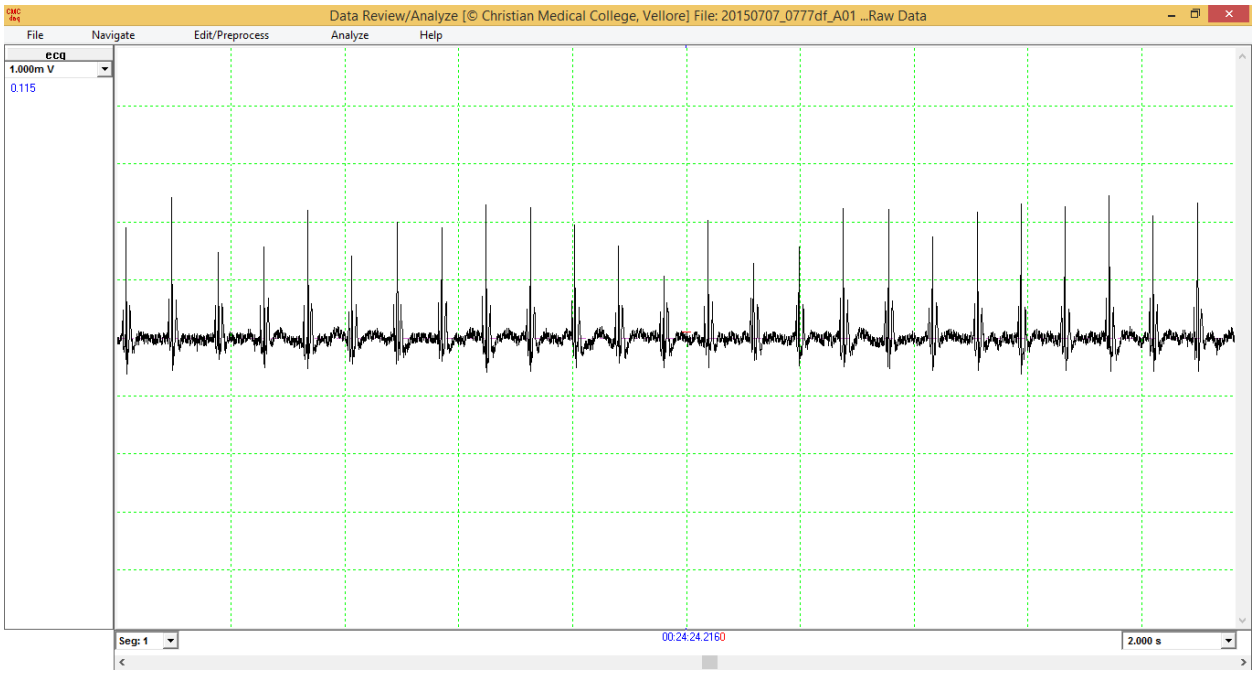


Figure 18: Sample of tracing after perfusing with 100 micromolar Ryanodine

Manual calculation of heart rate is done from the data recorded from surface electrogram and tabulated.

CONTROL GROUP – with DMSO 0.25%

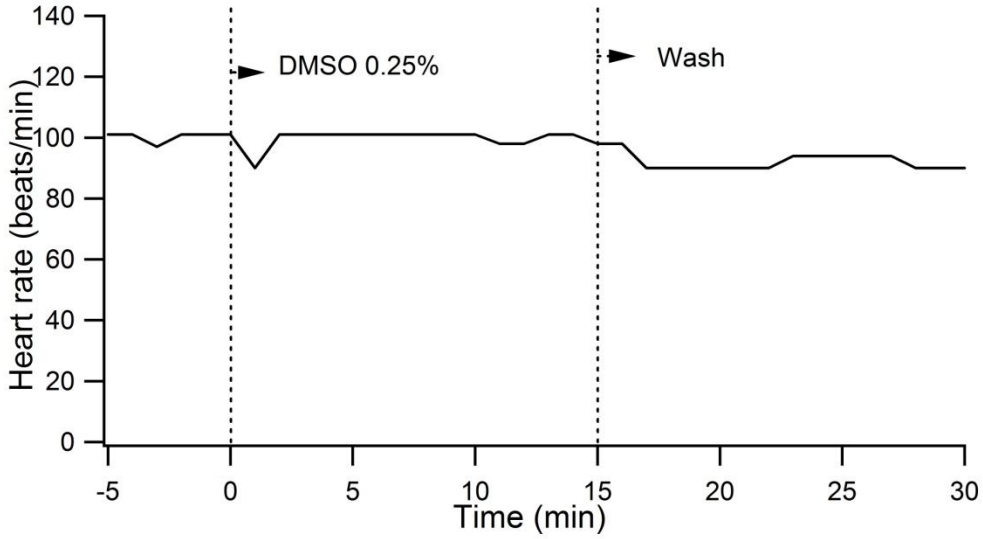
Time (min)	Heart rate (beats/min)					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
-5	101	124	116	120	113	113
-4	101	124	116	120	113	113
-3	97	124	116	120	116	113
-2	101	124	116	120	116	113
-1	101	120	116	120	113	113
0	101	124	116	113	113	113
1	90	105	116	113	109	113
2	101	116	116	120	113	113
3	101	120	116	120	113	113
4	101	116	116	120	109	105
5	101	105	113	120	109	113
6	101	105	113	128	109	113
7	101	105	113	128	113	113
8	101	109	113	120	113	113
9	101	105	116	120	105	105
10	101	124	116	120	109	105
11	98	124	116	120	109	113
12	98	124	109	120	105	105
13	101	105	113	120	105	105
14	101	124	113	120	105	105
15	98	124	109	128	105	113
16	98	105	109	128	109	105
17	90	105	109	128	109	105
18	90	105	109	128	113	105
19	90	105	105	128	116	113
20	90	105	109	128	116	120
21	90	109	105	128	113	113
22	90	105	113	128	113	113
23	94	105	105	128	113	113
24	94	109	105	120	113	113
25	94	109	105	120	113	113
26	94	105	109	128	113	113
27	94	109	113	128	113	113
28	90	105	109	120	113	113
29	90	105	113	128	113	113
30	90	105	113	128	113	113

INTERVENTION – with Ryanodine 100µm

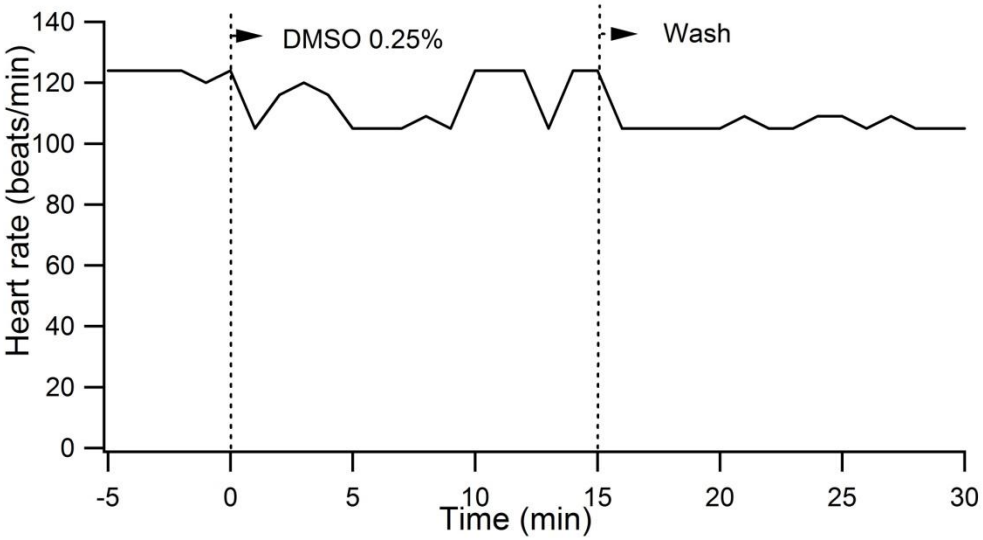
Time (min)	Heart rate (beats/min)					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
-5	128	105	124	188	143	150
-4	128	105	124	188	135	150
-3	128	105	124	188	135	150
-2	128	105	124	188	143	150
-1	128	105	120	188	143	150
0	128	113	120	188	143	150
1	124	113	116	150	143	128
2	128	113	109	135	128	150
3	109	113	101	128	120	128
4	109	98	105	105	120	105
5	105	94	105	105	120	105
6	90	83	101	98	105	90
7	83	79	101	98	90	83
8	83	68	98	98	90	83
9	79	60	79	90	90	75
10	79	60	75	90	83	75
11	79	60	75	98	83	83
12	71	60	75	90	83	83
13	68	56	75	90	83	75
14	71	56	75	90	90	75
15	68	60	75	90	90	83
16	68	60	75	90	90	83
17	68	60	75	90	90	75
18	68	60	75	98	90	75
19	68	60	75	98	113	75
20	68	60	79	98	113	75
21	64	60	75	98	105	75
22	68	60	79	90	98	75
23	64	60	79	98	105	68
24	68	60	79	98	98	68
25	64	56	79	98	90	75

Using IGOR pro software calculated heart rate was plotted against time for each control experiments

EXPERIMENT 1 :



EXPERIMENT 2 :



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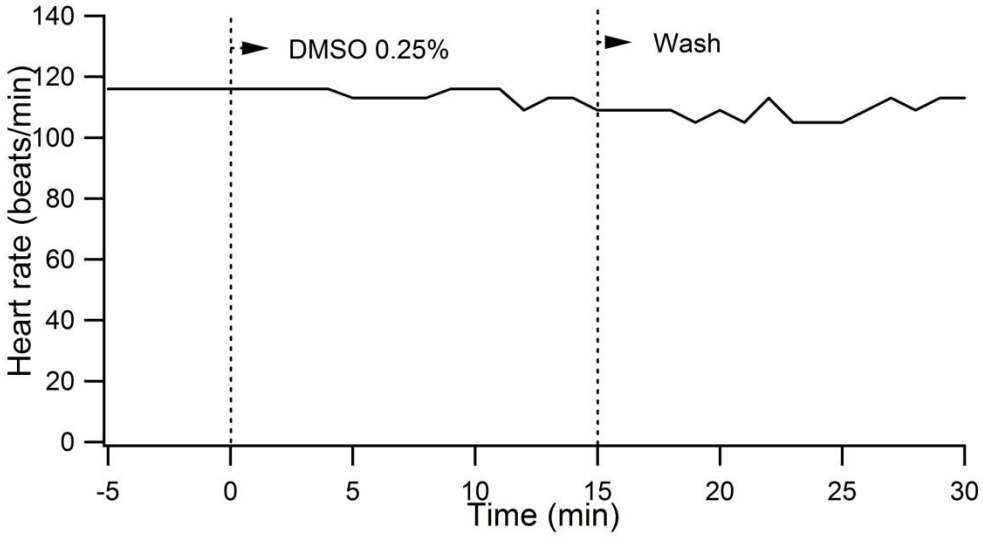

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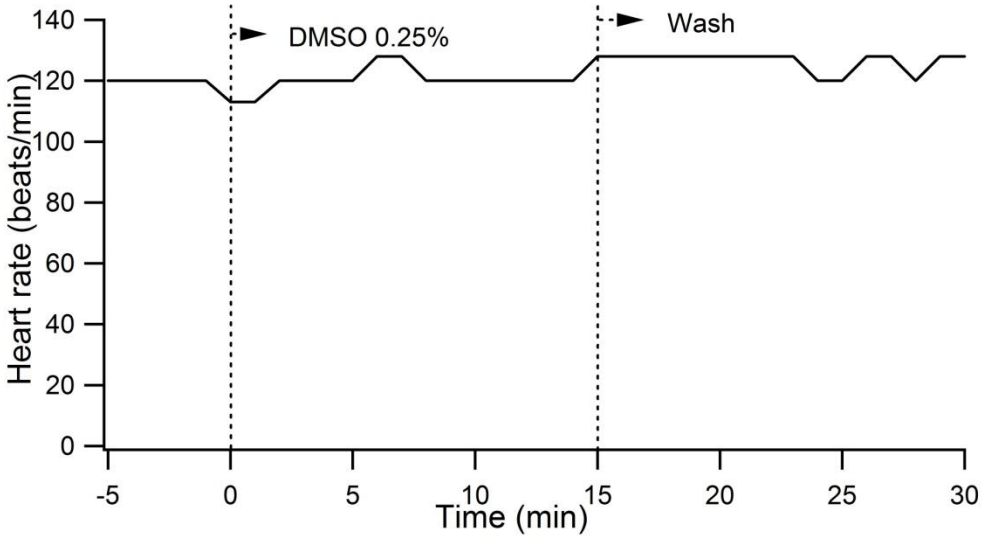
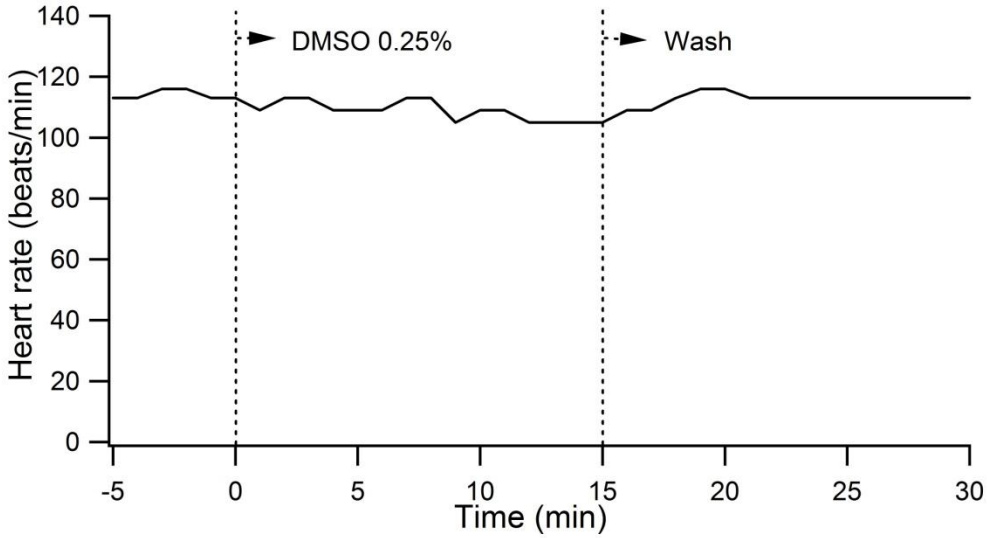
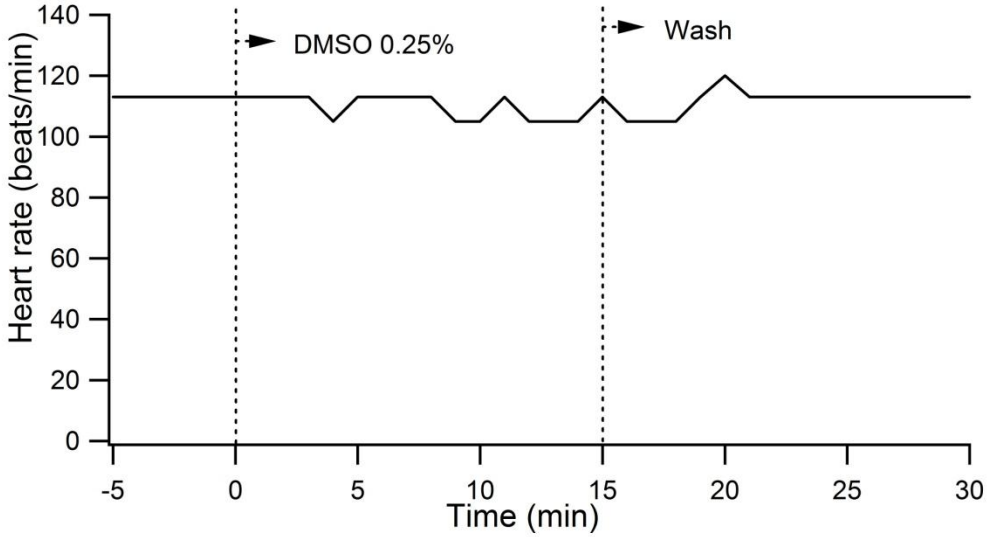


EXPERIMENT 5:



EXPERIMENT 6:



CONTROL GROUP :

In order to avoid the change in basal heart rates of different rats normalization to basal heart rate was done. For each minute mean and standard deviation is calculated. These values were plotted in a graph using IGOR Pro software.

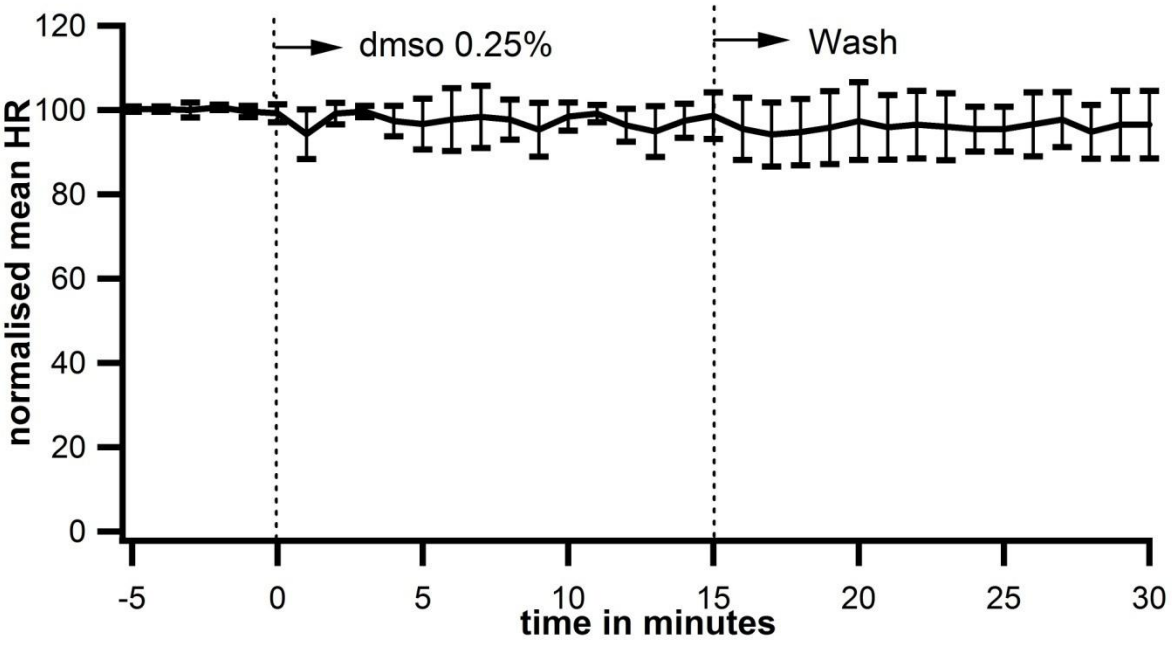


Figure 19:A plot of normalized heart rate against time using Langendorff mode of perfusion of isolated rat heart at 37 degree Celsius with oxygen in which DMSO 0.25% was added to serve as control. Significant change in heart rate didn't occur after 15 minutes of perfusion.

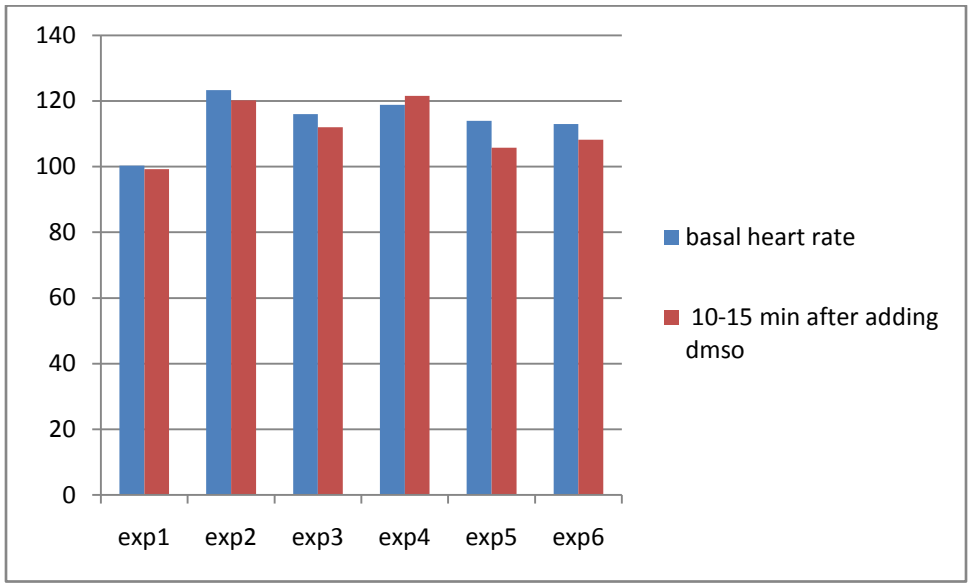


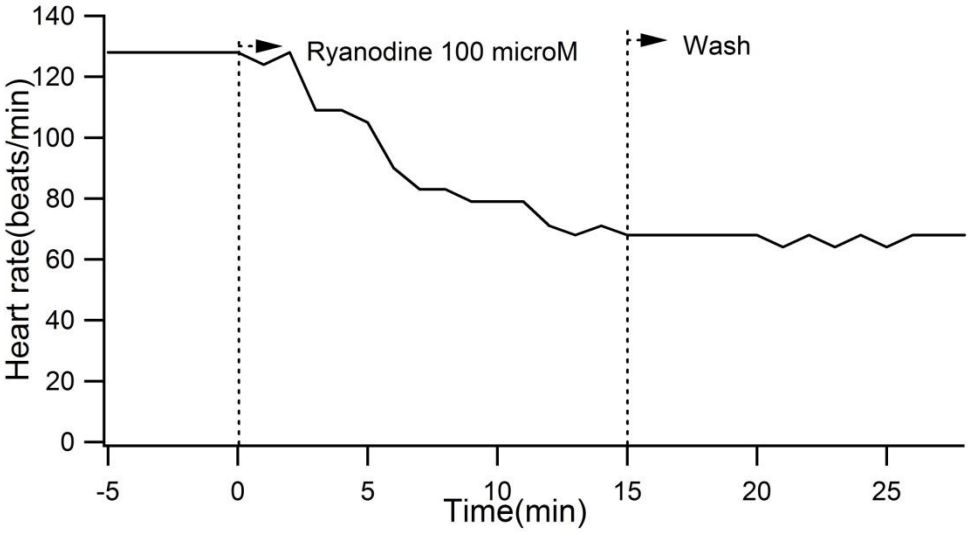
Figure 20: The effect of dmso on heart rate shown as a category plot

Statistical analysis:

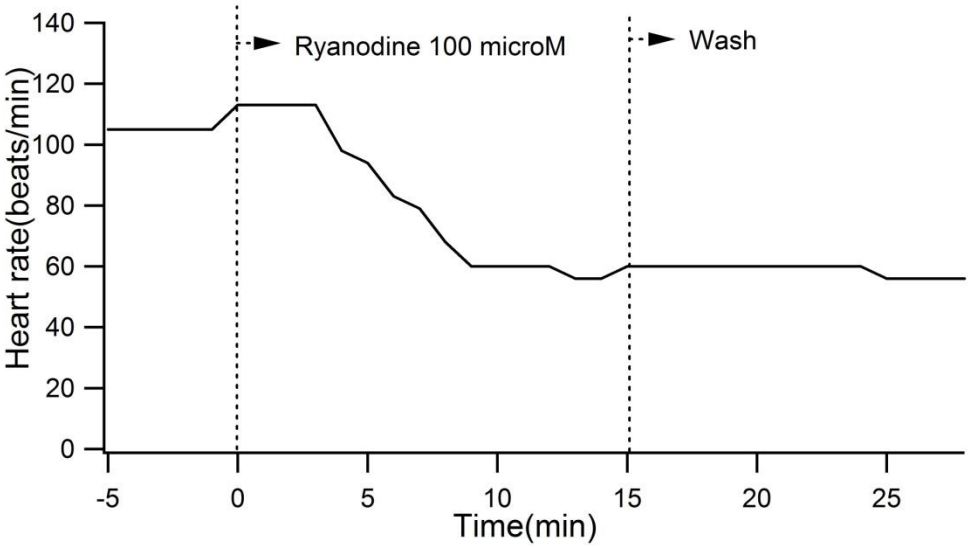
Percentage change in heart rate is calculated before and after addition of DMSO 0.25%. Post intervention values compared with pre intervention reading and analyzed with Wilcoxan Signed Rank Test. Analysis using SPSS software showed that in control group heart rate does not change with DMSO (p value 0.075).

Using IGOR pro software calculated heart rate was plotted against time for each intervention experiments

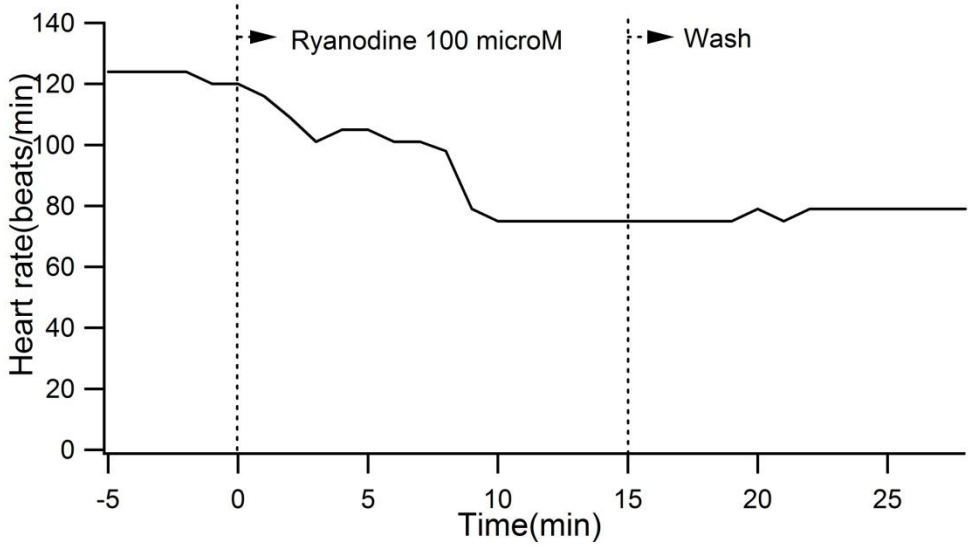
EXPERIMENT 1:



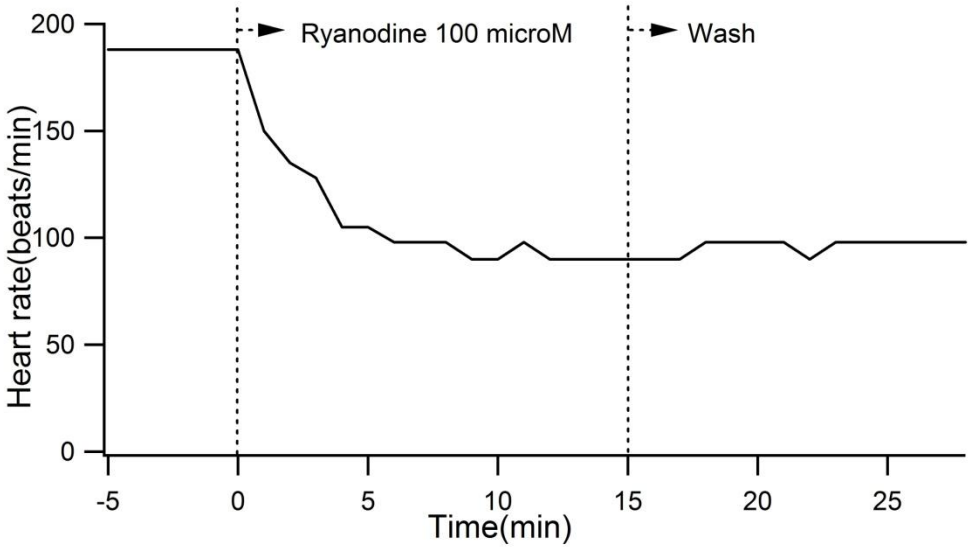
EXPERIMENT 2:



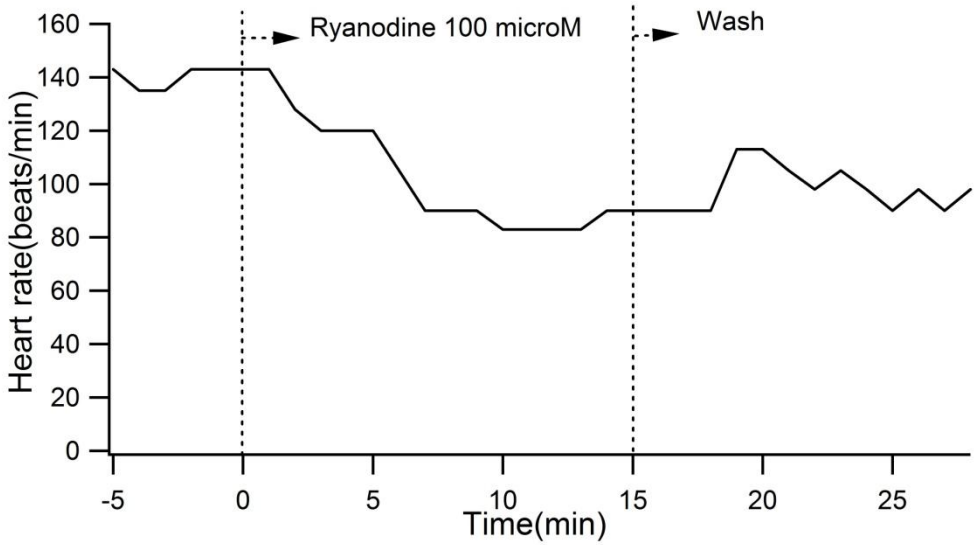
EXPERIMENT 3:



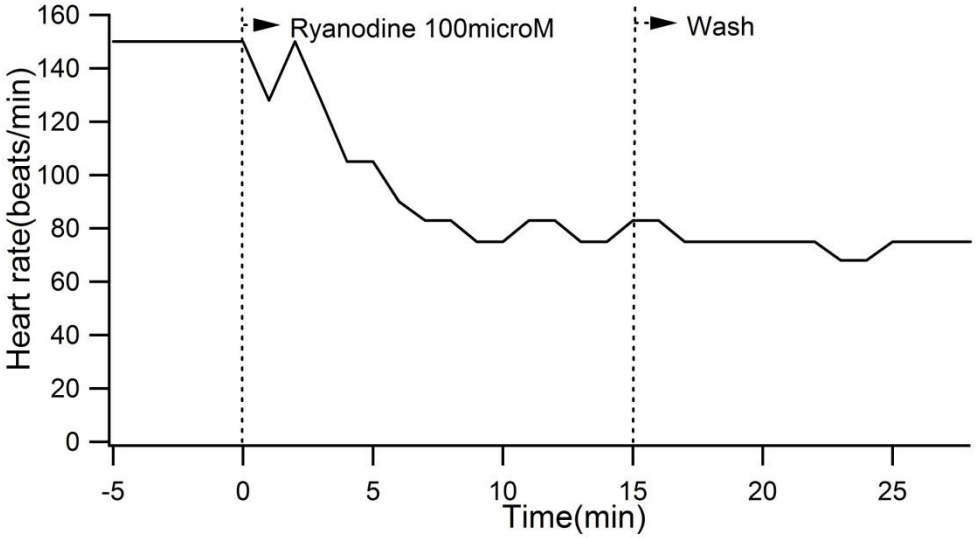
EXPERIMENT 4:



EXPERIMENT 5:



EXPERIMENT 6:



INTERVENTION GROUP

INTERVENTION:

In order to avoid the change in basal heart rates of the six rats normalization of basal heart rate was done. Mean and standard deviation is calculated for each minute. These values were plotted in a graph using IGOR Pro software

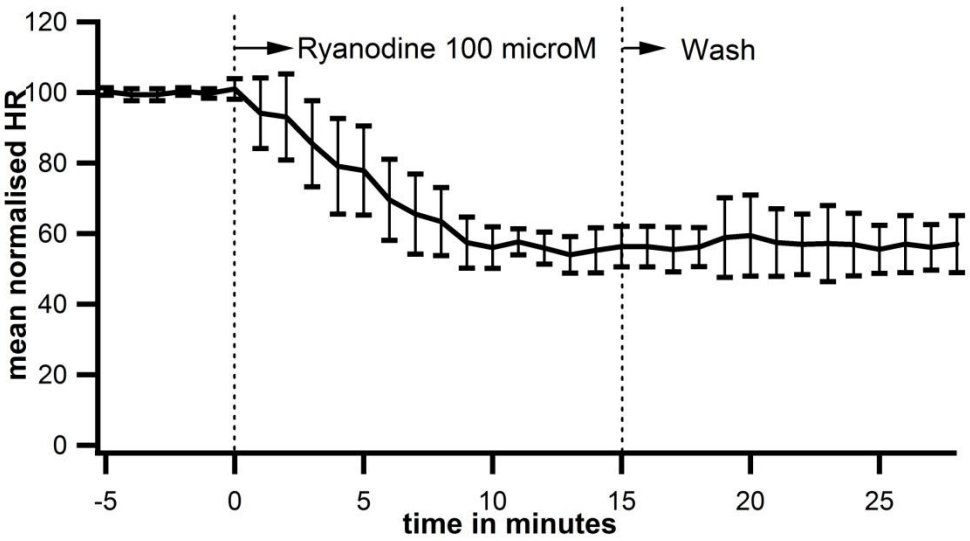


Figure 21 : With Ryanodine 100micromolar there is a decrease in heart rate. (p value=0.028)

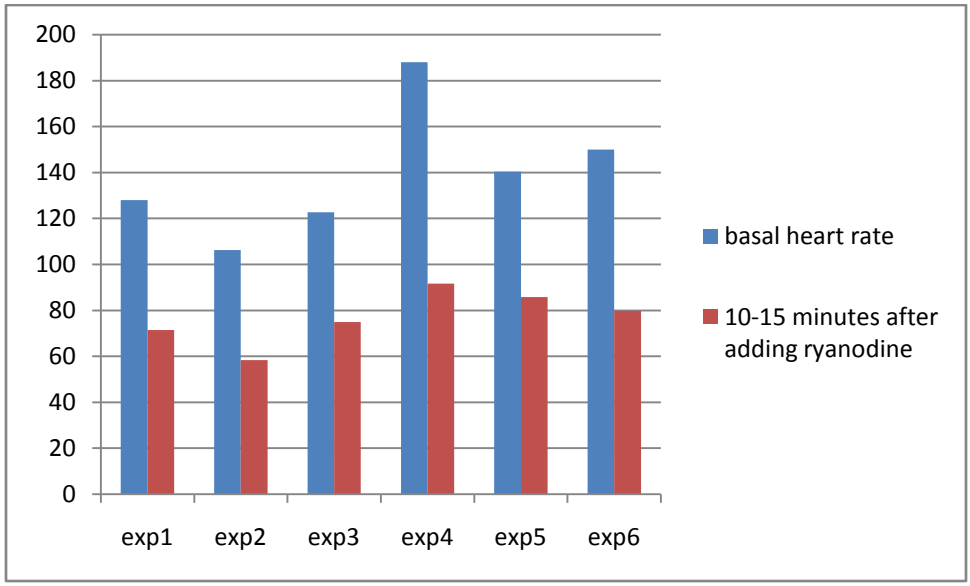


Figure 22: A plot showing decrease in heart rate with Ryanodine 100 micromolar (n=6)

Statistical analysis:

Comparison between basal heart rates and 10-15 minutes after Ryanodine 100 micromolar is done and the data is analyzed using SPSS software with Wilcoxan Signed Rank test. P value is found to be 0.028. This implies that decrease in heart rate with Ryanodine is significant.

COMPARING THE TWO GROUPS

Plotting of mean and standard deviation of normalized heart rates of the all six groups were done in a same graph for comparison

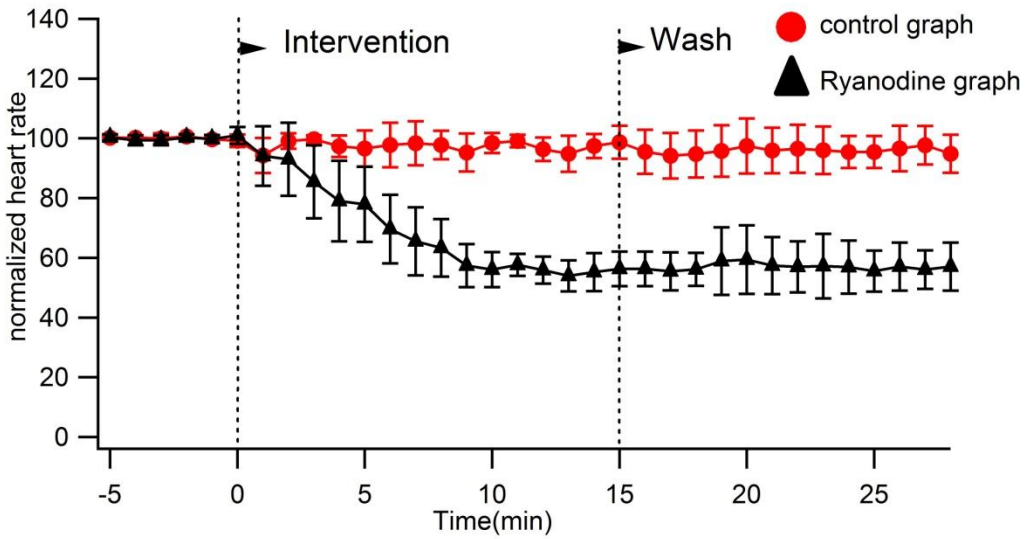


Figure 23: The mean and standard deviation of two groups (control group-red colour and Ryanodine group-black colour) are plotted in the same graph

DISCUSSION:

Heart is endowed with one major electrophysiological property called chronotropy. It is also called excitability or automaticity. It is the property of heart to generate impulses of its own. From old time onwards several researches have been done to understand the molecular basis of rhythm generation.

SA node is known as the natural pacemaker of heart. It has got ability to produce highest number of impulses of its own. It is located inside the right atrium near the entry of superior vena cava. Sinoatrial nodal cell action potential differs from that of working cardiomyocytes.

Sinoatrial nodal action potential

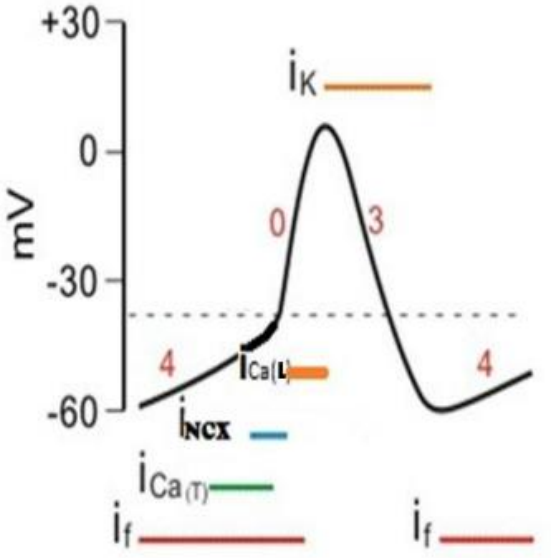


Figure 24: Sinoatrial node action potential and ion channels involved

The three phases of sinoatrial node action potential are Diastolic depolarization phase called phase 4, depolarization phase called phase 0 and repolarization phase called phase 3.

The sinoatrial nodal cells are submitted to a slow depolarization in phase 4 and they do not possess a stable resting membrane potential. Towards end of repolarization membrane is hyperpolarized with membrane potential reaching a value of -60mV. This causes opening of hyperpolarization activated cyclic nucleotide gated ion channels causing current known as funny currents (I_f). This produces initial part of diastolic depolarization upto -50mV by producing an inward current. T type calcium channel open at this potential and more depolarization of membrane occurs. This calcium entry via T type calcium channels cause calcium induced calcium release from sarcoplasmic reticulum via Ryanodine and IP3 receptors. This extruded calcium causes sodium calcium exchanger to act in forward causing an inward current which causes further depolarization. At membrane potential -40 mV opening of L type calcium channels occurs and this calcium entry cause further depolarization of membrane to reach the threshold potential(8).

Increased conductance via L type calcium channels constitute the Phase 0 of action potential. Since conductance of sodium is fast compared to that of calcium membrane depolarization is slow in case of sinoatrial nodal cell compared to ventricular myocytes. This action potential is also known as slow response action potential(63).

Phase 3 is the repolarization which is brought about by the opening of potassium channels generating an outward hyperpolarization current.

Simultaneously closure of calcium channels also occurs. Again membrane potential reaches -60mV and this cycle repeats.

The most important part of pacemaker potential is diastolic depolarization or phase 4. The spontaneous rhythmic discharge of impulses occurs because of this slow depolarization occurring in Phase 4. The two theories which explain the molecular mechanisms of diastolic depolarization are membrane clock theory and the calcium clock theory.

Membrane clock theory suggests that diastolic depolarization occurs because of funny currents generated by HCN channels. The HCN channels are highly expressed in SA nodal cells and purkinje cells compared to rest of myocardium. Thus funny currents act as pacemaker current (1).

Next theory is calcium clock theory. It states that during diastole there occurs spontaneous rhythmic release of calcium from the sarcoplasmic reticulum. This released calcium extrudes via sodium calcium exchanger producing an inward current. This current is responsible for diastolic depolarization(3). Thus this theory states that intracellular calcium act as clock that produces depolarization.

Recently many studies have been done to establish the role of sodium calcium exchanger in rhythm generation mechanism. It depends upon the release of calcium from the internal storage of cells. The major storage of internal calcium is sarcoplasmic reticulum. There are two receptors on the surface of sarcoplasmic reticulum

which are concerned with calcium release. Ryanodine receptors and Inositol 1,4,5-triphosphate(IP_3) receptors. Thus localized calcium release occurs via any of these channels and that calcium gets extruded via sodium calcium exchanger to produce diastolic depolarization.

IP_3 receptor is a calcium releasing channel present on the sarcoplasmic reticulum. Both calcium as well as IP_3 can cause activation of this channel. After binding with IP_3 even a small increase in calcium during diastole can cause release of calcium(25).

Ryanodine receptor is the other calcium release channel present on the sarcoplasmic reticulum. Voltage gated Ryanodine receptors are seen in skeletal muscles. In cardiac muscle it causes calcium induced calcium release. It causes contraction of muscle during systole.

The aim of present study is to determine the role of Ryanodine receptors in diastolic depolarization. There occurs calcium release from sarcoplasmic reticulum during diastole also which via sodium calcium exchanger contributes to diastolic depolarization. This calcium release can occur via Ryanodine receptors or IP_3 receptors. To test whether the calcium release occur via Ryanodine receptor we added Ryanodine receptor blocker Ryanodine 100 microM.

Ryanodine is an alkaloid which at nanomolar concentration locks the channel in partially opened state and causes full closure of channel at micromolar concentration. Ryanodine was found to be soluble in vehicle dimethyl sulfoxide (DMSO).

So in control experiments after perfusion with normal ringer 0.25% of DMSO was added to see its effect. DMSO didn't produce significant changes in heart rate (p value-0.075). In intervention group heart perfused with Ryanodine 100 micromolar produced significant reduction in heart rate (p value-0.028). Compared to control experiments Ryanodine 100 micromolar caused 55.56% reduction in heart rate (p value-0.004 with Mann whitney U test).

This suggests that Ryanodine receptors are important for calcium release during diastole. This is important for diastolic depolarization. But blockade of Ryanodine receptors didn't lead to stoppage of heart. This means that release of calcium can occur via other receptors which help to maintain heart rate at a lower level.

There are earlier studies which showed decrease in heart rate with blockers of IP_3 receptors. This suggests importance of IP_3 receptors in calcium release during diastole. This reveals the importance of action of both receptors together in maintaining normal cardiac rhythm.

The decrease in heart rate with Ryanodine in isolated cardiomyocytes is already shown. Few studies are done in isolated heart setup. Hence this study to assess the role of Ryanodine receptors in diastolic depolarization.In this study we are adding Ryanodine, a blocker of Ryanodine receptor and seeing its effect in heart rate.

SUMMARY:

The most important part of pacemaking is diastolic depolarization or phase 4 of SA nodal action potential. To explain the molecular mechanism behind the diastolic depolarization two important theories have been submitted. They include membrane clock theory and calcium clock theory.

The calcium clock theory proposes that during diastole calcium release occur from intracellular stores. Major source of intracellular calcium is sarcoplasmic reticulum. There occurs spontaneous rhythmic calcium release from sarcoplasmic reticulum. One calcium ion moves outward via sodium calcium exchanger in exchange for three sodium ions inward. This result in production of an inward current called I_{NCX} which produces depolarization during diastole. Because local calcium release act as a intracellular clock to produce depolarization this is called calcium clock theory.

This NCX is rising as a pacemaker current. There are mainly two types of calcium release channels on the sarcoplasmic reticulum- Ryanodine receptors and IP_3 receptors. The aim of this study was to assess the role of Ryanodine receptors in diastolic depolarization.

This study showed 55.56% reduction in heart rate with Ryanodine when compared to control. This shows that Ryanodine receptors are important for diastolic depolarization. But blockade of Ryanodine only caused reduction in heart rate suggests that they are not the absolute requirement. Other channels such as IP_3 receptors also play a role in

release of calcium from sarcoplasmic reticulum and can maintain heart rate at a lower level in the absence of Ryanodine receptor action. This study comes to the conclusion that both Ryanodine and IP3 receptors are important for rhythm generation.

CONCLUSION:

In isolated heart perfused with normal extracellular solution in Langendorff mode addition of Ryanodine 100 micromolar caused decrease in heart rate. It shows that Ryanodine receptor is important for diastolic depolarization of heart and therefore for pacemaking of heart.

LIMITATIONS

Our study showed that Ryanodine receptors are important in rhythm generation. It is already shown IP3 receptors are also important.This study should be done isolated myocardiocytes with each channel blockers.

FUTURE COURSE OF THIS STUDY

Compare the contribution of Ryanodine receptors and IP_3 receptors in diastolic calcium release and thus in rhythm generation. Study the effect of blocking both IP_3 and Ryanodine receptors simultaneously.

REFERENCES

1. Brown HF, Difrancesco D, Noble SJ. How does adrenaline accelerate the heart? Nature. 1979 Jul 19;280(5719):235–6.

2. Maltsev VA, Lakatta EG. Normal heart rhythm is initiated and regulated by an intracellular Calcium clock within pacemaker cells. Heart Lung Circ. 2007 Oct;16(5):335–48.

3. Bogdanov KY, Vinogradova TM, Lakatta EG. Sinoatrial Nodal Cell Ryanodine Receptor and Na+-Ca2+ Exchanger Molecular Partners in Pacemaker Regulation. Circ Res. 2001 Jun 22;88(12):1254–8.

4. Yaniv Y, Lakatta EG. Pacemaker gene mutations, bradycardia, arrhythmias and the coupled clock theory. J Cardiovasc Electrophysiol. 2013 Dec;24(12):E28-29.

5. Sutko JL, Ito K, Kenyon JL. Ryanodine: a modifier of sarcoplasmic reticulum calcium release in striated muscle. Fed Proc. 1985 Dec;44(15):2984–8.

6. Anderson RH, Razavi R, Taylor AM. Cardiac anatomy revisited. J Anat. 2004 Sep 1;205(3):159–77.

7. John E Hall JEH. GYTON & HALL Textbook of MEDICAL PHYSIOLOGY. TWEFTH EDITION.

8. Grant AO. Cardiac Ion Channels. Circ Arrhythm Electrophysiol. 2009 Apr 1;2(2):185–94.

9. Marionneau C, Couette B, Liu J, Li H, Mangoni ME, Nargeot J, et al. Specific pattern of ionic channel gene expression associated with pacemaker activity in the mouse heart. J Physiol. 2005 Jan 1;562(Pt 1):223–34.

10. Irisawa H, Brown HF, Giles W. Cardiac pacemaking in the sinoatrial node. Physiol Rev. 1993 Jan;73(1):197–227.

11. Rigg L, Heath BM, Cui Y, Terrar DA. Localisation and functional significance of ryanodine receptors during beta-adrenoceptor stimulation in the guinea-pig sino-atrial node. Cardiovasc Res. 2000 Nov;48(2):254–64.

12. Hüser J, Blatter LA, Lipsius SL. Intracellular Ca2+ release contributes to automaticity in cat atrial pacemaker cells. J Physiol. 2000 Apr 15;524(Pt 2):415–22.

13. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. Science. 1993 Oct 29;262(5134):740–4.

14. Cannell MB, Soeller C. Mechanisms Underlying Calcium Sparks in Cardiac Muscle. J Gen Physiol. 1999 Mar 1;113(3):373–6.

15. Guo T BD. Calcium signalling in cardiac ventricular myocytes. Ann N Acad Sci. 2005;1047;86-98.

16. Berridge MJ. Cardiac calcium signalling. Biochem Soc Trans. 2003;31(Pt 5):930-935.

17. Isshiki M, Ying Y, Fujita T, Anderson RGW. A Molecular Sensor Detects Signal Transduction from Caveolae in Living Cells. J Biol Chem. 2002 Nov 8;277(45):43389–98.

18. Torihashi S, Fujimoto T, Trost C, Nakayama S. Calcium oscillation linked to pacemaking of interstitial cells of Cajal: requirement of calcium influx and localization of TRP4 in caveolae. J Biol Chem. 2002 May 24;277(21):19191–7.

19. Brillantes AM, Bezprozvannaya S, Marks AR. Developmental and tissue-specific regulation of rabbit skeletal and cardiac muscle calcium channels involved in excitation-contraction coupling. Circ Res. 1994 Sep;75(3):503–10.

20. Flockerzi V, Bosse E, Biel M, Hullin R, Hofmann F. High voltage activated calcium channels: molecular composition and function. Eur Heart J. 1991 Aug;12 Suppl D:95–8.

21. Perez-Reyes E. Molecular physiology of low-voltage-activated t-type calcium channels. Physiol Rev. 2003 Jan;83(1):117–61.

22. Zhu X, Birnbaumer L. Calcium Channels Formed by Mammalian Trp Homologues. News Physiol Sci Int J Physiol Prod Jointly Int Union Physiol Sci Am Physiol Soc. 1998 Oct;13:211–7.

23. Tse G. Mechanisms of cardiac arrhythmias. J Arrhythmia. 2016 Apr;32(2):75–81.

24. Xu L, Tripathy A, Pasek DA, Meissner G. Ruthenium Red Modifies the Cardiac and Skeletal Muscle Ca2+ Release Channels (Ryanodine Receptors) by Multiple Mechanisms. J Biol Chem. 1999 Nov 12;274(46):32680–91.

25. Gilbert JC, Shirayama T, Pappano AJ. Inositol trisphosphate promotes Na-Ca exchange current by releasing calcium from sarcoplasmic reticulum in cardiac myocytes. Circ Res. 1991 Dec;69(6):1632–9.

26. Subramani S, Subbanna PKT. Calcium-transporters in myocardial cells. Indian J Physiol Pharmacol. 2006 Jun;50(2):99–113.

27. Rogers EF, Koniuszy FR, Shavel J, Folkers K. Plant Insecticides. I. Ryanodine, A New Alkaloid from Ryania Speciosa Vahl. J Am Chem Soc. 1948 Sep 1;70(9):3086–8.

28. Franzini-Armstrong, C. Studies of the triad. I. Structure of the junction in frog twitch fibers. J Cell Biol. 1970;47,488–499.

29. Lanner, J. T G DK.Joshi, AD.and Hamilton, SL. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb Perspect Biol. 2010;2.

30. Lanner JT, Georgiou DK, Joshi AD, Hamilton SL. Ryanodine Receptors: Structure, Expression, Molecular Details, and Function in Calcium Release. Cold Spring Harb Perspect Biol [Internet]. 2010 Nov [cited 2016 Mar 31];2(11). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2964179/

31. Endo, M., Tanaka, M. O Y. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibers. Nature. 1970;228,34–36.

32. Fabiato, A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol. 1983;245,C1–C14.

33. Meissner, G. G Darling, E.and Eveleth, J. Kinetics of rapid Ca2+ release by sarcoplasmic reticulum. Effects of Ca2+,Mg2+ and adenine nucleotide. Biochemistry (Mosc). 1986;25,236–244.

34. Palade, P., Mitchell, R. D. F S. Spontaneous calcium release from sarcoplasmic reticulum. General description and effects of calcium. J Biol Chem. 1983;258,8098–8107.

35. Block, B. A I T.Campbell, KP.and Franzini-Armstrong, C. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J Cell Biol. 1988;107,2587–2600.

36. Tanabe, T., Beam KG Adams, BA.Niidome, T.and Numa, S. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature. 1990;346,567–569.

37. Tanabe, T., Beam KG Powell, JA.and Numa, S. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature. 1988;336, 134–139.

38. Rios, E., and Brum, G. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. Nature. 1987;325, 717–720.

39. Dulhunty AF, Haarmann CS, Green D, Laver DR, Board PG, Casarotto MG. Interactions between dihydropyridine receptors and ryanodine receptors in striated muscle. Prog Biophys Mol Biol. 2002 Jul;79(1–3):45–75.

40. Eisner DA, Sipido KR. Sodium Calcium Exchange in the Heart Necessity or Luxury? Circ Res. 2004 Sep 17;95(6):549–51.

41. Vinogradova TM, Zhou Y-Y, Maltsev V, Lyashkov A, Stern M, Lakatta EG. Rhythmic ryanodine receptor Ca2+ releases during diastolic depolarization of sinoatrial pacemaker cells do not require membrane depolarization. Circ Res. 2004 Apr 2;94(6):802–9.

42. Herrmann S, Lipp P, Wiesen K, Stieber J, Nguyen H, Kaiser E, et al. The cardiac sodium-calcium exchanger NCX1 is a key player in the initiation and maintenance of a stable heart rhythm. Cardiovasc Res. 2013 Sep 1;99(4):780–8.

43. Samsó, M., Wagenknecht, T., A PD. Internal structure and visualization of transmembrane domains of the RyR1 calcium release channel by cryo-EM. Nat Struct Mol Biol. 2005;12,539–544.

44. Serysheva, I. I., Ludtke, S. J., Baker, M. L., Cong, Y., Topf, M., Eramian, D.,, Sali, A., Hamilton, S. L.,, and Chiu, W. Subnanometer resolution electron cryomicroscopy-based domain models for the cytoplasmic region of skeletal muscle RyR channel. Proc Natl Acad Sci USA. 2008;105, 9610–9615.

45. Samsó, M., Feng, W., Pessah, I. N., A PD. Coordinated movement of cytoplasmic and transmembrane domains of RyR1 upon gating. PLoS Biol. 2009;e85.

46. Ludtke, S. J., Serysheva, I. I., Hamilton, S. L., C W. The pore structure of the closed RyR1 channel. Structure13,. 2005;1203–1211.

47. Ahern, G. P., Junankar, P. R., D AF. Subconductance states in single-channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. Biophys J. 1997;72,146–162.

48. Fill M, Copello JA. Ryanodine Receptor Calcium Release Channels. Physiol Rev. 2002 Jan 10;82(4):893–922.

49. Meissner, G., Darling, E., E J. Kinetics of rapid Ca 2+ release by sarcoplasmic reticulum. Effects of Ca 2+ ,Mg 2+ , and adenine nucleotides. Biochemistry (Mosc). 1986;25,236–244.

50. . Gillard, E. F., Otsu, K., Fujii, J., Khanna, V. K., de Leon, S., Derdemezi, J., Britt, B. A., Duff, C. L., Worton, R. G., MacLennan, D. H. A substitution of cysteine for arginine 614 in the ryanodine receptor is potentially causative of human malignant hyperthermia. Genomics11. 1991;751–755.

51. Santulli G, Xie W, Reiken SR, Marks AR. Mitochondrial calcium overload is a key determinant in heart failure. Proc Natl Acad Sci U S A. 2015 Sep 8;112(36):11389–94.

52. Taur Y, Frishman WH. The cardiac ryanodine receptor (RyR2) and its role in heart disease. Cardiol Rev. 2005 Jun;13(3):142–6.

53. Kucerova D, Doka G, Kruzliak P, Turcekova K, Kmecova J, Brnoliakova Z, et al. Unbalanced upregulation of ryanodine receptor 2 plays a particular role in early development of daunorubicin cardiomyopathy. Am J Transl Res. 2015 Jul 15;7(7):1280–94.

54. Hilgemann DW, Delay MJ, Langer GA. Activation-dependent cumulative depletions of extracellular free calcium in guinea pig atrium measured with antipyrylazo III and tetramethylmurexide. Circ Res. 1983;53(6):779–793.

55. Welch W, Ahmad S, Airey JA, Gerzon K, Humerickhouse RA, Besch HR, et al. Structural determinants of high-affinity binding of ryanoids to the vertebrate skeletal muscle ryanodine receptor: a comparative molecular field analysis. Biochemistry (Mosc). 1994 May 24;33(20):6074–85.

56. Procita L. The Action of Ryanodine on Mammalian Skeletal Muscle Ix Situ. J Pharmacol Exp Ther. 1956 Aug 1;117(4):363–73.

57. Lai FA, Misra M, Xu L, Smith HA, Meissner G. The ryanodine receptor-Ca2+ release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. J Biol Chem. 1989;264(28):16776–16785.

58. Carroll S, Skarmeta J, Yu X, Collins KD, Inesi G. Interdependence of ryanodine binding, oligomeric receptor interactions, and Ca2+ release regulation in junctional sarcoplasmic reticulum. Arch Biochem Biophys. 1991 Oct;290(1):239–47.

59. Jenden DJ, Fairhurst AS. The pharmacology of ryanodine. Pharmacol Rev. 1969 Mar;21(1):1–25.

60. Sutko JL, Airey JA, Welch W, Ruest L. The pharmacology of ryanodine and related compounds. Pharmacol Rev. 1997 Mar;49(1):53–98.

61. Viero C, Thomas NL, Euden J, Mason SA, George CH, Williams AJ. Techniques and methodologies to study the ryanodine receptor at the molecular, subcellular and cellular level. Adv Exp Med Biol. 2012;740:183–215.

62. Thomas NL, Williams AJ. Pharmacology of ryanodine receptors and Ca2+-induced Ca2+ release. Wiley Interdiscip Rev Membr Transp Signal. 2012 Jul 1;1(4):383–97.

63. de Carvalho AP, Hoffman BF, de Paula Carvalho M. Two Components of the Cardiac Action Potential. J Gen Physiol. 1969 Nov 1;54(5):607–35.

ANNEXURES

IRB Approval Letter

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

November 08, 2014

Dr. Teena Maria Jose
PG Registrar
Department of Physiology
Christian Medical College, Vellore 632 002

Sub: **Fluid Research Grant Project:**
Are ryanodine receptors important for diastolic depolarization in heart?
Dr. Teena Maria Jose, PG Registrar, Dr. Sathya Subramani, Dr. Jesi. W, Physiology, CMC, Vellore.

Ref: IRB Min No. 9073 [OTHER] dated 06.10.2014

Dear Dr. Teena Maria Jose,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Are ryanodine receptors important for diastolic depolarization in heart?" on October 6th 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Cvs of Drs. Teena Maria Jose, Sathya Subramani, Jesi W
3. No of documents 1-2

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on October 6th 2014 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Chandra Singh	MS, MCH, DMB	Professor, Urology, CMC.	Internal, Clinician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Vivek Mathew	MD (Gen. Med.) D.M (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC	Internal, Clinician
Dr. Simon Pavamani	MBBS, MD,	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC	Internal, Clinician
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Dept of Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Jacob John	MBBS, MD	Associate Professor, Community health	Internal, Clinician
Dr. T. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mrs. Pattabiraman	B. Sc, DSSA	Social Worker, Vellore	External, Lay Person
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert
Dr. Anuradha Rose	MBBS, MD	Assistant Professor, Community Health, CMCH.	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse

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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Mrs. Sheela Durai	MSc Nursing	Addl. Deputy Nursing Superintendent, Professor of Nursing in Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Legal Expert, Vellore	External, Legal Expert
Dr. Shirley David	M.Sc, PhD	Professor, Head of Fundamentals Nursing Department, CMC.	Internal, Nurse
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP(Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology, Additional Vice Principal (Research), Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

This proposal will also need to be submitted to the Institutional Animal Ethics Committee (IAEC) for approval. The animal requirements and budget will have to be discussed with the Animal House Staff prior to submission of the proposal to the Institutional Animal Experimentation Committee.

IRB Min No: 9073 [OTHER] dated 06.10.2014

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Fluid Grant Allocation:

A sum of 79,500/- INR (Rupees Seventy Nine Thousand Five Hundred only) will be granted for 2 years. The funds will be released only after the IAEC clearance.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

DR. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc. Dr. Sathya Subramani, Physiology, CMC, Vellore.

IRB Min No: 9073 [OTHER] dated 06.10.2014

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Institutional Animal Ethics Committee Approval Letter

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
CHRISTIAN MEDICAL COLLEGE, VELLORE

Dr. Alfred Job Daniel
Principal and Chairman
email: princi@cmcvellore.ac.in

Dr. Vinay Timothy Oommen
Secretary
email: vinayoommen@cmcvellore.ac.

21st November, 2014

Teena Maria Jose
Department of Physiology
CMC Vellore

Dear Teena Maria Jose

Your research proposal titled **"Are ryanodine receptors important for diastolic depolarization in heart."** has been approved by the Institutional Animal Ethics Committee (IAEC).

After discussion, **16 Wistar Rats have been approved for Year I,**

The IAEC approval number for the study is **13/2014**

You are required to maintain all records as per form D, ensure humane treatment of animals and submit a **final report** to the IAEC.

With best wishes,

Yours sincerely,

Dr. Alfred Job Daniel,
Principal & Chairperson
Institutional Animal Ethics Committee

Cc:
Dr. Vinay Timothy Oommen
Secretary, IAEC