ROLE OF INOSITOL 1,4,5-TRIPHOSPHATE ON HEART RATE IN ISOLATED HEART MODELS OF WISTAR RATS

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

Department of Physiology

Christian Medical College, Vellore

Tamilnadu

April 2015

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CERTIFICATE

This is to certify that the thesis entitled "**Role Of Inositol 1,4,5-Triphosphate On Heart Rate In Isolated Heart Models Of Wistar Rats**" is a bonafide, original work carried out by Dr.Jesi.W, 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- 2015.

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**Role Of Inositol 1,4,5-Triphosphate On Heart Rate In Isolated Heart Models Of Wistar Rats**" 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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ABSTRACT:

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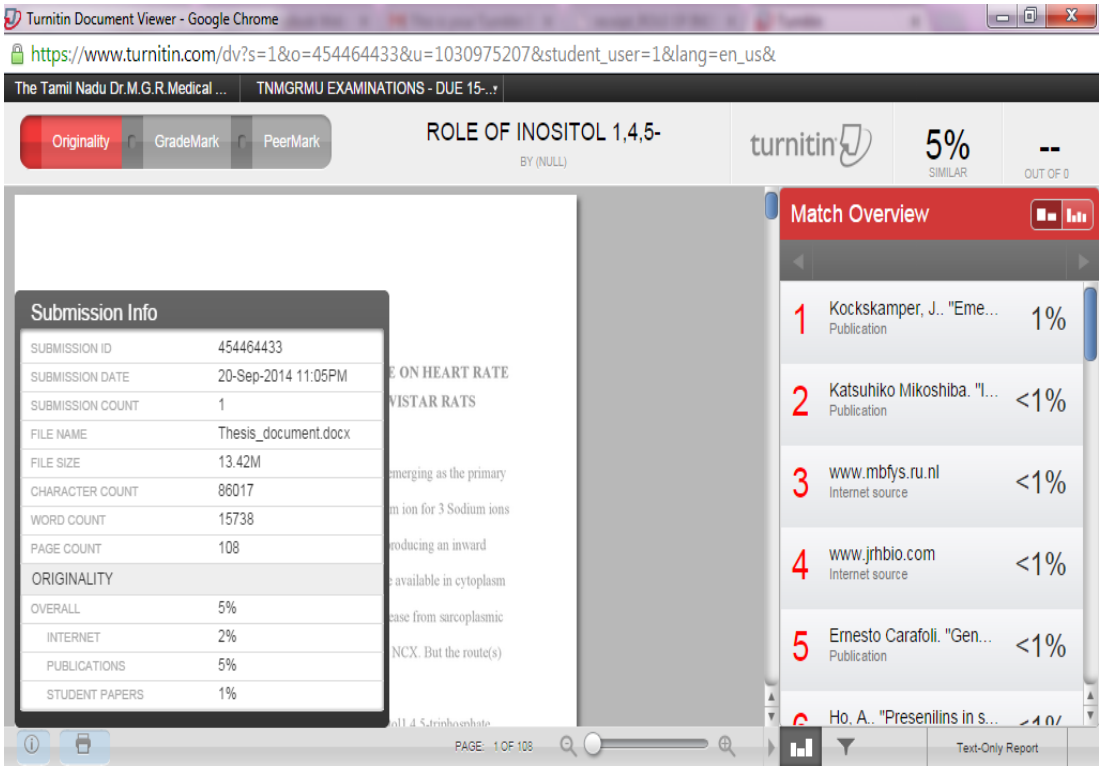
In this study we wanted to see the role of Inositol1,4,5-triphosphate receptor(IP3R) in diastolic calcium release. If it is involved, stimulating IP3R should increase heart rate and decreasing IP3 formation should decrease heart rate. In this study rat hearts were isolated and perfused with normal extracellular solution in Langendorff mode and the heart rate was recorded. Phenylephrine, an IP_3 agonist was added to the perfusate and its effect on heart rate was recorded(n=6). Neomycin which blocks the production of IP_3 was added along with Phenylephrine in another set of experiments and the effects on heart rate was recorded(n=6). The change in heart rate was compared and analysed using Wilcoxon Signed Rank test.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The sodium calcium exchanger (NCX) is emerging as the primary pacemaking mechanism in heart. It extrudes 1 Calcium ion for 3 Sodium ions taken into the cell (forward mode of NCX), thereby producing an inward current which depolarizes the cell. Calcium should be available in cytoplasm to drive NCX in forward mode. Diastolic calcium release from sarcoplasmic reticulum (SR) provides for calcium to drive forward NCX. But the route(s) of calcium release from SR is/are to be confirmed.

In this study we wanted to see the role of Inositol1,4,5-triphosphate receptor (IP3R) in diastolic calcium release. If it is involved, stimulating IP3R should increase heart rate and decreasing IP3 formation should decrease heart rate. In this study rat hearts were isolated and perfused with normal extracellular solution in Langendorff mode and the heart rate was recorded. Phenylephrine, an IP_3 agonist was added to the perfusate and its effect on heart rate was recorded (n=6). Neomycin which blocks the production of IP_3 was added along with Phenylephrine in another set of experiments and the effects on heart rate was recorded (n=6). The change in heart rate was compared and analysed using Wilcoxon Signed Rank test.

Our results showed a 44% increase in heart rate with Phenylephrine ($p = 0.028$). With Neomycin the Phenylephrine-induced increase in heart rate was blocked. This confirms that the positive chronotropic effect of Phenylephrine is through IP_3. The study confirms that IP_3 has a positive chronotropic effect. This opens a whole new portal of pharmaco-therapeutics in the management of cardiovascular pathologies like arrhythmias.

INTRODUCTION:

The research on the molecular mechanism behind the cardiac pacemaker activity has lead to the identification of ion channels involved in generating cardiac action potential and their encoding genes. Sinoartial node is the primary pacemaker of the heart. The Sinoatrial node action potential is different from the action potential of other working cardiomyocytes. The Sinoatrial node action potential has a diastolic depolarisation during phase 4 which is responsible for the spontaneous production of action potential. The ionic mechanism behind this diastolic depolarisation is responsible for the pacemaker activity of heart and this has studied extensively.

In 1979, the funny currents (I_f) was discovered by Noble et al and these currents were called as the pacemaker currents. These funny currents through HCN (hyperpolarisation activated cyclic nucleotide gated) channels get activated on hyperpolarisation. So at the end of repolarisation phase when the membrane potential is more negative, these channels get activated producing an inward current and thereby diastolic depolarisation (Brown et al., 1979). Since this HCN channel which is present in the membrane is responsible for the diastolic depolarisation, this theory is called the membrane clock theory.

Later studies using confocal imaging and immunoflorescence showed that an intracellular calcium clock activates the membrane ion

channels and produce action potential. This intracellular calcium clock is described as a spontaneous, regular and rhythmic local calcium release (LCR) from sarcoplasmic reticulum which occurs before the next action potential. This local calcium release activates sodium calcium exchanger in forward mode producing an inward current and depolarisation (Bogdanov et al., 2001). Since the intracellular calcium release initiates action potential this theory is called ‘Calcium Clock’ theory.

Recent studies have shown that in cardiac pacemaker cells the calcium clock and the membrane clock work together to produce diastolic depolarisation. This is called as ‘coupled clock’ theory (Yaniv and Lakatta, 2013).

The local calcium release which activates sodium calcium exchanger in forward mode is from sarcoplasmic reticulum. The sarcoplasmic reticulum has two calcium release channels namely: Ryanodine receptors and IP_3 receptors (Inositol1,4,5-triphosphate receptors). The sarcoplasmic reticulum releases calcium both during systole and diastole. The sarcoplasmic reticular calcium release during systole is responsible for contraction and the sarcoplasmic reticular calcium release during diastole is responsible for pacemaking activity of the heart. Which of the calcium channels of the sarcoplasmic reticulum is responsible for diastolic calcium release is not yet clear.

The Ryanodine receptor is activated by calcium to produce a calcium induced calcium release during systole (Griffiths and MacLeod, 2003). IP_3 receptors are activated by calcium and IP_3 (Bootman et al., 2009). Hence when IP_3 is bound to its receptor, even a very little amount of calcium as seen during diastole can activate the receptor to release more calcium (Gilbert et al., 1991). Hence IP_3 may have a more significant role than Ryanodine receptor in diastolic calcium release.

This study aims at assessing the role of IP3 receptor in diastolic depolarisation and thereby heart rate. We have used an isolated rat heart model perfused in Langendorff mode with normal extracellular solution at 37 degree Celsius and constantly oxygenated. The heart is calculated from the surface ECG recorded using surface electrodes. The concentration of IP_3 is altered in the cardiac tissue using Phenylephrine (IP_3 agonist) and Neomycin (IP_3 antagonist) and the change in heart rate is assessed.

REVIEW OF LITERATURE

REVIEW OF LITERATURE:

HEART:

Heart is a vital organ in our body. It does the work of pumping blood throughout the body. Thus it carries over the functions of transport of gases, waste products and hormones, regulation of homeostasis and also helps in immunity. People have been very curious to know about heart and its functions right from ancient times. In 4th century B.C., the Greek scientist and philosopher Aristotle described heart as the most important organ of the body and first to be formed based on his observation of chick embryos. After Aristotle many other great scientists like Galen and Leonardo da Vinci have studied heart and described about it. Later in 1628, the English physician William Harvey published his article called 'On the circulation of the blood' which formed the basis for all the modern research and understanding of heart and its function.

Heart is a muscular organ made of four chambers. The cardiac muscle is an involuntary, striated muscle. The cardiac muscle is different and more complex compared to the skeletal muscle. The fibres of the cardiac muscle form a structural network and acts as a syncytium. The impulses that activate the heart are neither produced nor conducted by the nervous system as in skeletal muscles. The excitatory impulses of heart are produced by a group

of specialized cells present in the right atrium called the Sinoatrial node. The rhythmic impulses produced by the Sinoatrial node are distributed throughout the heart by a special conduction system present in the heart. The conduction system includes the Sinoatrial node, inter-atrial fibres, Atrioventricular node, Bundle of His, Purkinje fibres and inter-ventricular fibres. The wall of the heart is made of three layers: the endocardium which forms the inner layer, the middle myocardium and the epicardium which forms the outer layer.

- **Properties of heart**

The heart has five electrophysiological properties. In 1897, Theodor Wilhelm Engelmann, a German physiologist introduced the Greek terms for describing the electrophysiological properties of heart. They are:

- Chronotropy
- Inotropy
- Dromotropy
- Lusitropy
- Bathmotropy

Chronotropy is the property of the heart to produce electrical impulse on its own. It is also called as automaticity.

Inotropy is the property of the heart to contract to the electrical impulse. It is also called as contractility.

Dromotropy is the property of the heart to conduct the electrical impulse. It is also called as conductivity.

Lusitropy is the property of the heart to relax.

Bathmotropy is the property of the heart to respond to stimulus. It is also called as excitability.

Cardiac Action potential:

Rapid activation of a group of muscles which produces and distributes an electrical impulse is necessary for the normal sequence of synchronous contraction of the atrium and ventricle. This electrical impulse must be able to change the heart rate rapidly and to respond to any change in autonomic tone. All these roles are fulfilled by the cardiac action potential. A normal cardiac action potential has 5 phases.

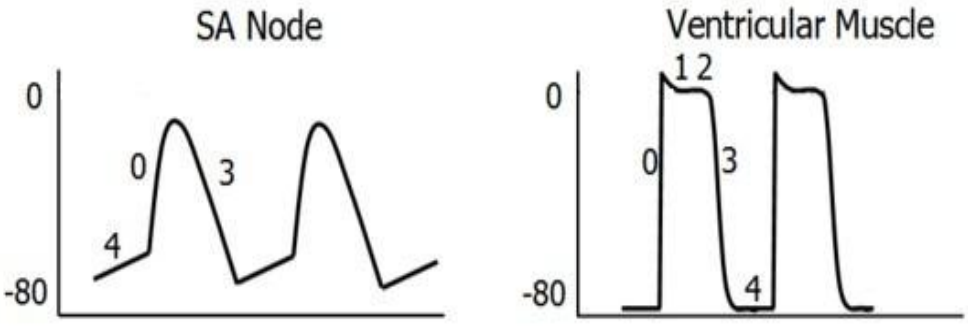


Figure 1 : Action potentials of a Sinoatrial node cell and ventricular muscle cell.

1. Phase 4: This is also called as the resting potential. It is around -90 mV in working myocardial cells. It is more depolarised in the pacemaker cells present in the Sinoatrial node. In the pacemaker cell it is around -50 to -65 mV and it undergoes a slow depolarization.
2. Phase 0: This is a phase of rapid depolarization. This phase is important for the rapid propagation of the impulses at the velocity of 1m/s. In Sinoatrial node this phase is slower compared to the working myocardial cells. Hence there is slow propagation of impulse with velocity of 0.1 to 0.2 m/s (Grant, 2009).
3. Phase 1: This is a phase of rapid repolarisation. This phase sets the potential for the next plateau phase.
4. Phase 2: This phase is a plateau phase and this is the longest phase. This is seen only in the working myocardial cells and not in the Sinoatrial nodal cells. This phase marks calcium entry into the cell.
5. Phase 3: This is a phase of rapid repolarisation. This phase restores the membrane potential back to the resting membrane potential (Grant, 2009).

Ion channels responsible for different phases of action potential:

The action potential generation and their difference in different regions of the heart are due to the difference in distribution and permeability of ion channels in the heart.

Sinoatrial nodal Action Potential:

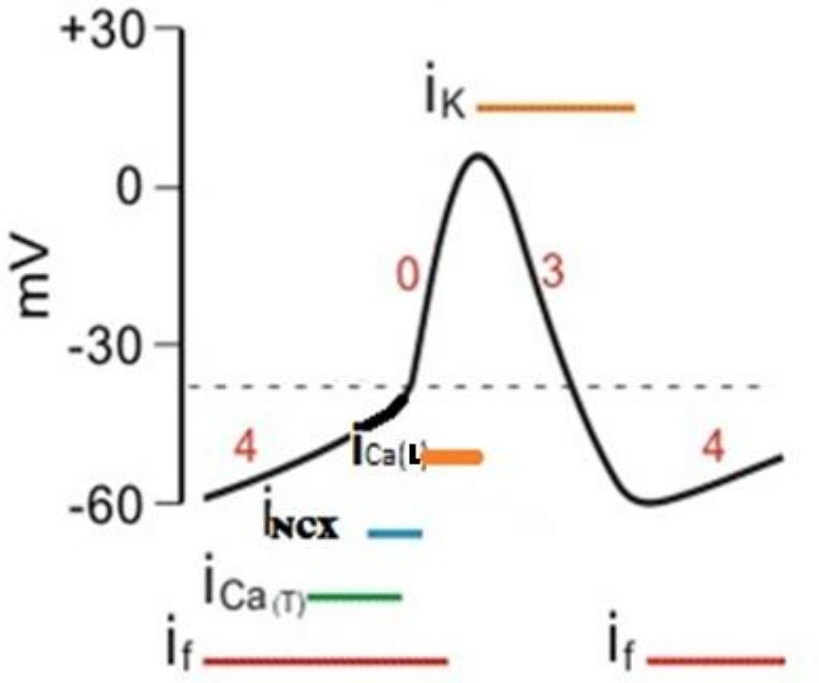


Figure 2 : Action potential in the Sinoatrial nodal cell and the ion channels involved in each phase

The cells in Sinoatrial node donot have any true resting potential, instead they generate spontaneous and regular action potentials.

During phase 4, the Sinoatrial node action potential undergoes a slow depolarisation which is called as ‘Diastolic Depolarisation’. At the end of repolarization, the membrane potential reaches a negative value of about -60 mV. At this potential there is opening of an ion channel called HCN (Hyperpolarization-activated cyclic nucleotide-gated) channels which produces an inward current called the funny currents. This produces little depolarization of the membrane. At around -50 mV the T-type calcium channels get activated and produce more depolarization. The calcium ions entering the cell through this calcium channel activate calcium-induced calcium release from the sarcoplasmic reticulam stores through Ryanodine receptors and IP_3 (Inositol 1,4,5-triphosphate)receptors. This increase in intracellular calcium drives the sodium calcium exchanger in forward mode and produces a depolarizing inward current. When the membrane potential reaches -40 mV, it activates the L- type calcium channels. Opening of this channel causes calcium to enter into the cell and depolarises the cell till the threshold potential is reached (Grant, 2009) .

Phase 0 of the Sinoatrial node action potential is due to increased conductance of calcium ions through the open L-type calcium channels. Since the activation of calcium channels is not as fast as sodium channels, the rate of depolarization is slow compared to the working myocardial cells action potential. Hence this is also called as the ‘Slow Response’ action potential (de Carvalho et al., 1969).

Phase 2 is absent in the Sinoatrial nodal action potential.

In Phase 3, the repolarization phase of the Sinoatrial node action potential occurs because of the potassium conductance through the voltage gated potassium channels producing an outward hyperpolarizing current. At this time the calcium channels become inactivated and closed. Once the cell is completely repolarised and reaches -60 mV, the cycle is repeated spontaneously.

Working cardiomyocytes/Ventricular Action potential:

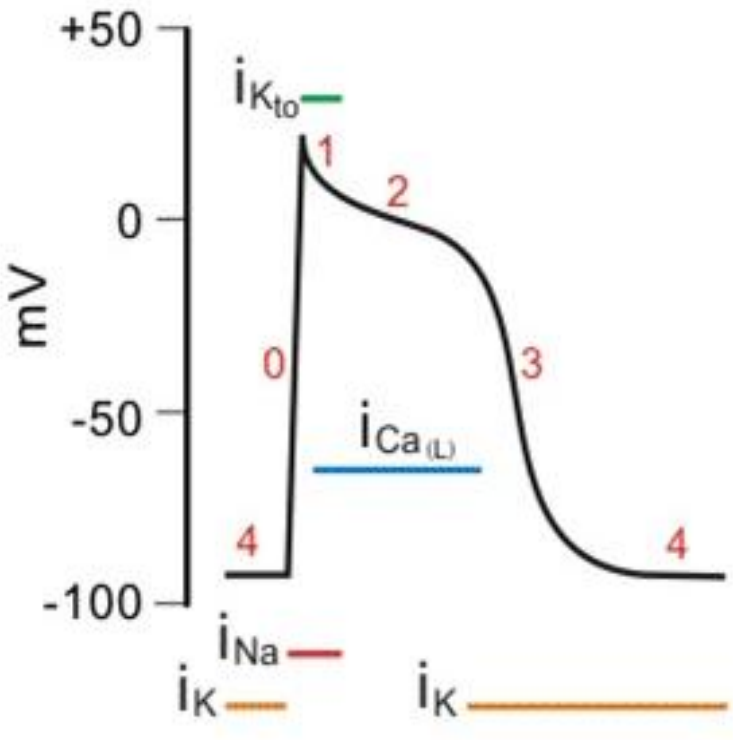


Figure 3 : Action potential tracing in a working myocardial cell and the ion channels involved in each phase

Atrial myocytes and ventricular myocytes are the examples of working myocardiocytes or non-pacemaker cells.

Unlike Sinoatrial nodal cells these cells have true resting membrane potential. This resting membrane potential remains near the equilibrium potential of potassium ion which is around -90 mV.

In Phase 4, the resting membrane potential of the working cardiomyocytes is around -90 mV. This is because of the potassium conductance as the cell is more permeable to potassium at this potential.

Phase 0 occurs when these cells are depolarised by the action potential from the adjacent cell. There is a rapid depolarization at phase 0. This is due to the opening of sodium channels and increased sodium conductance. This is faster than the calcium conductance seen in the Sinoatrial nodal cells. Hence this action potential is referred to as ‘Fast Response’ action potential (de Carvalho et al., 1969).

Phase 1 is the initial repolarisation phase. This is because of opening of a transient outward potassium channel which produces a hyperpolarizing current and is short lived.

Phase 2 is a plateau phase. This is because of the calcium conductance through the open L-type calcium channels. This plateau phase increases the duration of action potential.

Phase 3 is the repolarisation phase which occurs due to the opening of potassium channels and closing of calcium channels. This repolarisation takes the membrane to the resting membrane potential.

Chronotropy

Chronotropy is the property to produce electrical impulse on its own. Pacemaker cells are the cells with this property of Chronotropy or automaticity. Pacemaker cells are described as cells which can spontaneously depolarize from its resting membrane potential, producing an action potential which can then contract the cell. The electrical coupling of the neighbouring cells which act as a syncitium, helps in conduction of this action potential produced by the pacemaker cells.

Self excitability seen in other cells like GIT:

Pacemaker cells which have the property of Chronotropy or automaticity are also found in the Gastro-intestinal tract. The pacemaker cells present in the gut is called as Intestitial cells of Cajal. These cells generate slow waves which determine the frequency of the phasic contractions of stomach, small intestine and colon. These cells were first described by Santiago Ramon y Cajal in 1911 and he called them ‘primitive neurons’ (Cajal, 1911). These cells are spontaneously active in their resting membrane

potentials and hence can produce action potentials. The ionic basis of the pacemaker mechanism in these cells is still under study. A study shows that the initiation of pacemaker activity is by the release of calcium from the internal stores through IP_3 receptor which produces unitary current and depolarisation (Sanders et al., 2006).

Pacemaker in heart

Heart has pacemaker activity i.e. it can produce impulse and beat on its own without any neuro-humoral stimulation. This was observed as early as second century AD when Galen saw that even after isolating the heart from the body, it was beating for some time. All the cells in the conductive system have the property of rhythm generation. But Sinoatrial node can produce impulses at highest rate (60-100 per minute) compared to other regions (AV node:40-55/min, purkinje system: 25-40/min) in the conductive system. Hence it suppresses the other areas and acts as a primary pacemaker.

Diastolic depolarisation

The pacemaker cells have a phase called Diastolic depolarisation which is a depolarising phase at the end of repolarisation that brings the membrane to threshold potential for producing action potential.

Theories of pacemaking:

The ionic and molecular mechanism behind the pacemaker activity of the heart has been studied for a long time and many theories have been proposed. The discovery of hyperpolarisation activated current (I_f) in sinoatrial nodal cells and purkinje cells was a breakthrough in this research. Recently the importance of Sodium calcium exchanger and spontaneous intracellular calcium release in pacemaking has been highlighted.

Membrane clock Theory:

In 1979 Noble et al proposed that the diastolic depolarisation is due to the funny currents (I_f) through the HCN channels which gets activated on hyperpolarisation and produce an inward current (Brown et al., 1979).The funny current is a mixed cationic current carried by sodium and potassium. HCN4 gene codes for the funny currents and this is found to be expressed more in Sinoatrial node and it is not expressed in the surrounding atrium (Marionneau et al., 2005) showing that it is uniquely expressed in Sinoatrial node. The funny current is also called as pacemaker current. This pacemaker current is activated by hyperpolarisation and deactivated by depolarisation, working continuously like a clock. Hence this theory is called as membrane clock theory.

Calcium clock Theory:

Studies using confocal imaging and immunoflorescence have shown that intracellular calcium cycling (calcium clock) activates the membrane ion channels and produces action potentials. The calcium clock is described as a spontaneous, rhythmic, regular, intracellular local calcium release (LCR) from sarcoplasmic reticulum that appears before the next action potential. This LCR activates sodium calcium exchanger in forward mode to produce inward current and thereby depolarisation (Bogdanov et al., 2001). Thus an intracellular calcium clock that generated LCR initiates the action potential in pacemaker cells and this theory is called as calcium clock theory.

Coupled clock theory:

The coupled clock theory states that in a cardiac pacemaker cell the calcium clock and the membrane clock do not operate individually but work together through many interactions modulated by intracellular calcium, membrane voltage, Protein kinase A and calcium-calmodulin dependent protein phosphorylation (Yaniv and Lakatta, 2013).

Calcium signalling

Calcium is the most important second messenger which is involved throughout the history of a cell. It helps in producing new life by fertilisation, helps in development and differentiation process, involved in all control

mechanisms like secretion, metabolism, learning and memory and it is also involved in cell death. Thus calcium signalling is highly versatile.

Sources of calcium :

Mechanisms of Calcium influx across plasma membrane:

The normal intra-cellular concentration of calcium is about 100 nM/L. Calcium signalling which is necessary for various processes like contraction, secretion, etc. occurs when there is momentary increase in the intracellular concentration (Calcium transient) of up to five fold. This increase in intracellular calcium is through influx of calcium across the plasma membrane or release of calcium from the intracellular stores.

The plasma membrane calcium influx pathways are: L-type calcium channels, T-type calcium channels, P,Q and N-type of calcium channels, Store operated calcium channels (ORAI/STIM1), Sodium calcium exchanger (Reverse mode) and MNDA glutamate receptor (in neurons)

The calcium release pathways from the intracellular stores:

The intracellular calcium stores are mobilizable calcium pools found stored in organelles inside the cell like endoplasmic reticulum and mitochondria. The calcium is released from the endoplasmic reticulum

through two receptors namely, the Ryanodine receptor and the IP_3 receptor. Another calcium release pathway from mitochondria is SCaMPER (sphingolipid Ca2+-release-mediating protein of endoplasmic reticulum) (Mao et al., 1996). The calcium stored in the mitochondria is released through two pathways, one is a sodium dependent - mitochondrial sodium calcium exchanger and another is a sodium-independent calcium efflux pathway (Hoppe, 2010).

Calcium clearing mechanisms

Excess calcium or persistent high levels of calcium inside the cell is toxic. Hence all cells have calcium limiting mechanisms and calcium clearing mechanisms. Example of calcium limiting mechanism is calcium dependent inactivation of voltage gated L-type calcium channels and calcium dependent inactivation of Ryanodine receptors. The calcium clearing mechanisms move calcium either across the plasma membrane or into the intracellular stores. Calcium clearing mechanism across the plasma membrane is through sodium calcium exchanger (NCX) in forward mode and Plasma membrane calcium ATPase (PMCA). Movement of calcium into the endoplasmic reticulum is through Sarco-endoplasmic reticular calcium ATPase (SERCA). Calcium uptake into mitochondria is through mitochondrial calcium uniporter (Hoppe, 2010).

Elementary calcium signals:

The elementary calcium signals are visualised by confocal microscopy and they form the basis for calcium signalling.

Local Calcium events regulate many processes like release of secretory vesicles, activation of ion channels and nuclear calcium signal for gene transcription. Since these local Calcium events are spatially limited and declines rapidly it has advantage compared to global Calcium events. Many elementary calcium release events that are non-propagatory have been seen in many excitable and non-excitable cells (Berridge, 1997; Bootman et al., 2001). The elementary calcium release event representing the calcium release by an individual Ryanodine receptor is called as 'Calcium quarks' and by a cluster of Ryanodine receptors is called 'Calcium sparks'. These Calcium sparks summate spatially and temporally to produce a global raise in calcium transient which is responsible for excitation contraction coupling (Blatter et al., 1997).

IP_3 receptor dependent calcium release event from a single receptor is termed 'Calcium blips'. Elementary calcium release event from a cluster of IP_3 receptor is termed 'Calcium puffs'. These have been observed in non-excitable cells like oocytes (Parker et al., 1996) and vascular endothelial cells (Hüser and Blatter, 1997) . But these Calcium puffs are not seen in cardiac myocytes. This might be because of the lower density of IP_3 receptors

compared to Ryanodine receptor in these cardiac myocytes. In a study, this problem is solved by using a Ryanodine receptor blocker- tetracaine in permeabilised cat atrial myocytes. In these cells after blocking Ryanodine receptors, the Calcium puffs were seen and they were completely abolished by heparin which is an IP_3 receptor blocker (Györke et al., 1997) .

The calcium puffs have significantly slower kinetics compared to the Calcium sparks. Also the calcium puffs have 75-80% smaller amplitude compared to calcium sparks. The calcium puffs are about 3 times long lasting and the rise was about twofold prolonged compared to the calcium sparks. But the spatial orientation and spread of both calcium sparks and calcium puffs were not significantly different (Berridge, 1997).

All these data show that IP_3 receptor mediated calcium release is significantly smaller compared to Ryanodine receptor mediated calcium release in cardiac cells. But still they can have potential role in these cells. The calcium puffs can summate with the calcium sparks to increase the global calcium transient. Also the calcium puffs themselves can activate nearby Ryanodine receptors to produce more calcium sparks thereby amplifying the sarcoplasmic calcium release.

Global Ca signals - Ca waves:

Increase in intracellular calcium can be spontaneous or can be stimulated by any neuro-humoral mechanism. This increase in intracellular calcium can occur at single foci or multiple foci and can lead to an elevation of intracellular calcium propagating throughout the cytoplasm in a wave-like pattern. This is called as calcium waves. These calcium waves are seen in both excitable and non-excitable cells (Lipp and Niggli, 1996) and they play a role in many signal processing like excitation contraction coupling. Even in cells with disrupted plasma membrane, local contractions were reported. This shows the role of calcium waves in contraction (Fabiato and Fabiato, 1975).

Calcium clock theory and Calcium in diastolic depolarisation

Calcium clock theory states that the local calcium release from the sarcoplasmic reticulum activates the sodium calcium exchanger in forward mode. The stoichiometry of the sodium calcium exchanger is 1 calcium:3 sodium. It exchanges one calcium ion out of the cell for 3 sodium ions into the cell (Hinata and Kimura, 2004). Thus it produces a net inward current causing depolarisation of the cell. This increases the slope of the diastolic depolarisation phase resulting in action potential. Thus studies say that calcium release from sarcoplasmic reticulum is responsible for diastolic

depolarisation. The calcium release from sarcoplasmic reticulum can be through either Ryanodine receptor or IP_3 receptor.

Ryanodine receptor

Ryanodine is a plant alkaloid which is also a specific ligand for a large conductance calcium release channel present in the membrane of endoplasmic reticulum. Hence those calcium release channels are named as Ryanodine receptors. The elementary calcium release events caused by release of calcium from single Ryanodine receptor is called 'Calcium quarks" and from a cluster of Ryanodine receptors is called 'Calcium sparks". In skeletal muscles the Ryanodine receptors are activated following the depolarisation mediated activation of the L-type calcium channels. These two channels are co-localised in the T-tubule sarcoplasmic junctions and hence the Ryanodine receptor in skeletal muscle is voltage gated, where the voltage is sensed by the calcium channels. The cardiac Ryanodine receptors are activated by intracellular calcium which enters the cell through the calcium channels in the plasma membrane. This is called as calcium-induced calcium release (Griffiths and MacLeod, 2003).

Ryanodine receptors are tetramers. They have a molecular weight of 2.3 million Daltons. There are 3 subtypes of Ryanodine receptors- RyR1, RyR2, RyR3. The Ryanodine receptor1 (RyR1) is present in skeletal muscle

sarcoplasmic reticulum(Marks et al., 1989). The Ryanodine receptor 2 (RyR2) is present in cardiac muscle sarcoplasmic reticulum and it has 66% sequence identity with RyR1(Nakai et al., 1990). The third isoform of Ryanodine receptor (RyR3) is present in brain. This RyR3 is almost similar to RyR2 (Hakamata et al., 1992). Ryanodine receptor is the major sarcoplasmic calcium release channel present in the cardiac myocytes. Its concentration is 500 – 1000 fmol/mg of protein in ventricular myocytes in various mammalian species (Bers and Stiffel, 1993). This accounts for around 1.5 to 35 million Ryanodine receptors in a 30 pl ventricular myocytes (Bers, 2001).

Functional role of Ryanodine receptor:

Ryanodine receptor releases calcium from the sarcoplasmic reticulum during systole and thus plays a role in excitation contraction coupling (Bers, 2002). Some studies have shown that Ryanodine also releases calcium from the sarcoplasmic reticulum in diastole and thereby play a role in rhythm generation (Maltsev and Lakatta, 2007). But during diastolic depolarisation when the intracellular calcium is low, what causes activation of Ryanodine receptor is not known. Some studies show that the calcium entry through the T-type calcium channels during the diastolic depolarisation activates the Ryanodine receptors (Huser et al., 2000).

IP_3 receptor

Evidence from recent studies have shown the important role of IP_3 mediated Calcium release in cytoplasmic and nuclear calcium signalling. In 1953 a response called 'phosphatidylinositol response' was discovered by Hokin and Hokin and it was widely accepted that binding of some ligands cause breaking of the phosphoinositol present in cell membrane and this is coupled to increased intracellular calcium release (Hokin M R and Hokin L E, 1953).

The IP_3 receptor was discovered by Mikoshiba in 1989. It was identified as a ligand activated calcium channel (Furuichi et al., 1989). IP_3 receptor is ubiquitously present in all organisms and in all types of cells. The IP_3 receptor is found to be localized to the endoplasmic reticulum by immunogold method (Otsu et al., 1990). These IP_3 receptors change their conformation in the presence of calcium. Calcium binding changes the conformation of the IP_3Receptor from a square shaped closed state to a windmill shaped open state (Hamada et al., 2003) and open their pores on binding to IP_3(Chan et al., 2010).

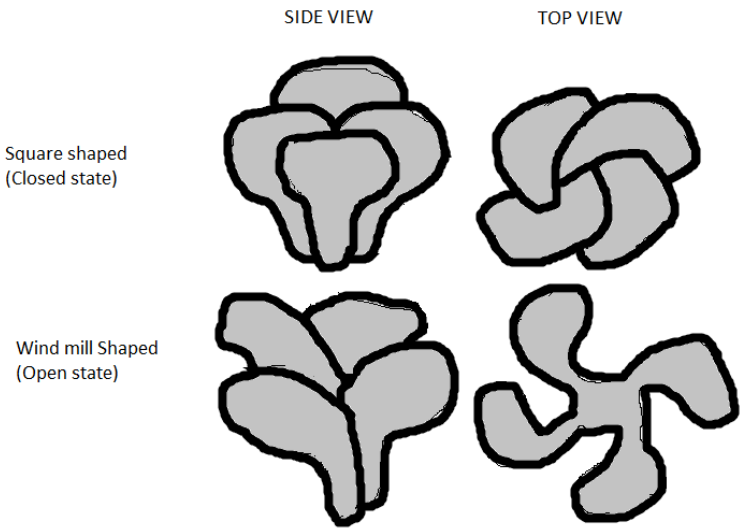


Figure 4: IP_3 receptor conformational change from the closed square shape to the open wind-mill shape by calcium

There are three isoforms of IP_3 receptors (IP_3R) namely IP_3 R1, IP_3R2, IP_3R3(Furuichi et al., 1994)It has been found that type 1 IP_3R are dominant in human atrial cardiomyocytes and in rat purkinje myocytes. The atrial and ventricular myocytes of most of the species express predominantly the Type 2 IP_3R and to a lesser extent they express the type3 IP_3R (Gorza et al., 1993).

The different isoforms of IP_3Receptors differ in their IP_3 binding affinities. IP_3R2 has the highest binding affinity to IP_3, which is followed by IP_3 R1 and then IP_3R3. This difference in binding affinities of the three isoforms to IP_3 is due to the suppressor and ligand binding domain interaction within the receptor (Iwai et al., 2007). They also differ in activation and

inactivation by calcium, phosphorylation and regulation by ATP (Bezprozvanny, 2005).

The IP_3R1 was cloned from rodent and human T cells(Furuichi et al., 1989; Harnick et al., 1995). The second type of IP_3 receptor was cloned from rat cerebellum and it shares 69%sequence identity with IP_3R1(Südhof et al., 1991). The third type of IP_3 receptor was found in pancreatic islets, kidney, gastrointestinal tract and other tissues. They have 62% sequence identity with IP_3R1 and 64% sequence identity with IP_3R2 (Blondel et al., 1993). These isoforms differ from each other by their IP_3 binding affinities. IP_3 R2 has high affinity towards IP_3 binding compared to other isoforms, which is followed by IP_3R1 and then IP_3R3 (Iwai et al., 2007).

The structure of IP_3 receptor is homotetrameric or heterotetrameric. Type 1 IP_3 receptor is a polypeptide made of 2749 amino acids. It has five functionally different regions – the amino terminal IP3 suppressor domain, the IP3 binding domain, the central modulatory domain, the carboxy terminal channel domain and the coupling domain(Bosanac et al., 2005). The IP_3 binding core domain is the region of specific IP_3 binding. The amino terminal domain with 225 amino acid residues is the suppressor region for IP_3 binding and deletion of this region increases the affinity of the receptor to IP_3 binding(Yoshikawa et al., 1996).

The IP_3 binding core domain has two regions namely the α domain and the β domain. In between these two domains is a positively charged pocket where

IP_3 molecule binds. The 11 amino acid residues in the IP_3 binding core domain are responsible for IP_3 binding and all of them except Gly-268 are maintained in all isoforms of IP receptor. Calcium binding sites are identified in two separate locations in IP_3 binding core domain(Bosanac et al., 2002).

In the amino terminus, the first 220 amino acid residues just before the IP_3 binding region plays a major role in suppression of IP_3 binding and in interaction of regulatory proteins. The suppression region has a head sub domain in the form of β-trefoil and an arm sub domain in the form of helix-turn-helix. There is a conserved region in the head sub domain which interacts with the IP_3 binding core domain and also with the binding sites of other cellular signalling proteins like calmodulin and thus it communicates with these proteins (Bosanac et al., 2005).

With the help of biochemical studies and NMR, it has been found that the aromatic residue present in Tyr-167 in IP_3 R1 and Trp-168 in IP_3R3 is responsible for coupling between channel gating and ligand binding (Chan et al., 2010).

A three dimensional electron microscopic structure of the receptor shows a 4-fold symmetry indicating the tetrameric organization of the receptor. Two regions are seen in this three dimensional view – one is the cytoplasmic portion of the receptor which is larger and another smaller

transmembrane portion (Bosanac et al., 2002). All these studies on the structure of IP_3Receptor have been done on IP_3 R1 isoform and IP_3R3 isoform.

Activation of IP_3R:

Ryanodine receptors require intracellular calcium for its activation. Hence they depend on the voltage gated calcium channel activity during every action potential (Bers, 2002).

IP_3Receptors are activated by both IP_3 and calcium (Bootman et al., 1995). It is understood that binding of IP_3R by IP_3 ligand helps in activation of the receptor by calcium ion (Foskett et al., 2007). During systole the IP_3R is bound with IP_3 and it waits for the calcium to bind and activate the receptor. But this IP_3 binding to the receptor not only activates the IP_3Receptor but also increases the sensitivity of the receptor towards calcium. Thus IP_3 bound receptor can be activated even in the presence of minimal level of calcium like diastolic calcium (Gilbert et al., 1991). This activation of IP_3R by diastolic calcium levels can be responsible for the pacemaking effects and pro-arrhythmogenic effects of IP_3R (Ter Keurs and Boyden, 2007).

IP_3Receptor is ubiquitously present in all organisms and in all types of cell. But it is present in a very small number compared to Ryanodine receptors which are also present in endoplasmic reticulum and nuclear envelope and also has the function of calcium release. Microsomal

preparations of hearts of various species showed that Ryanodine Receptor density was 3-4 pmol/mg protein in human ventricle (Nimer et al., 1995) and 5.5-7pmol/mg protein in dog, mouse, rat and rabbit ventricles (Jeyakumar et al., 2001). But the IP_3R densities were 0.09pmol/mg protein in bovine ventricle (Gorza et al., 1993), 0.35 pmol/mg protein in rat ventricular myocytes (P. Lipp et al., 2000) and 0.66 pmol/mg protein in canine ventricular myocytes(Kijima and Fleischer, 1992). This shows the ratio of IP_3R to Ryanodine receptor to be 1:100(Bootman et al., 2009).

The major IP_3 sensitive intracellular site of Calcium store is endoplasmic reticulum. But studies have shown that IP_3 can release calcium from other non-endoplasmic reticular stores like golgi bodies and nuclear envelope (Rizzuto and Pozzan, 2006). In some cells like B lymphocytes, IP_3 receptor is present in the plasma membrane and it gates the calcium influx from the extracellular fluid (Vazquez et al., 2002) .

INOSITOL 1,4,5-TRIPHOSPHATE:

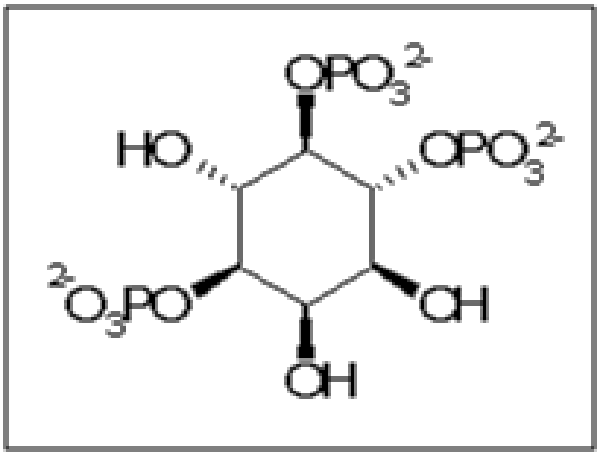


Figure 5: Structure of an IP_3 molecule

IP_3 is produced by the stimulation of plasma membrane receptors which are bound to G proteins – Gq. The stimulation of these receptors activates Gq which in turn activates the membrane bound enzyme called Phospholipase C (PLC). Phospholipase C hydrolyses Phosphatidylinositol 4,5-bisphosphate (PIP2) into IP3 and DAG (Diacyl glycerol). PIP2 is a minor phospholipid present in the plasma membrane. But it is the major form of polyphosphoinositide present in the plasma membrane. The concentration of PIP2 in cardiomyocyte plasma membrane is around 10-30 µM or 150-450 pmol/mg of protein (Nasuhoglu et al., 2002). PIP2 itself acts as an important signalling structure for regulation of ion channels and transporters like Plasma membrane calcium ATPase (PMCA), Sodium calcium exchanger (NCX) and epithelial Sodium channels (ENaC). It also helps in anchoring cytoskeletal proteins with the membrane (Hilgemann et al., 2001). PIP2 is the precursor of PIP3 which is also involved in cell signalling.

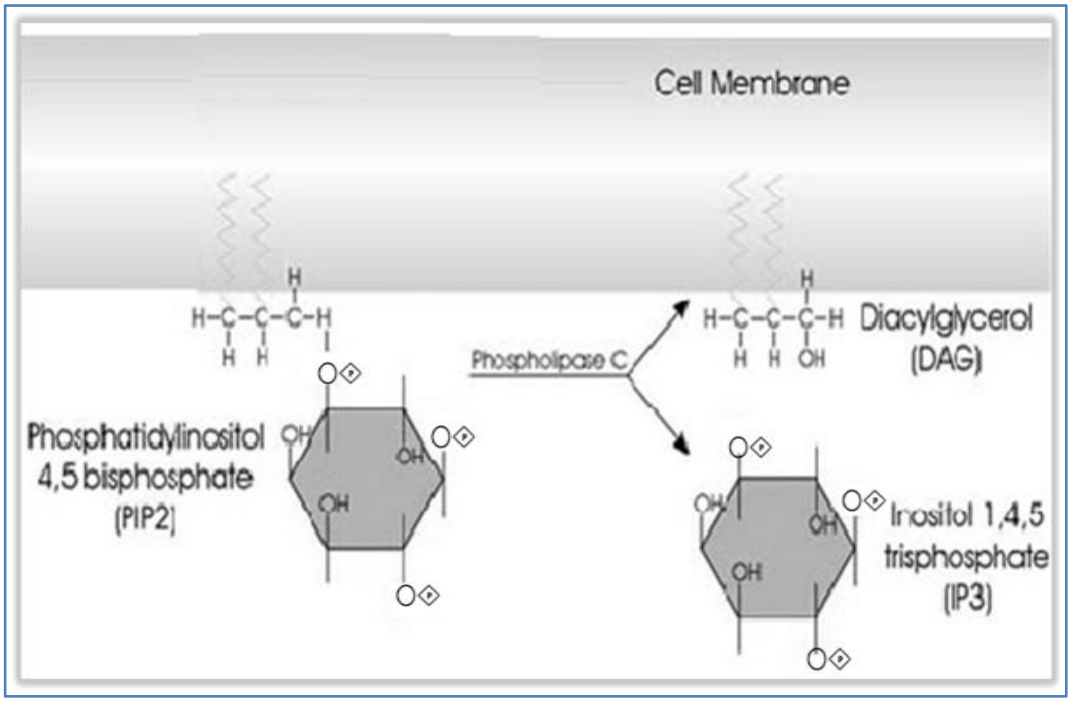


Figure 6 : Mechanism of IP_3 production from PIP2

There are at least 13 different isoforms of PLC which are grouped into 6 subfamilies: β, δ, ε, η, γ and ζ. Of these subfamilies, β, γ, δ and ε are present in cardiomyocytes. PLC can be activated by various factors such as: Ras which activates PLCε, PIP2 and calcium which activates PLCδ, receptor tyrosine kinase which activates PLCγ and heptahelical G protein coupled receptor which activates PLCβ (Rhee, 2001). Thus various factors like hormones, neurotransmitters and stimuli like stretch can act on their receptors and activate PLC and cause an increase in IP_3 concentration. Many studies have been done to study the kinetics of IP_3 turnover. These studies show that stimulation of cardiomyocytes with endothelin causes a rise in IP_3 concentration from around 15nM to 35nM in 400ms. This increase in

IP_3concentration then gradually comes back to baseline within ten minutes (Cooling et al., 2007).

Among the two products produced by the hydrolysis of PIP2, IP_3 is hydrophilic. Hence it enters the cytoplasm and goes and binds to its receptor present in the endoplasmic reticulum. DAG is insoluble, so remains attached to the membrane and activates the enzyme Protein Kinase C (PKC) and is involved in some signal processing.

Involvement in different process in different tissues

- **$IP_3$is involved in antigen specific T lymphocyte activation:**

Cell mediated immunity acts through T lymphocytes. It is a very important defence mechanism of our body against pathogens like virus. The antigen is presented to the T cell by APC (antigen presenting cells). When the T cells come in contact with this antigen it gets activated. Antigen specific activation of T lymphocytes occurs when the T Cell Receptor (TCR) binds with the antigen. This TCR activates tyrosine kinase which in turn activates Phospholipase C (PLC) present in the cell membrane. PLC acts on PIP2 to produce IP_3 and DAG. This IP_3 goes and binds to its receptor present in the endoplasmic reticulum and releases calcium. This increase in intracellular calcium causes transcriptional activation of Interleukin 2 which is a T cell

growth factor(Jayaraman et al., 1995). Thus IP_3 is involved in activation of T cell and hence in immunity.

- **IP3 is involved in apoptosis caused by various stimuli:**

Apoptosis is programmed cell death. The thymocytes undergo apoptosis by glucocorticoids, irradiation, antibodies to CD3 and removal of certain growth factors. All these factors cause increase in calcium concentration and this increase in calcium activates endonuclease which cleaves the DNA fragments causing apoptosis. Studies have shown that thapsigargine which inhibits SERCA (sarco endoplasmic reticulum calcium ATPase) can initiate apoptosis(Jiang et al., 1994) and chelation of intracellular calcium can prevent apoptosis(Story et al., 1992). These results confirm the role of intracellular calcium in apoptosis. The T cells lacking IP_3R1 were resistant to apoptosis (Jayaraman and Marks, 1997). This shows that IP_3 induced increase in intracellular calcium is essential for apoptosis.

- **IP3 and SOC:**

When IP3 induced calcium release depletes the store, this depletion of store opens up a channel in the plasma membrane to allow influx

of calcium and refill the stores. This process was first proposed by Putney in 1986 and later he named it as Store operated Calcium Entry (SOCE)(Putney, 1990, 1986). The current through this SOC was first best described in mast cells by Hoth and Penner as a highly calcium selective, inward rectifying current and not voltage selective (Hoth and Penner, 1992). They named it as Calcium Release Activated Calcium Current (CRAC). Ever since many SOC currents which are less calcium selective and even completely non-selective calcium entry have been described in many other cells. Studies have found that this CRAC current has two components. One is through the molecular structure of SOC: Orai1 present in the plasma membrane together with the calcium sensor STIM1 present in endoplasmic reticulum. Another feature of CRAC channel is the conformational coupling between the IP_3 receptor present in the endoplasmic reticulum and the TRPC1 channel present in the plasma membrane. This conformational coupling is similar to the one seen between voltage gated calcium channels and Ryanodine receptors seen in skeletal muscles.

- **IP_3 in cardiovascular system:**

IP_3 mediated calcium release from the endoplasmic reticulum plays a major role in pharmaco-mechanical coupling in the smooth muscles of the blood vessels. Thus they are important in maintaining the vessel tone and thereby the blood pressure.

- **IP_3 in heart**

All three isoforms of IP_3 receptors are found in isolated atrial and ventricular cardiomyocytes. Among these three isoforms, Type II isoform is most predominant in cardiomyocytes (P Lipp et al., 2000). The atrial cardiomyocytes show a six fold higher expression of IP_3R compared to the ventricular cardiomyocytes (P Lipp et al., 2000).

IP_3 involvement in other effects in heart:

- **Role of IP_3 in excitation transcription coupling in atrial and ventricular myocytes:**

Nuclear calcium dynamics is different from the cytoplasmic calcium dynamics. This is because of the presence of a nuclear envelope present around the nucleus. This nuclear envelope has an inner nuclear membrane and an outer nuclear membrane with a space between them. This nuclear envelope is contiguous with the sarco-endoplasmic reticulum. The double layered nuclear envelope fuses at many places to form pores. These pores have structures called nuclear pore complex (NPC). These pores acts as a portal for the movement of ions like calcium between the cytoplasm and nucleoplasm (Wu et al., 2006). Similar to sarco-endoplasmic reticulum, the nuclear envelope also has many calcium handling and ion transporting proteins like the Ryanodine receptor type 1, Sodium calcium exchanger,

sodium potassium ATPase and sodium proton exchanger. The calcium pump SERCA is not present in the nuclear envelope (Wu et al., 2006; Zima and Blatter, 2004). Studies have shown the presence of IP_3R in the nuclear envelope. Also studies have shown a depletion of nuclear calcium on stimulation of these IP_3 receptors in the nuclear envelope (Wu et al., 2006). The change in the local nuclear calcium kinetics can activate many transcription factors. Thus IP_3 is found to be responsible for the regulation of nuclear calcium released from peri-nuclear stores in both atrial and ventricular myocytes and therefore responsible for regulation of gene transcription (Kockskämper et al., 2008a). This regulation of gene transcription by nuclear calcium is called as excitation-transcription coupling (Wu et al., 2006).

- **Role of IP_3 in ECC in atrial and ventricular myocytes**:

In cardiac myocytes ECC is a process of action potential mediated activation of voltage gated calcium channels present in the plasma membrane, which in turn activates calcium induced calcium release (CICR) from the sarcoplasmic reticulum. This increases the cytosolic calcium and causes muscle contraction (Bers, 2001). IP_3 receptors are present in both atrial and ventricular cardiomyocytes. Atrial myocytes express more density of IP_3R compared to the ventricular receptors (Zima and Blatter, 2004). The

predominant form of IP_3R expressed in cardiomyocytes is type 2 IP_3R. Their channel activity is dependent on intracellular IP_3 concentration and intracellular calcium concentration (Michell et al., 1981). Many studies have shown the role of intracellular IP_3 in modulating the cytosolic calcium transients in both atrial and ventricular cardiomyocytes. IP_3 produces increase in intracellular calcium by releasing calcium from the endoplasmic reticulum. But the quantum of calcium release by IP_3 R is very small compared to Ryanodine receptor and also the number of IP_3R is less compared to that of Ryanodine receptor (Bootman et al., 2009). The role of Ryanodine receptor in the ECC is a well established fact. But the role of IP_3R in ECC has been a controversy. But studies have shown that IP_3R has a role in ECC. In permeabilised rat ventricular myocytes, application of IP_3 increased the frequency of calcium sparks to 21% suggesting the role of IP_3 in positive inotropic effect in heart (Zima and Blatter, 2004).

The IP_3R are present co-localised with the Ryanodine receptors in the sub-sarcolemmal space in the cardiomyocytes (P Lipp et al., 2000). Activation of IP_3 R by membrane permeable IP_3 ester showed seven fold increase in number of spontaneous calcium sparks in rat cardiomyocytes. Thus the small eventless calcium release from IP_3R can activate the Ryanodine receptor and can modulate the excitation contraction coupling in heart (P. Lipp et al., 2000).

Other studies have shown that endothelin is strongly cardiotonic and it acts by stimulating the production of IP_3 and mobilising the intracellular stores(Vigne et al., 1989). Also Histamine acts through the H1 receptor and exerts a positive inotropic effect. This was studied in guinea pig left atrium and it was found that the positive inotropic effect was through increase in IP_3 production (Sakuma et al., 1988).

- **IP_3 receptor in developing cardiomyocytes:**

One of the first organs found to develop in an embryo is the heart. As heart develops, the embryo acquire the ability to produce spontaneous rhythmic calcium signals and to contract. Many studies done in either prenatal cells or ESdCs (Embryonic Stem cell derived Cardiomyocytes) have shown that IP3 receptors have a significant role in the pacemaking activity and thereby cardiogenesis. The spontaneous calcium oscillations start as soon as the heart tube is formed. This drives contraction, necessary gene transcription and regulation of structural arrangement of the cardiomyocytes (Fu et al., 2006). Hence inhibition of these calcium oscillations can prevent the development of heart (Pucéat and Jaconi, 2005).

Type 2 Ryanodine receptor is responsible for the calcium oscillations seen in the adult cardiomyocytes and neonatal cardiomyocytes. Studies have shown that at day 10 of embryonic life (E10) mice deficient of

type 2 Ryanodine receptor die with cardiac defects because of immature excitation contraction coupling (Takeshima et al., 1998). However some calcium oscillations are seen in this Ryanodine receptor deficient mice as early as E 5-9.5 (Takeshima et al., 1998)and these oscillations were insensitive to inhibition of voltage gated calcium channels or NCX (sodium calcium exchanger), but were affected by depletion of internal stored calcium (Sasse et al., 2007). Thus indicating that in embryonic cardiomyocytes there is periodic release of calcium from sarcoplasmic reticulum which is not through Ryanodine receptor.

Many studies show that IP_3 receptors have a role in these calcium oscillations seen in early embryonic stage of cardiomyocytes. For example, a study has shown that the calcium oscillations seen in rat embryonic cells were sensitive to 2-APB (2-aminoethoxy diphenyl borate) which is an IP_3 receptor blocker (Sasse et al., 2007). Another study has shown that IP_3 receptors are expressed in early embryonic cells as early as day 5 in contrast to Ryanodine receptor which is not expressed until day 8.5 or later (Rosemblit et al., 1999). These data show that IP_3 receptors are much earlier expressed than the Ryanodine receptors in embryonic cardiomyocytes and their timing of expression correlates with the onset of periodic calcium oscillations seen in these cells.

Embryonic stem cell derived cardiomyocytes are used to study the cardiac development because of its ease of availability and genetic manipulation compared to the pre-natal cells (Banach et al., 2003). Studies using embryonic stem cell derived cardiomyocytes have shown that in these cells IP_3 receptors are expressed during proliferating stage and during differentiation stage (Kolossov et al., 1998). Also these cells start showing spontaneous rhythmic calcium oscillations and contractions even before they are terminally differentiated. Usually the mechanism for spontaneous calcium oscillations in adult cell is the HCN (hyperpolarisation activated cyclic nucleotide gated channel) channel mediated current (Yasui et al., 2001). However the spontaneous calcium oscillations are seen even earlier than the appearance of HCN mediated current and they are not affected by I_f inhibitors (Méry et al., 2005). Also these calcium oscillations are not affected by complete depolarisation, implying that membrane potential oscillations are not responsible for these spontaneous calcium oscillations (Viatchenko-Karpinski et al., 1999). But when these embryonic stem cell derived cardiomyocytes were transfected with antisense DNA against IP_3 receptor type 1 or when IP_3 receptor antagonist were applied there was significant reduction of the spontaneous calcium oscillations and contractions (Mery et al., 2005).Stimulation of IP_3 production had positive chronotropic effect in these embryonic stem cell derived cardiomyocytes (Kapur and Banach, 2007).

But these effects diminished as the cells differentiated suggesting that as these cells differentiated, the calcium handling is regulated by other mechanisms also (Fu et al., 2006). But IP_3 receptor is not completely lost. They are found in neo natal rats cardiomyocytes (Luo et al., 2008) and they are expressed till adult cells.

- **Role of IP_3 in pathological conditions of heart:**
  - **Role of IP_3 in arrhythmia:**

Studies have shown the role of IP_3R in arrhythmia. Atrial fibrillation is the most common arrhythmia found in clinical settings. The main mechanism behind arrhythmia is abnormal cytosolic calcium oscillations. The intracellular calcium oscillation is maintained by two important receptors present in the endoplasmic reticulum. They are Ryanodine receptors and IP_3R. The incidence of atrial fibrillation increases with age and also the expression of IP_3 receptors increases with age in heart (Cao et al., 2002). Studies have shown that there is increased expression of IP_3R in the atrial tissues of patients with chronic atrial fibrillation (Yamada et al., 2001). Thus up-regulated IP_3R can modulate intracellular calcium homeostasis and produce a delayed after depolarization leading to arrhythmia in heart (Zima and Blatter, 2004). Also studies have shown that IP_3 deficient rats are protected from pro-arrhythmogenic stress (Li et al., 2005). Also the levels of

endothelin and angiotensin II are also increased in cardiac tissues with atrial fibrillation (Zerkowski et al., 1993). Both these are known to act by increasing IP_3 levels and produce arrhythmia in isolated human atrial tissues. Thus angiotensin II and endothelin mediated increase in IP_3 levels may be responsible for atrial fibrillation. Several studies have shown that ACE inhibitors and AT_1 receptor blockers can reduce the incidence of developing atrial fibrillation in patients at risk (Ehrlich et al., 2006).

- **Arrhythmia related to Ankyrin-B mutation:**

Ankyrin-B is an adaptor protein molecule with molecular weight of 220 kDa. It is responsible for the post translational stability and also for targeting the IP_3 receptor to the sarcoplasmic reticulum near the junction of T-tubules (Mohler et al., 2004a). Mutation leading to lack of Ankyrin-B causes decrease in IP_3 receptor in the sarcoplasmic reticulum- T-tubule junctions and are associated with arrhythmias (Mohler et al., 2004b). When IP_3 receptors are absent as seen in mutation of Ankyrin-B, there is increase in sarcoplasmic reticulum calcium load. This causes activation of Ryanodine receptors and produce arrhythmia (Mohler et al., 2003). Thus both activation of IP_3 receptor and loss-of-function mutation of IP_3 receptor is responsible for the development of arrhythmia.

- **IP_3 is also found to be responsible for the hypertrophy of cardiomyocytes**.

  Endothelin is an endothelium derived vaso-constricting agent. It is produced by aortic endothelial cells and acts on cardiac myocytes. This endothelin is found to increase MLC2,alpha-actin and troponin I which are the muscle specific genes involved in hypertrophy of cultured neonatal rat cardiomyocytes(Ito et al., 1991) and this endothelin is found to act through IP_3(Ito et al., 1991; Kockskämper et al., 2008b)

- **Reperfusion arrhythmia**:

  Reperfusion of heart after a period of ischemia produces arrhythmias and even sudden cardiac death. During reperfusion followed by ischemia there is increase in IP_3 concentration in the cardiac myocytes (Jacobsen et al., 1996). This is because of increased release of norepinephrine (NE) from the sympathetic nerves within the heart which stimulate a lot of α-adrenergic receptors and produce increased concentration of IP_3. This increased concentration of IP_3 is responsible for arrhythmia, ventricular tachycardia and even ventricular fibrillation (Woodcock et al., 2000). Studies have shown that agents that prevent IP_3 generation can prevent these reperfusion arrhythmias (Du et al., 1995).

- **Diabetic cardiomyopathy:**

  Altered calcium homeostasis is seen in diabetic cardiomyocytes. The calcium transients are reduced and prolonged in these cells. This is because of the changes seen in calcium handling proteins in diabetic cardiomyopathy like: reduced expression of SERCA, Ryanodine receptors and sodium calcium exchangers, increased expression of phospholamban and IP_3 receptors. As a result the sarcoplasmic calcium release and reuptake is altered (Choi et al., 2002). Studies have shown that Insulin increases IP_3 concentration and triggers arrhythmia in diabetic mice (Fauconnier et al., 2005). Also the levels of PIP2 and activity of PLC enzyme are reduced (Tappia et al., 2004). Taken together, altered IP_3 generation and IP_3-induced sarcoplasmic calcium release contributes to the arrhythmias seen in diabetic cardiomyopathy.

- **Heart Failure**:

  Heart failure is associated with activation of sympathetic nervous system, endothelin system and rennin-angiotensin system. The levels of noeepinephrine, Angiotensin II and Endothelin are elevated in cardiac tissue in heart failure and also their receptors expression is also altered. Expression of α_1-adrenergic receptor is not changed but there is decreased expression of β_1-adrenergic receptors leading to a relative increase in α_1-adrenergic receptor (Bristow et al., 1988). The angiotensin

receptor- AT_1 is down regulated (Regitz-Zagrosek et al., 1998) and the endothelin receptor-ET_A is up-regulated (Pieske et al., 1999; Zolk et al., 1999). Also there is increased activation of PLC enzyme and PIP2 metabolism and therefore increased IP3 concentration in heart failure. Also the IP_3 receptor expression is increased in both human and animal model of heart failure (Tappia et al., 1999; Ziegelhoffer et al., 2001). The Ryanodine receptor expression is decreased (Ai et al., 2005). Thus all these data suggest that IP_3mediated calcium release is responsible for the development and progress of the disease in heart failure.

- **IP3 in Chronotropy**

The heart beat is initiated in a specialised group of cells called pacemaker cells. These pacemaker cells in adult heart are found to have high expression of pacemaker channels known as HCN (hyperpolarisation activated cyclic nucleotide gated channels) channels(Accili et al., 2002). The cell contractions and rhythmic calcium oscillations are seen as early as 7th day of embryonic life(Porter and Rivkees, 2001). But in this early embryonic stage there is less expression of HCN channels(Stieber et al., 2003). Studies have shown that in these early stages of embryonic life, the calcium oscillations and the cell contractions are through rhythmic shunting of calcium in and out of the endoplasmic reticulum. This was confirmed by studies where calreticulin

decreased pacemaking by buffering the endoplasmic reticular calcium leading to decreased release of calcium from endoplasmic reticulum (Méry et al., 2005). The calcium oscillations from endoplasmic reticulum was mediated through IP_3R, as addition of IP_3 antibody or Xestospongin C (an IP_3 receptor blocker) also affects the pacemaking activity in these early embryonic cells(Méry et al., 2005). This IP_3 mediated rhythmic calcium oscillations from endoplasmic reticulum is essential for pacemaker activity in early cardiogenesis and foetal life.

A study in neonatal rat cardiomyocytes isolated from hearts of 2 to 5 day old rats, showed an IP_3 mediated increase in chronotropy by the action of Stromal cell derived factor-1α (SDF-1α) which is a chemokine acting on CXCR4 receptor. This receptor is a G protein coupled receptor, hence activates PLC and increases the production of IP_3. This increase in chronotropy was shown as increase in cell beating frequency. The increase in cell beating frequency and increase in calcium transient measured by calcium imaging by SDF-1α were sensitive to 2-APB which is an IP_3 antagonist. Thus showing that IP_3 has a positive chronotropic effect in neonatal rat cardiomyocytes. (Hadad et al., 2013)

The role of IP_3 in chronotropy of heart has been shown in a study on embryonic stem cell derived cardiomyocytes (ESdCs) which are used in cardiac replacement therapy. Spontaneous calcium transients were found in the day 9 cells at the onset of beating and these were dependent on L type

calcium channels. But the calcium transients found on day 16 were dependent on Calcium induced calcium release (CICR). Similarly calcium extrusions in day 9 cells were predominantly by sodium calcium exchanger (NCX) and in day 16 cells were mostly by SERCA. Also the calcium transients were suppressed by IP_3 receptor blocker and by PLC inhibitor, but not with caffeine. This shows that the calcium transients responsible for the beating of cells were IP_3 dependent. Thus the study shows that there is developmental change in calcium handling mechanisms and IP_3R has a role in chronotropy of heart in ESdCs (Kapur and Banach, 2007).

Tools for studying cardiomyocytes IP_3 signalling:

Many tools have been used to study the IP_3 signalling in cardiomyocytes. They are: a) by measuring intracellular IP_3 levels b) by pharmacological manipulation of IP_3 generation, degradation of IP_3 receptors and c) by genetic manipulation of the IP_3 cascade and producing a transgenic animal model.

a) The method of studying IP_3 signalling by measuring IP_3 levels has been of great interest for many years. It is done by analysing the whole cell extracts by various methods like mass spectrometry, gas chromatography or ion exchange chromatography (Dean and Beaven, 1989). These methods cannot be used to study the spatio-temporal organisation of the IP_3

signalling system inside the cell. New florescent probes are also used to study the IP_3 dynamics. Example for this type of fluorescent probe is 'LIBRA' – a biosensor derived from IP_3 binding site of type-3 IP_3 receptor (Tanimura et al., 2004). A recent study shows the use of a new biosensor which can measure intracellular IP_3 with temporal and spatial resolution. These sensors are called FIRE-1 and FIRE-3 (Fluorescent IP3 responsible Element Type 1 and 3). These biosensors are promising tools for studying IP_3 dynamics with high spatio-temporal resolution in a living cell, but it can buffer some IP_3 which has to be taken into account (Remus et al., 2006).

b) Pharmacological manipulation of IP_3 signalling cascade is a more conventionally used method to study IP_3 signalling. The commonly used pharmacological agents are blockers of IP_3 formation and they act by blocking PLC (Neomycin, the PLC inhibitor U73122), IP_3 receptor blocker or antagonist (Heparin, 2-aminoethoxy diphenyl borate [2-APB],xestospongins, curcumin). Like all other pharmacological agents these also lack specificity. They have other intercellular targets and many other unwanted side effects. Some lack membrane permeability (Taylor and Broad, 1998). Recently there is development of the membrane permeable form of IP_3 agonist (Thomas et al., 2000). Also the caged IP_3

released on photolysis has become a very good tool in studying IP_3 dynamics (Li et al., 1998).

c) The other method of studying cardiac IP_3 function is the molecular technique. In this method the IP_3 signalling cascade is altered genetically and transgenic animals are developed. Example for this method of study is using an adenoviral mediated IP_3 affinity trap which is also known as an IP_3 sponge (Domeier et al., 2008). It has been used to abolish all the IP_3 mediated response similar to pharmacological IP_3R blocker. Another study gives an even better tool than the IP_3 sponge. The study shows the usage of IP_3 metabolising enzyme called IP35'phosphatase. This has an advantage that it need not be present in 1:1 stoichiometry to completely abolish IP_3 activity. A recent study show that the endothelin mediated positive inotropic and pro-arrhythmic effects were completely absent in transgenic mouse atrial myocytes lacking Type 2 IP_3R. This effect of endothelin was specific to IP_3R because a normal inotropic response to β- adrenergic stimulation was seen in these transgenic mouse cells (Li et al., 2005).

- **Phenylephrine:**

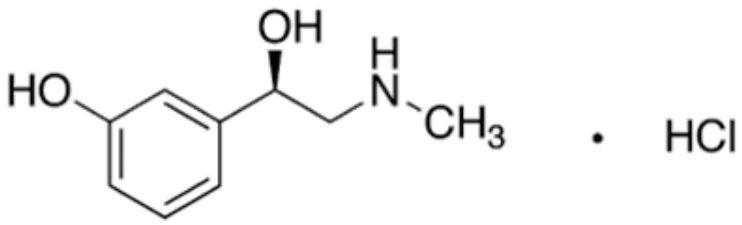


Figure 7: Structure of a Phenylephrine molecule

Phenylephrine is a selective α_1 adrenergic receptor agonist. The α_1 adrenergic receptor is a G-protein coupled receptor which is associated with the Gq type of G-protein. On activation of this receptor Gq gets activated which in turn activates Phospholipase C (PLC) which is a membrane bound enzyme. This activated PLC hydrolysis PIP2 to IP_3 and DAG and further activates protein kinase C (PKC). Thus α_1 adrenergic receptor acts by increasing the concentration of IP_3 and activating PKC. α_1 adrenergic receptors are abundantly found in vascular smooth muscle. They are also found in other sites like gastrointestinal and urinary sphincters, dilator muscle of iris and arrectorpili of hair follicles. Hence Phenylephrine which is an agonist of this α_1 adrenergic receptor produces vasoconstriction, dilatation of pupil and increased tone of urinary and gastrointestinal sphincters.

- **Phenylephrine in heart:**

It is known that catecholamine exert their effect on heart through beta adrenergic receptors and cause a positive inotropic effect. But heart also has alpha adrenergic receptors and studies have shown that alpha adrenergic agonist also cause increase in force of contraction in heart (Scholz et al., 1986). The mechanism of action of alpha adrenergic agonist -Phenylephrine was studied in left auricular tissues of rat heart and it was found to have a positive inotropic effect by increasing the concentration of IP_3(Scholz et al., 1988).

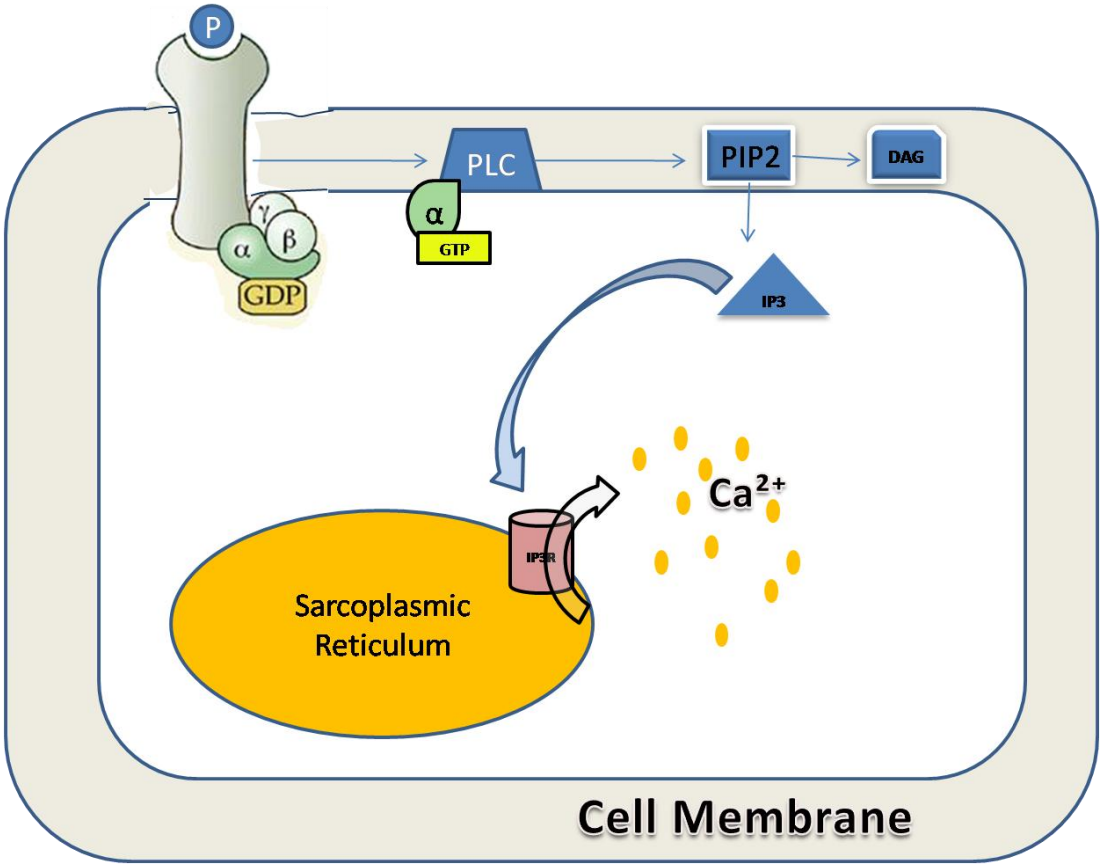


Figure 8: Mechanism of action of Phenylephrine. (P-Phenylephrine; PLC-Phospholipase C; PIP2- Phosphoinositol bisphosphate; DAG- Diacyl glycerol; IP3- Inositol 1,4,5-triphosphate; IP3R- IP3 receptor)

Neomycin

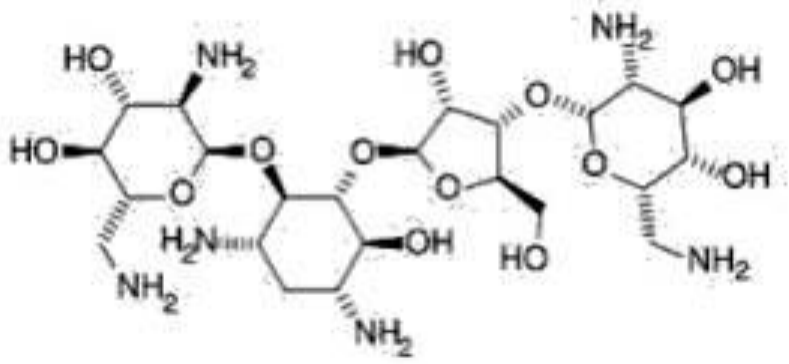


Figure 9 : Structure of a Neomycin molecule

Neomycin is an aminoglycoside antibiotic. It was discovered in 1949 by an American microbiologist Selman Waksman and his student Hubert Lechevalier. It is produced naturally from an actinobacteria, *Streptomyces fradiae.* Neomycin is active against both gram positive and gram negative bacteria. It binds with the 30s ribosome of the organism and interferes with mRNA binding and produces non-functional or toxic proteins which destroys the organism. It also blocks the action of Phospholipase C and thus decreases production of IP_3 and affects intracellular calcium signalling (Walz et al., 2000). Neomycin also non-selectively inhibits the L,N and Q type of calcium channels and inhibits the neuronal response mediated by these channels (Keith et al., 1992) and causes depression of neuromuscular functions (Pittinger and Adamson, 1972). Neomycin used as antibiotic has side effect of ototoxicity and nephrotoxicity. These toxicities are due to the binding of this antibiotic to

membrane phospholipids and inhibition of polyphosphoinositide turnover (Lodhi et al., 1979; Schacht, 1976)

- **Neomycin and IP3**

Neomycin is a polyvalent cation. It gets attached to the phosphatidyl inositol present in the cell membrane (Gabev et al., 1989). This attachment of Neomycin to phosphatidylinositol prevents the action of Phospholipase C (PLC). So PLC cannot produce IP_3 and DAG from PIP2, leading to decrease in IP_3 production(Marche et al., 1987). Thus Neomycin acts as an IP_3antagonist and is used in experiments to block the effect of IP_3.

Rat experiments:

Animal research has played a most important role in every medical breakthrough. Nearly every scientist who got Nobel Prize since 1901 for Physiology and Medicine has relied on animal experiments for their research. Animals are very similar to human beings in organ system and its function. Animals suffer from diseases like TB, asthma, flu etc which are similar to those in humans. Also many medicines like antibiotics and painkillers are the same for animals and human beings. Hence it is very useful to use animals for experiments and research. Human share around 95% of their gene with a mouse, thus making mouse a very effective model for human

body and disease condition. Hence more than 95% of animal experiments are done on rats and mouse. Other animals like dogs, cats, primates, birds, fish and some invertebrates are also used for experiments.

Rats are the most commonly used animal because of their availability, small size, ease of handling, low cost and fast reproduction rate. Developing genetic models and knockout model in rat is difficult compared to mouse. But rats are good models for drug testing.

Isolated heart study

The perfused isolated hearts from animals are extensively used for metabolic and hemodynamic studies. It is a predominant *in vitro* technique used in physiological and pharmacological research. The isolated perfused heart studies are useful in studying the intrinsic properties of heart like contractility, automaticity and conductivity without the neuro-humoral interference seen in an intact animal.

- **Langendorff setup**

The technique for studying isolated heart was first described by Oscar Langendorff in 1985(Langendorff, 1895). This technique of Langendorff has been modified and widely used for isolated heart studies ever

since. It is usefully to study the isolated heart of homeothermic animals with coronary arteries. Homeothermic animals are also called as warm blooded animals in which the internal body temperature is maintained constant regardless of the external temperature. In animals like frog where there is no coronary perfusion this setup is not required. The hearts of frog and fishes take oxygen and nutrients by diffusion through the numerous pores present in their cardiac musculature. The principle of this Langendorff setup is to perfuse the heart with a solution containing all substrates, nutrients and oxygen through a cannula inserted into the ascending aorta. The pressure of the flow of perfusate will keep the aortic valves closed and therefore the perfusate will flow through the open coronary ostium present in the base of aorta and will enter into the coronary system. This technique has been used in basic cardiovascular research for studying the basic properties of heart like inotropy and chronotropy, for obtaining individual cardiomyocytes by enzymatic digestion and for testing various drugs on isolated heart.

This study aims at finding the role of calcium release through IP_3 from the sarcoplasmic reticulum during the diastolic depolarisation and thus the effect of IP_3on heart rate. For this isolated perfused hearts of Wistar rats have been used in Langendorff mode and heart rate was calculated from the ECG recorded using surface electrodes. Phenylephrine and Neomycin were used to alter the IP_3 dynamics.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES:

Aim:

To study the role of Inositol 1,4,5-triphosphate (IP_3) in rhythm generation in isolated Wistar rat hearts.

Objectives:

1) To study the changes in heart rate in an isolated rat heart perfused with normal extracellular solution on addition of Phenylephrine (IP_3 agonist) and
2) To test whether those changes in heart rate by Phenylephrine can be blocked by Neomycin, an IP_3 antagonist.

MATERIALS AND METHODS

MATERIALS AND METHODS:

MATERIALS AND SOLUTIONS USED:

1. Langendorff perfusion setup
2. *MasterFlex* Peristaltic Pump
3. *EQUIBATH* Circulating Water Bath
4. Oxygen Supply
5. CMCdaq Data Acquisition Device
6. Perfusing Solution – Normal Extracellular Solution
7. Drugs - Ketamine
    - Phenylephrine
    - Neomycin

SOLUTION PREPARATION:

Normal Extracellular solution was used to perfuse the isolated heart in the Langendorff setup. This Normal Extracellular solution had all the electrolytes, glucose and pH similar to the extracellular fluid.

For the preparation of solution, the amount of salts needed to be added in one litre of Normal Extracellular Solution was calculated and weighed accurately using an electronic balance (Precisa XB 320M). This was added to one litre of distilled water and thoroughly mixed and dissolved using a magnetic stirrer. The solution was prepared freshly and used for all the experiments.

After all the salts were added and mixed thoroughly using the magnetic stirrer, the pH of the solution was checked using an electronic pH meter(METTLER TOLEDO MP220 pH meter). First the pH meter was calibrated using known pH solutions of 7.0 and 9.2. Then the pH of the solution was checked and was adjusted to 7.4 using 1 molar NaOH solution.

COMPOSITION OF NORMAL EXTRACELLULAR SOLUTION:

Salts	Concentration (mmol/L)
NaCl	135
KCl	5.0
NaH_2PO_4	0.4
$MgCl_2$	2
$CaCl_2$	1
HEPES	5
Glucose	10
pH	7.40
Osmolarity	307

STOCK SOLUTIONS OF DRUGS:

The stock solutions of each of the drugs used were prepared and stored. The stock solutions were prepared with thousand times concentration than the required concentration in the experiment.

Phenylephrine (MW-203.67) stock solution was prepared by mixing 20.367 mg of salt in 10ml of distilled water. This makes a stock of 10mM. This was stored in the freezer at -20 degree Celsius. From this stock solution 150µl was taken and added to 150ml of Normal extracellular solution during the experiment. This will dilute it thousand times to give a required concentration of 10µM in the experiment.

Neomycin (MW-908.88) stock was prepared by mixing 18.17 g of salt in 10ml of distilled water. This 2M stock was prepared and stored in refrigerator at 4 degree Celsius until use to maintain its efficiency. From this stock solution 150µl was added to 150ml of Normal extracellular solution during the experiment to make it to a concentration of 2 mM.

The salts used in the preparation of Normal extracellular solution were purchased from the following companies:

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

Drugs:

1. Phenylephrine : Sigma-Aldrich
2. Neomycin : Sigma-Aldrich

All these salts and drugs were stored at appropriate conditions as mentioned in their data sheets and they were used within their expiry date.

LANGENDORFF SETUP:

The technique for studying isolated heart was first described by Oscar Langendorff in 1985 (Langendorff, 1895). This technique of Langendorff has been modified and widely used for isolated heart studies ever since. It is useful to study the isolated heart of homeothermic animals with coronary arteries. In animals like frog where there is no coronary perfusion this setup is not required. The principle of this Langendorff setup is to perfuse the heart with a solution containing all substrates, nutrients and oxygen through a cannula inserted into the ascending aorta. The pressure of the flow of perfusate will keep the aortic valves closed and therefore the perfusate will flow through the open coronary ostium present in the base of aorta and will enter into the coronary system.

This technique has been used in basic cardiovascular research for studying the basic properties of heart like inotropy and chronotropy, for obtaining individual cardiomyocytes by enzymatic digestion and for testing various drugs on isolated heart.

The Langendorff setup used in this study included two graduated reservoirs and one bubble trap for continuous perfusion. All these reservoirs and the bubble trap were maintained at constant temperature of 37 degree Celsius using a water bath and the perfusion solution was oxygenated continuously.

Figure 10 : Langendorff Perfusion Setup

PERFUSION

The perfusion of the solution was done at a constant rate of 10ml per minute using a *Master Flex* peristaltic pump. This rate of perfusion gives a perfusion pressure of 60mm of Hg which is needed to perfuse the coronaries.

Figure 11 : Peristaltic Pump

OXYGENATION:

The perfusion solution was continuously oxygenated with 100% oxygen from an oxygen cylinder connected to the reservoir. The oxygenation was at a high flow rate and it was maintained throughout the experiment.

TEMPERATURE MAINTENANCE:

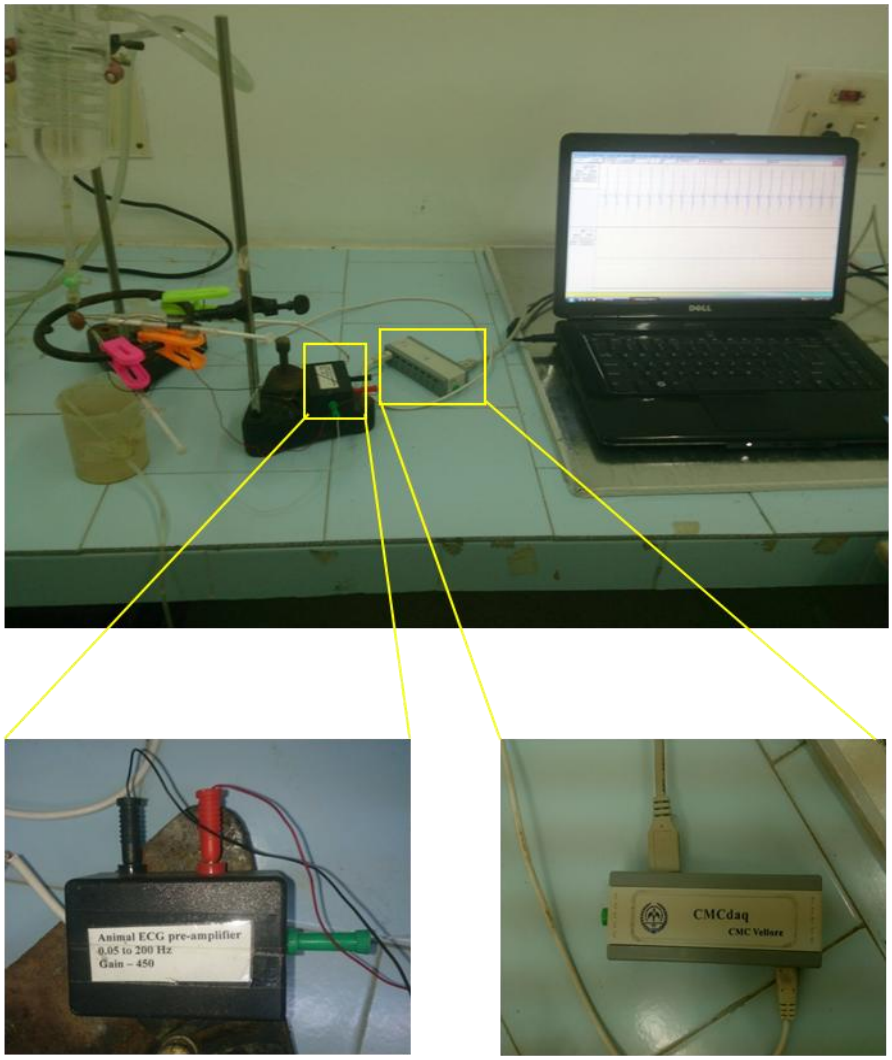
The perfusing solution was maintained and perfused at constant temperature of 37 degree Celsius using a circulating water bath. This water bath circulates water of 37 degree Celsius through the outer compartment of the reservoirs and the bubble trap and thus maintains the temperature of the perfusion solution throughout the experiment.

RECORDING HEART RATE:

The heart was mounted in the Langendorff setup and perfused with Normal Extracellular solution and allowed to beat on its own pace.

Surface electrocardiogram was recorded using three electrodes touching the surface of the heart connected to CMCdaq ECG amplifier (designed by the Department of Bioengineering, Christian Medical College, Vellore). The negative electrode was placed on the right atrium and the positive electrode was placed on the left ventricle. The third electrode was used as ground electrode to cancel noise in the recording. This placement of

electrodes was similar to Lead II ECG placement. The electrodes were connected to CMCdaq ECG amplifier which in turn was connected to the computer. The ECG tracings were recorded using CMCdaq software in the computer. The heart rate was calculated from the surface electrocardiogram tracings obtained.



ECG preamplifier **CMCdaq**

Figure 12: Recording the Surface ECG from the isolated rat heart using ECG preamplifier and CMCdaq

EXPERIMENT PROTOCOL:

Ethical clearance for the use of rats and for the protocol used in this study was obtained from the Institutional Animal Ethics Committee. (IAEC N0: 16/2013). The procedures were performed under anaesthesia and all efforts were made to minimize suffering.

Wistar rats weighing between 200grams to 300 grams were used in this study. Both male and female rats were used. These rats were housed and maintained in the animal house till the experiments were conducted. A total of 18 rats were used for the study and 6 rats were used for standardization.

Figure 13: Wistar Rat

ANAESTHESIA:

For anaesthesia, intraperitoneal injection of Ketamine was given to the rat at a dose of 100milligram per kilogram of body weight. The animals were then fixed to a plastic board with micro-pore tapes attached on all four limbs. Complete anaesthesia was confirmed by pinching the sole and then the procedure was started.

PROCEDURE:

The region of chest was wiped with distilled water prior to incision. A sub-costal incision was given just below the xiphi sternum and the skin and the muscle layer beneath were cut to open the abdominal cavity. Then the diaphragm was cut open to expose the chest cavity. The ribs were cut on both sides and the heart was completely exposed.

Figure 14: Sub-coastal incision below the xiphi sternum

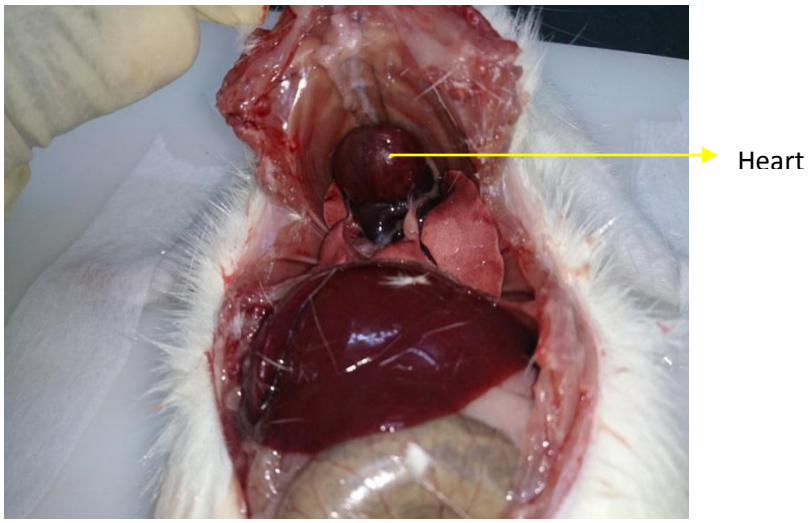


Figure 15: Exposure of heart

The aorta was identified and a bulldog artery clamp was applied around the aorta. Then the heart and the bulldog clamp were lifted together and the heart was excised carefully along with a sufficiently long piece of aorta using a fine scissors.

Immediately after removing the heart from the chest cavity, it was placed in a petri dish containing the ice cold Normal Extracellular solution. The blood inside the heart was removed by slow massaging. If this blood is not removed, it might get clotted inside the vessels and chamber of heart and interfere in perfusion and contraction of heart. Immediately the aorta was cannulated and the heart was mounted in the Langendorff setup and the coronaries were perfused with Normal Extracellular solution maintained at 37 degree Celsius and at the rate of 10ml per minute.

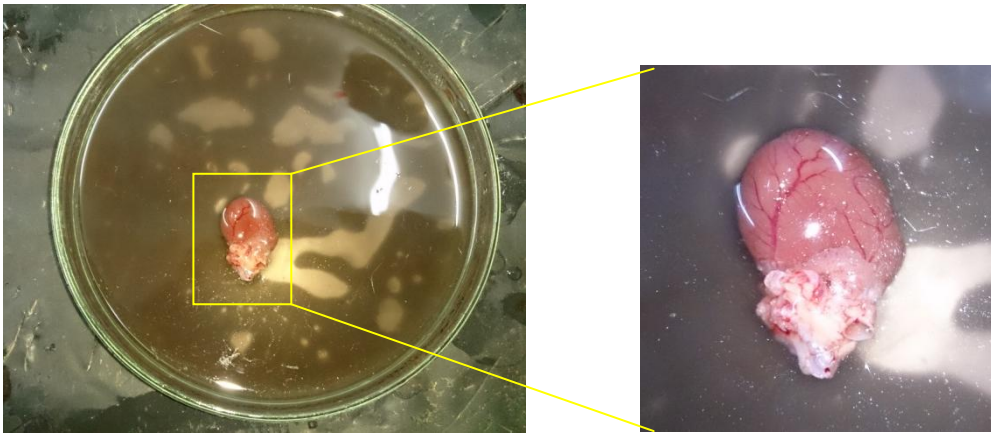


Figure 16: Heart isolated from the rat kept in a petri dish containing cold normal extracellular solution

The perfusing solutions were stored in the reservoirs and then they pass through the bubble trap before entering the aorta. This bubble trap prevents entry of air bubble into the aorta from the perfusion system. The heart is allowed to beat on its own without any external pacing. Surface electrocardiogram was recorded using surface electrodes, and CMCdaq amplifier connected to a computer. The analog data was acquired through computerized CMC data acquisition system.

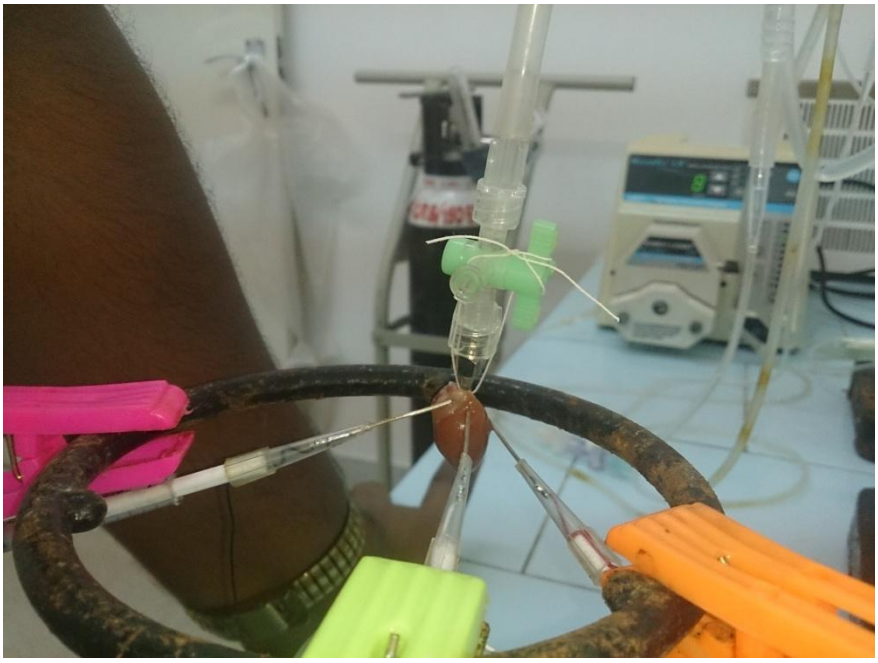


Figure 17: Recording of surface electrogram from isolated perfused rat heart

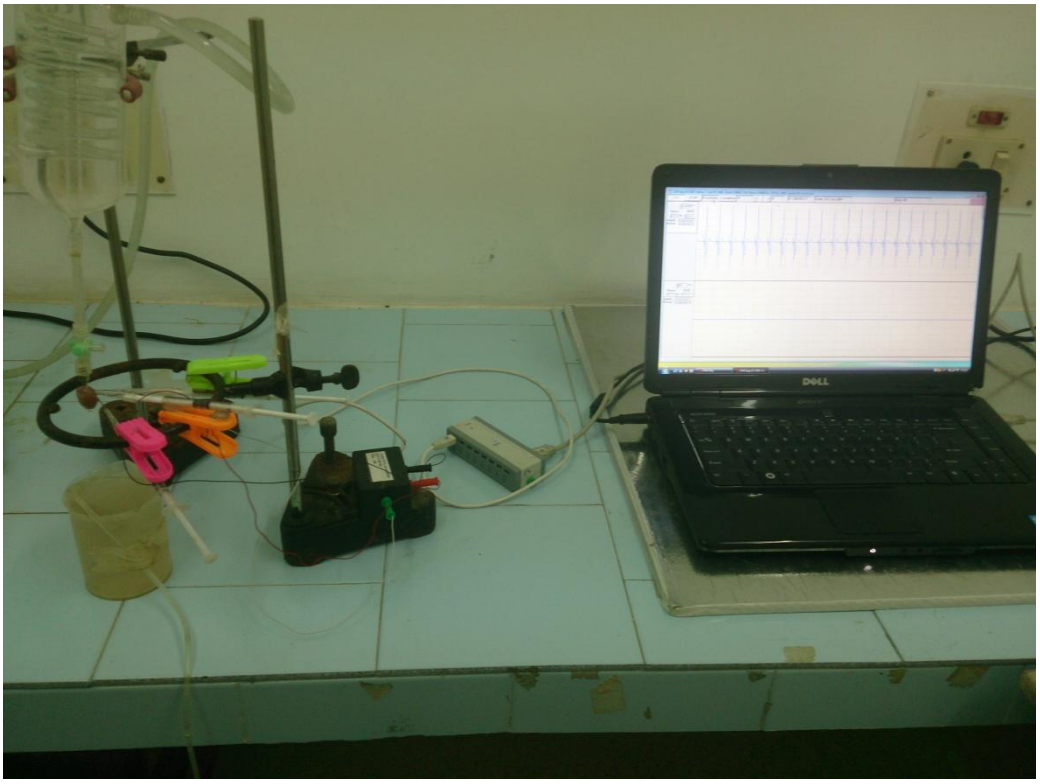


Figure 18 : CMCdaq computerized data acquisition system

DRUG DOSE STANDARDISATION:

6 rats were used for standardization of drug dosage. Different doses of the 2 drugs – Phenylephrine(1µM, 10µM, 20µM) and Neomycin (2mM, 2.5mM, 5mM) , were tried on different rats and the dose was standardized. The final standardized dose was 10µM for Phenylephrine and 2mM for Neomycin.

EXPERIMENTAL DESIGN:

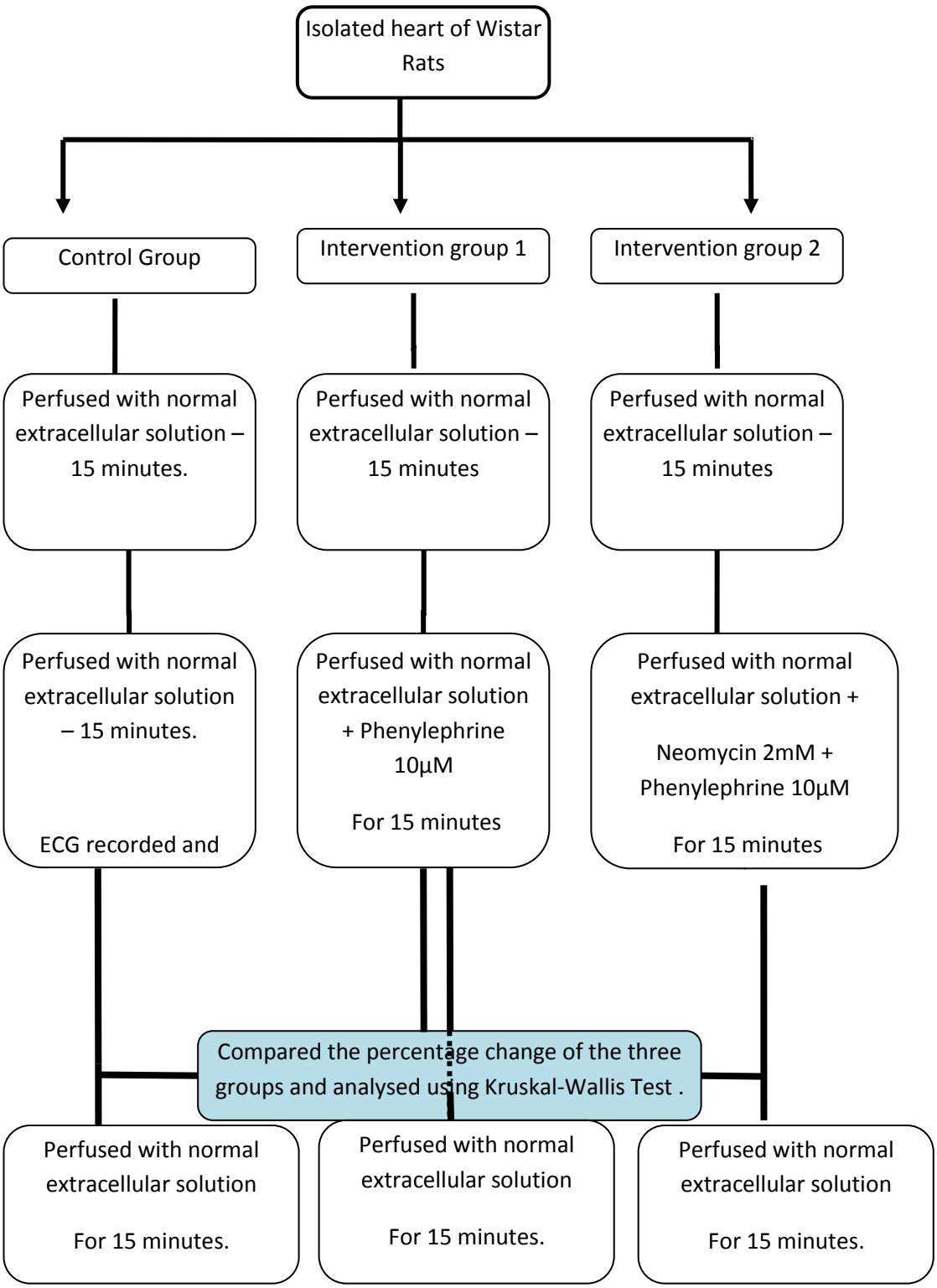
The experiment protocol had 3 groups.6 rats were used in each group. The total number of animals used was 18. Group1 was a control group where the heart was isolated from the rat and immediately mounted in the Langendorff setup and perfused with the modified extracellular solution for 45 minutes at 37 degree Celsius and constantly oxygenated. This was done to see if there was any trend of increase or decrease of heart rate with time. The heart rate was computed from the surface ECG recorded using the CMCdaq amplifier and software.

Group2 was an intervention group with Phenylephrine. The hearts were isolated from the rat and mounted and perfused in Langendorff mode with modified extracellular solution at 37 degree Celsius with constant oxygenation for 15 minutes or more till the heart rate stabilized. Then Phenylephrine 10μM was added to the perfusate and perfused for 15minutes. After 15 minutes the heart was again perfused with the modified extracellular solution for another 15 minutes to wash out the phenylephrine. Surface ECG was recorded throughout the experiment and heart rate was calculated from the ECG.

Group3 was another intervention group with Neomycin and Phenylephrine. In this set of experiments the rat hearts were isolated and perfused in Langendorff mode with the modified extracellular solution (37

degree Celsius and oxygenated) till the heart rate stabilized. Then Neomycin 2mM was added to the perfusate and after 2 minutes Phenylephrine 10μM was also added to the perfusate and perfused for 15 minutes. This was followed by wash with the modified extracellular solution for 15 minutes. The heart rate was calculated from the recorded surface ECG.

FLOWCHART DESCRIBING THE EXPERIMENTAL DESIGN



ANALYSIS:

The surface ECG was recorded continuously throughout the experiment using the surface electrodes connected to the CMCdaq ECG amplifier and the CMCdaq software. The heart rate was calculated from the recorded surface ECG. The heart rate for every minute was calculated manually by counting the peaks (R wave) from the ECG and tabulated. The change in heart rate during intervention was also calculated and expressed as percentage change.

STATISTICAL ANALYSIS:

The heart rate before and after the drug intervention within a group was compared. Since they were non-parametric paired data, they were analysed statistically using Wilcoxon Signed Rank test.

The change in heart rates between the groups was also compared. These were non-parametric unpaired data of three independent groups (control group, intervention 1 group and intervention 2 group). They were analysed using Kruskal Wallis Test.

Data were analysed using SPSS software (version17.0).

A p value of less than 0.05 was considered as significant.

IGOR Pro software (version 5.4) was also used to analyse and plot graphs.

RESULTS

RESULTS:

Representative sample of the raw tracing of the surface ECG recorded

Figure 19: Representative ECG tracing obtained with normal extracellular solution perfusion

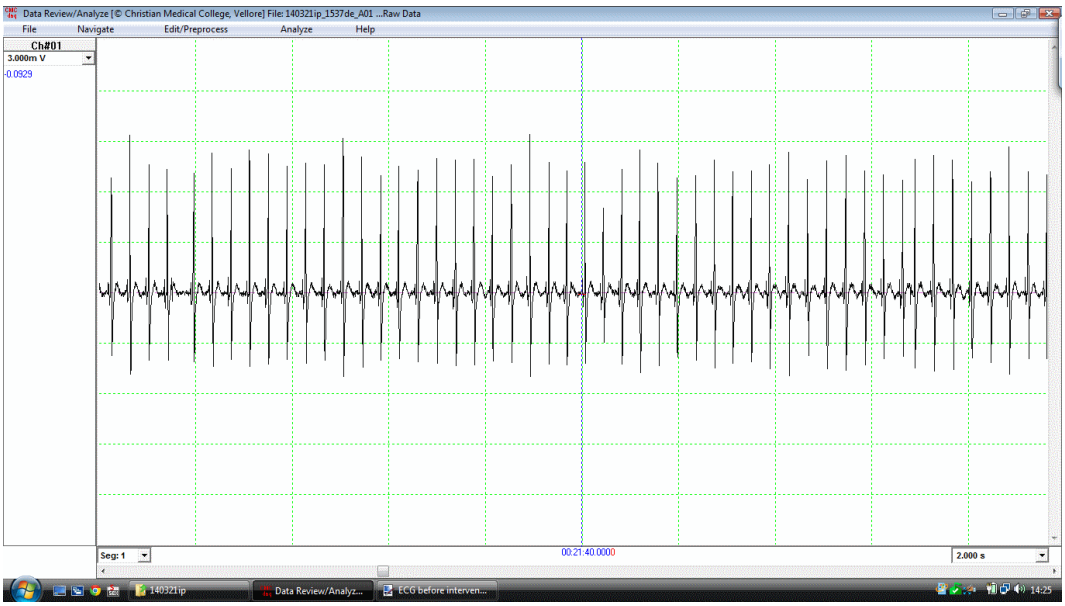


Figure 20: Representative ECG tracing obtained by adding Phenylephrine 10µM in normal extracellular solution perfusion

Heart rate was calculated manually from the recorded surface ECG and tabulated as follows:

CONTROL GROUP:

Time (min)	Heart rate (beats/min)					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
-5	190	160	170	90	170	110
-4	190	150	170	90	170	120
-3	190	160	170	100	170	100
-2	190	160	170	100	170	100
-1	190	160	170	100	170	100
0	190	150	170	100	170	110
1	190	150	170	110	170	110
2	190	150	170	110	160	110
3	180	150	170	110	160	110
4	180	140	170	110	160	110
5	190	150	160	120	160	110
6	180	150	170	120	160	110
7	190	150	170	110	170	110
8	180	150	170	100	170	110
9	180	150	170	100	160	100
10	180	150	170	100	160	100
11	190	150	170	110	160	100
12	190	140	160	120	160	100
13	180	150	160	120	160	100
14	180	140	160	120	160	100
15	180	150	160	120	160	110
16	170	140	160	130	160	110
17	180	150	160	130	160	100
18	180	150	160	130	160	100
19	180	150	160	130	150	110
20	180	150	160	130	160	110
21	170	150	150	130	160	110
22	170	150	160	130	160	110
23	170	150	160	130	160	100
24	170	150	160	130	150	110
25	170	150	160	130	160	110

INTERVENTION GROUP 1:

(Phenylephrine 10µM)

Time (min)	Heart rate (beats/min)					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
-5	130	160	160	110	130	150
-4	130	160	150	110	130	140
-3	130	160	150	110	130	140
-2	140	160	150	110	120	140
-1	140	160	150	110	120	150
0	134	160	152	110	126	144
1	160	180	160	110	150	170
2	170	180	170	120	160	180
3	190	180	170	140	160	190
4	200	180	170	160	160	180
5	190	190	180	180	160	180
6	200	180	170	190	160	180
7	200	210	170	200	160	190
8	210	210	180	200	170	180
9	210	220	170	200	180	180
10	210	220	180	220	160	180
11	220	230	170	220	160	170
12	220	230	180	210	160	160
13	220	230	190	210	160	160
14	220	240	190	210	160	170
15	220	230	190	210	160	180
16	210	260	200	210	150	180
17	210	260	210	210	160	180
18	210	250	200	220	150	180
19	200	230	200	210	160	180
20	200	240	200	210	140	180
21	200	230	190	210	120	180
22	200	230	200	200	120	170
23	190	210	190	200	120	170
24	180	220	190	200	120	160
25	180	200	190	200	110	140

INTERVENTION GROUP 2:

(Neomycin 2mM + Phenylephrine 10µM)

Time (min)	Heart rate (beats/min)					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
-5	90	140	130	140	150	110
-4	80	140	130	130	130	110
-3	90	130	120	130	150	110
-2	90	130	120	130	150	110
-1	100	110	100	130	150	90
0	90	130	120	132	146	106
1	110	100	90	130	100	90
2	100	100	100	130	60	90
3	80	90	90	110	70	90
4	80	80	90	100	60	90
5	80	90	90	80	60	90
6	80	90	80	80	60	90
7	80	90	80	80	60	90
8	80	80	80	80	60	90
9	80	80	80	80	60	100
10	80	80	80	80	60	90
11	80	80	60	80	60	100
12	80	80	60	70	60	100
13	80	80	70	70	60	90
14	80	90	70	70	60	70
15	90	100	130	70	60	70
16	100	90	140	70	60	90
17	110	80	160	70	60	100
18	110	90	180	70	60	110
19	110	90	190	70	70	110
20	110	90	190	70	70	130
21	110	160	180	70	70	140
22	110	120	180	80	80	130
23	110	130	160	90	100	90
24	110	160	170	90	100	70
25	100	150	170	100	110	70

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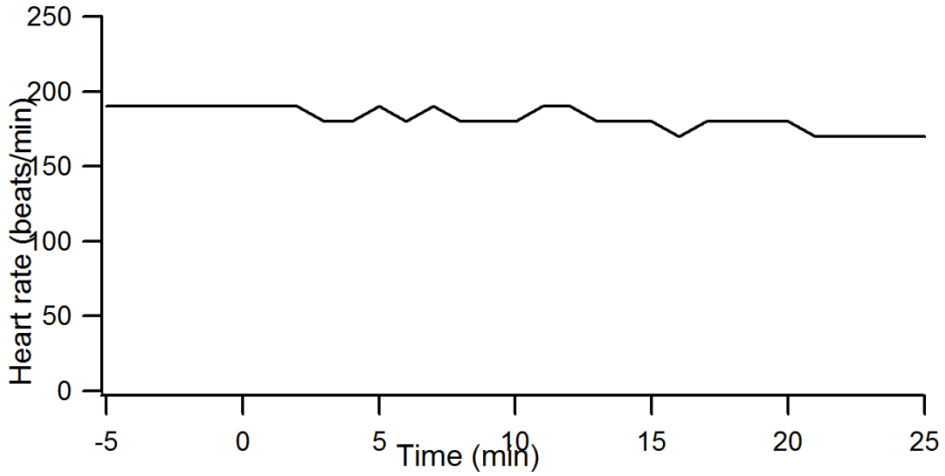
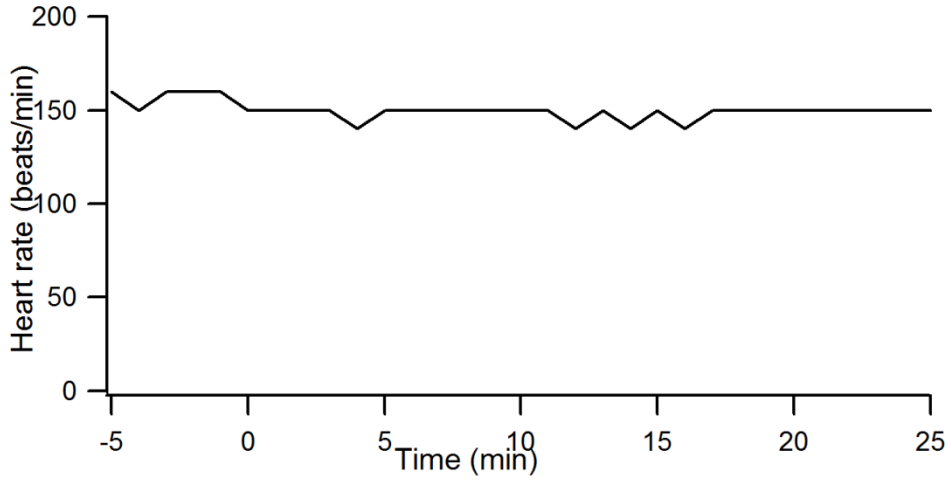
CONTROL GROUP:

The calculated and tabulated heart rates were plotted against time using IGOR pro software for each experiment in the control group.

EXPERIMENT 1:



EXPERIMENT 2:



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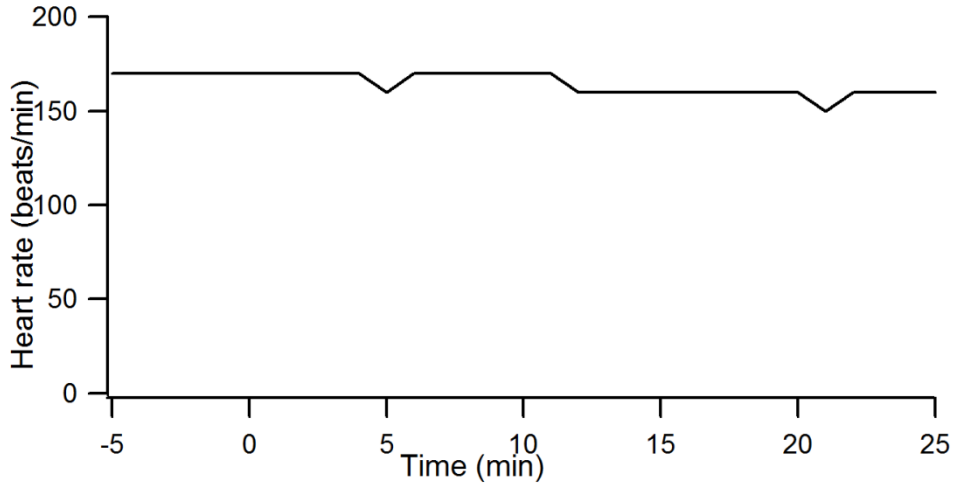
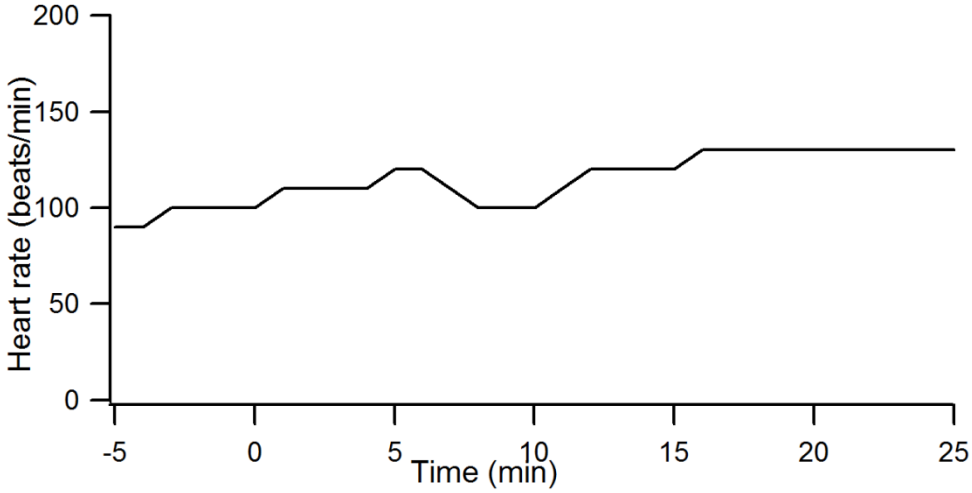
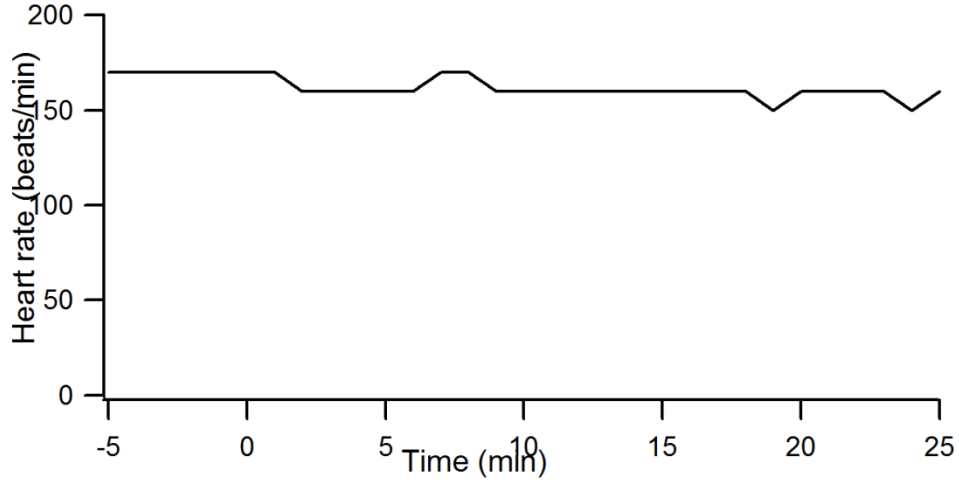


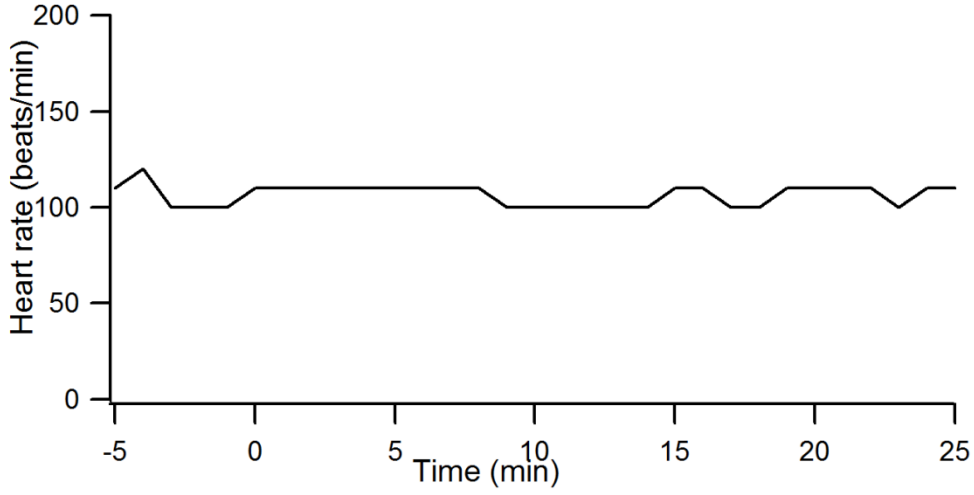
EXPERIMENT 4:



EXPERIMENT 5:



EXPERIMENT 6:



CONTROL GROUP:

Since the basal heart rates of each rat were different, all the values were normalized to the basal heart rate and the mean and standard deviation for each minute was calculated. A graph was plotted with this mean and standard deviation using IgorPro software to see the effect of time on heart rate.

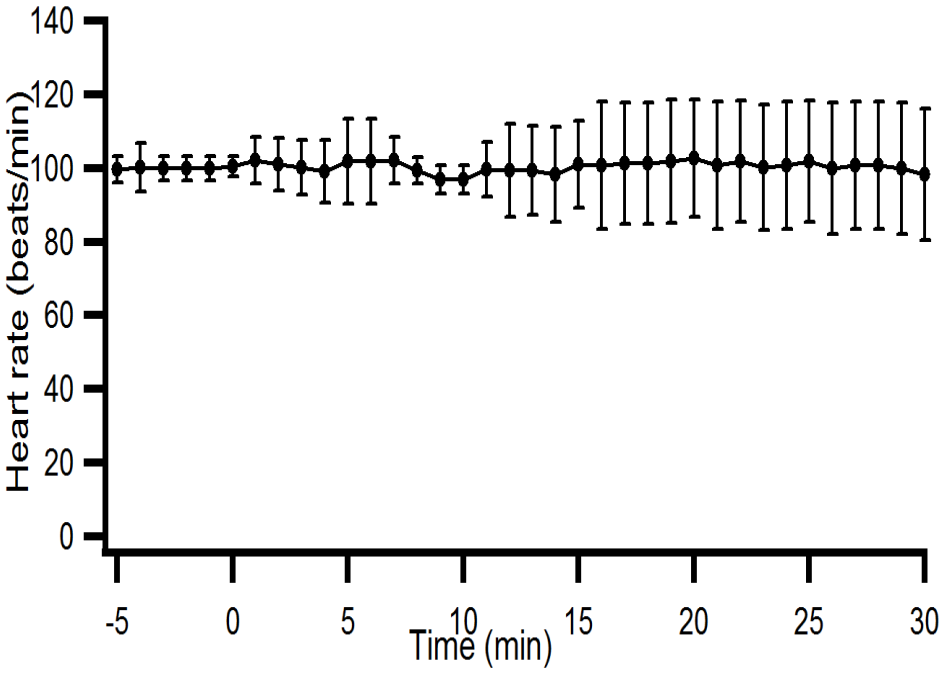


Figure 21: A plot of heart rate of control rats versus time in isolated rat hearts in Langendorff mode perfused with normal extracellular solution at 37 degree Celsius and constantly oxygenated. There is no change in heart rate over 30 minutes.

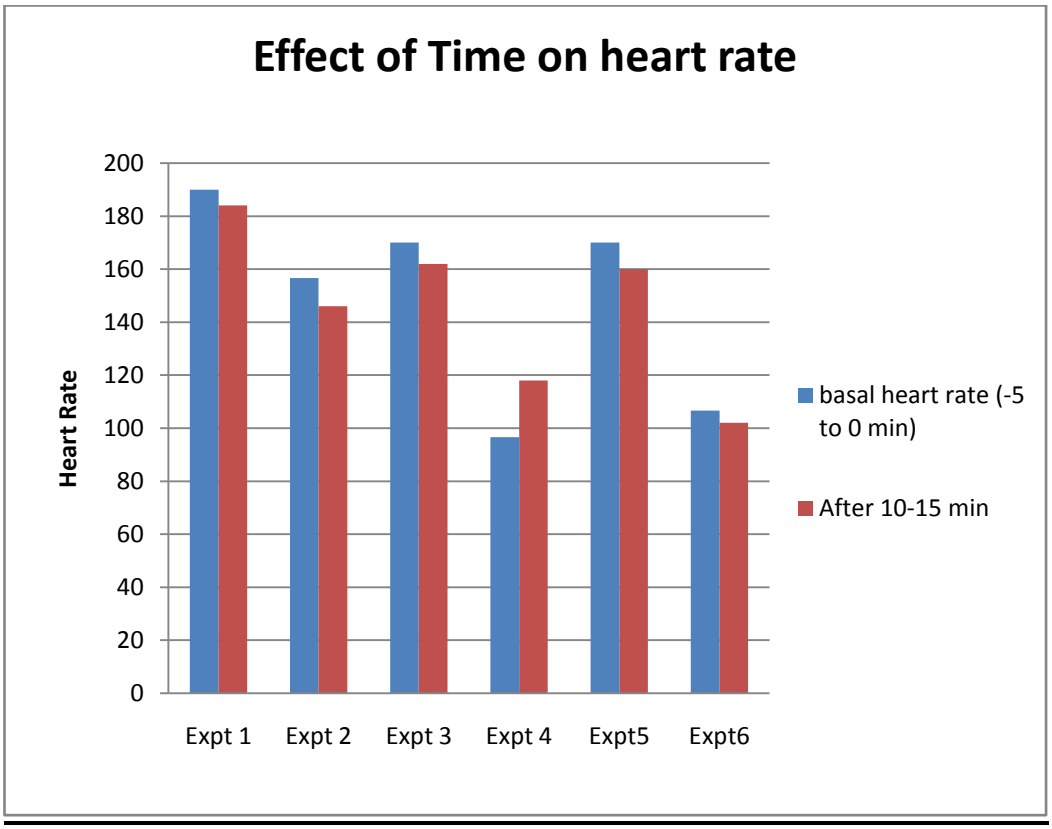


Figure 22: A category plot showing the change in heart rate with time in all six experiments

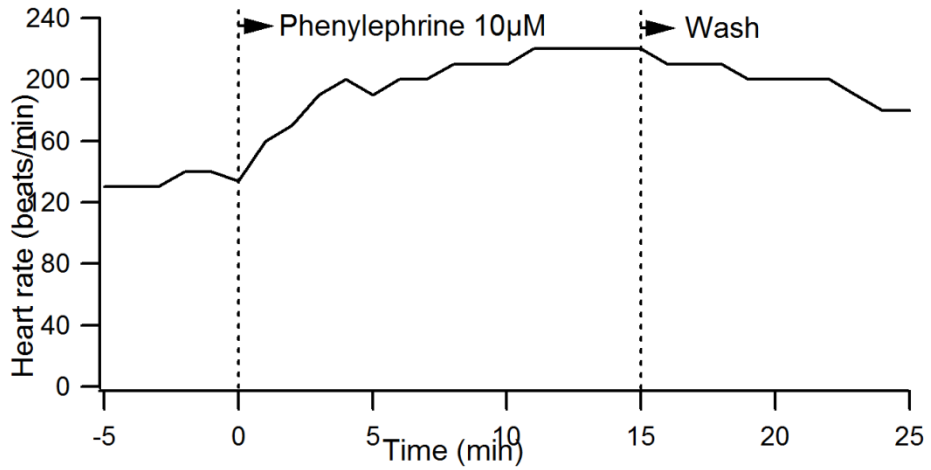
Statistical Analysis:

The basal heart rates of all six control experiments at the initial 5 minutes were compared to the heart rates after 10 to 15minutes by Wilcoxon Signed Rank Test using SPSS software. The *P* value was 0.345. This shows that there was no significant difference between these two groups. Thus the heart rate did not change with time.

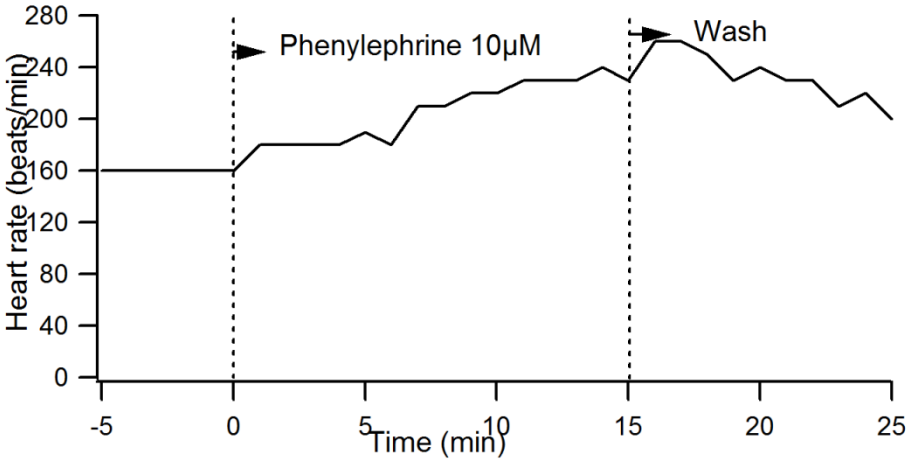
INTERVENTION GROUP (Phenylephrine 10µM)

The heart rates were calculated from the surface ECG and tabulated. These heart rates were plotted against time using IGORPro software for all the six experiments in the intervention group 1.

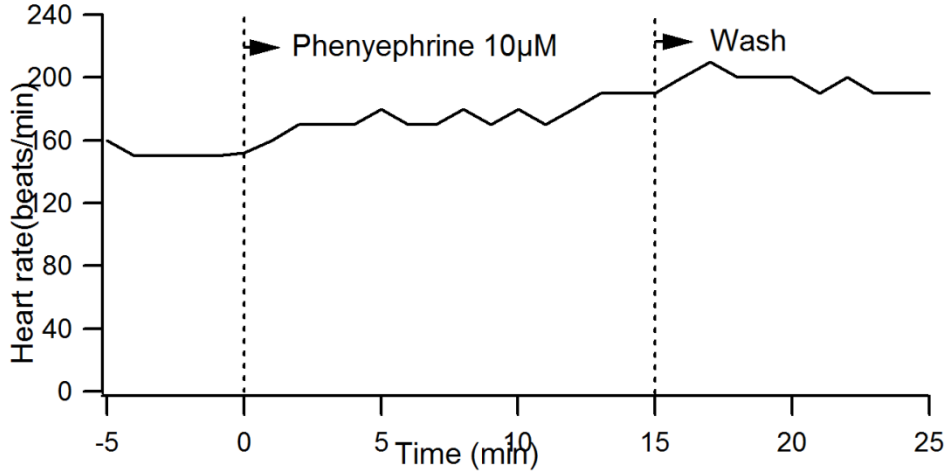
EXPERIMENT 1:



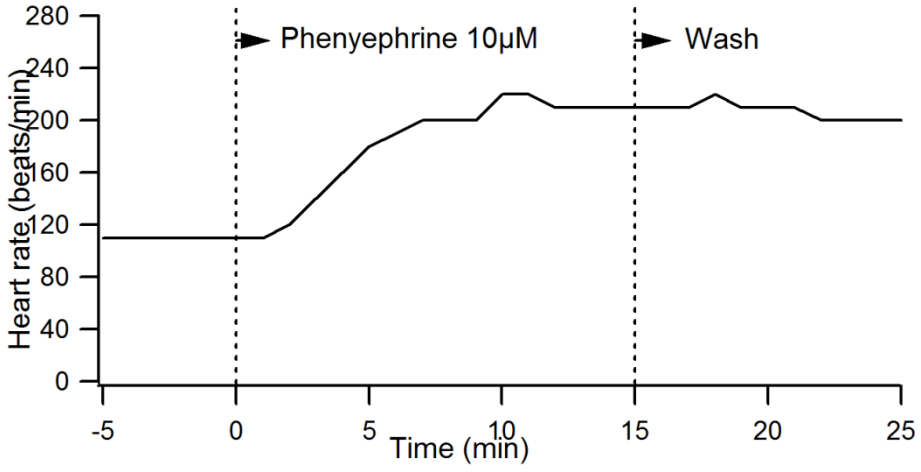
EXPERIMENT 2:



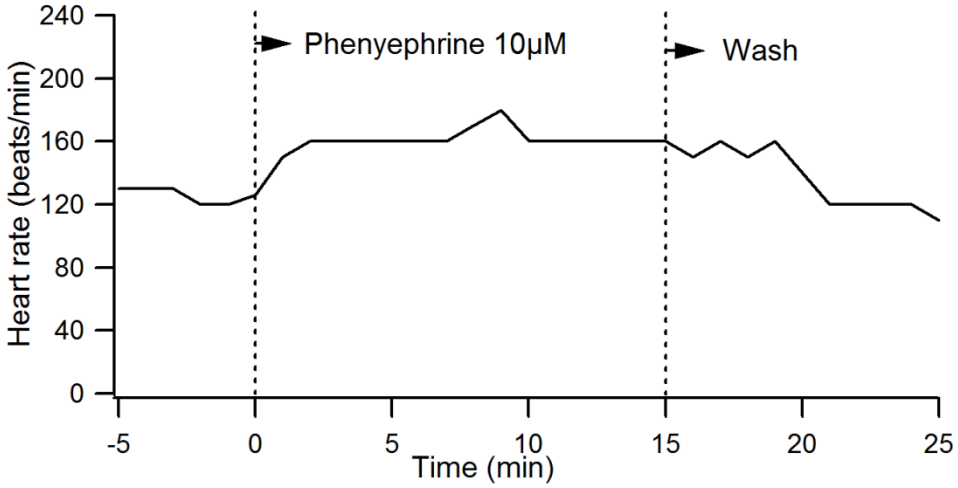
EXPERIMENT 3:



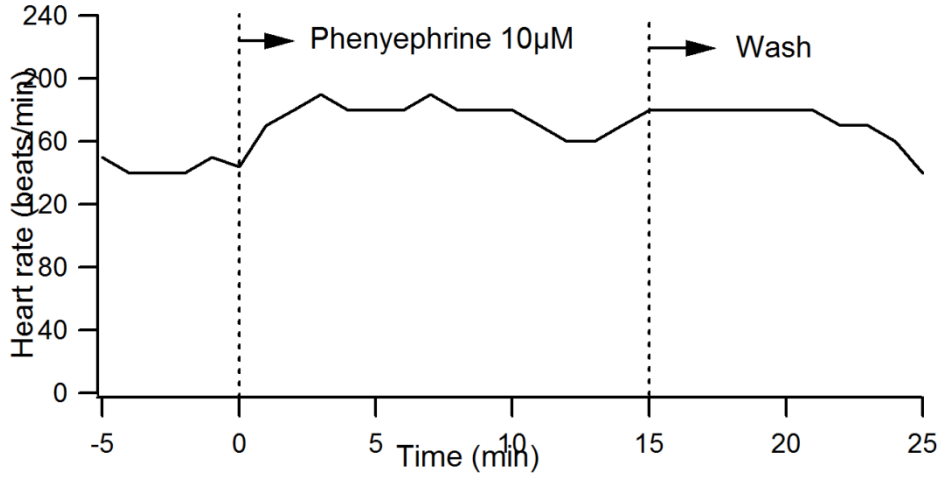
EXPERIMENT 4:



EXPERIMENT 5:



EXPERIMENT 6:



EFFECT OF PHENYLEPHRINE:

All the six rats had different basal heart rates and it was difficult to compare them. So the heart rates were normalized to the basal heart rate and a mean and standard deviation was calculated. This was plotted against time using IGORPro software.

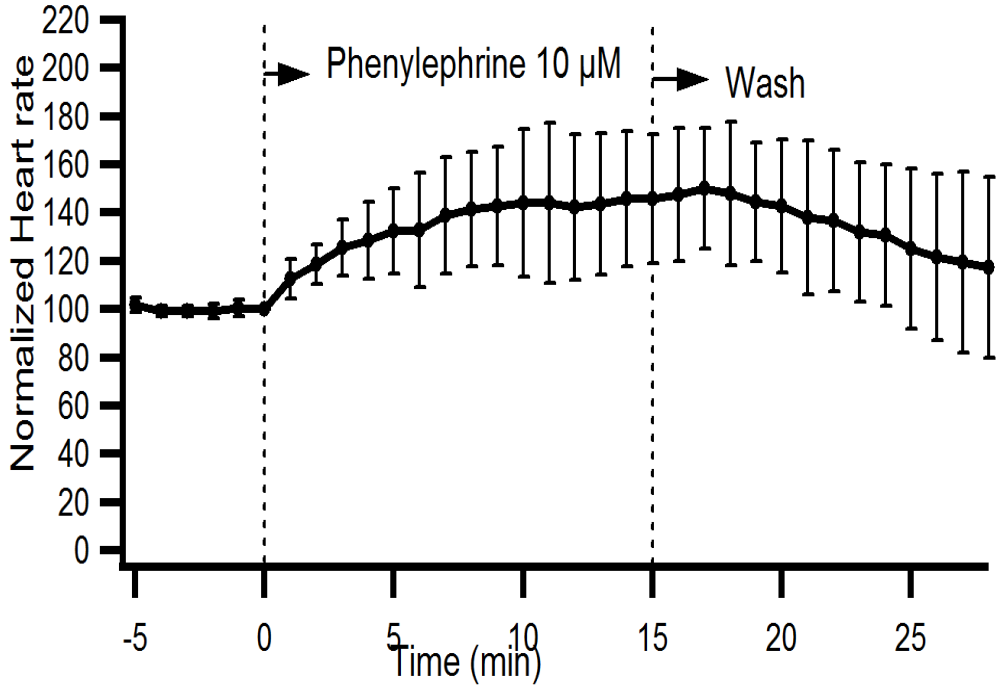


Figure 23: Phenylephrine 10µM produced a 44% increase in heart rate in isolated rat heart perfused in Langendorff mode. (n=6)

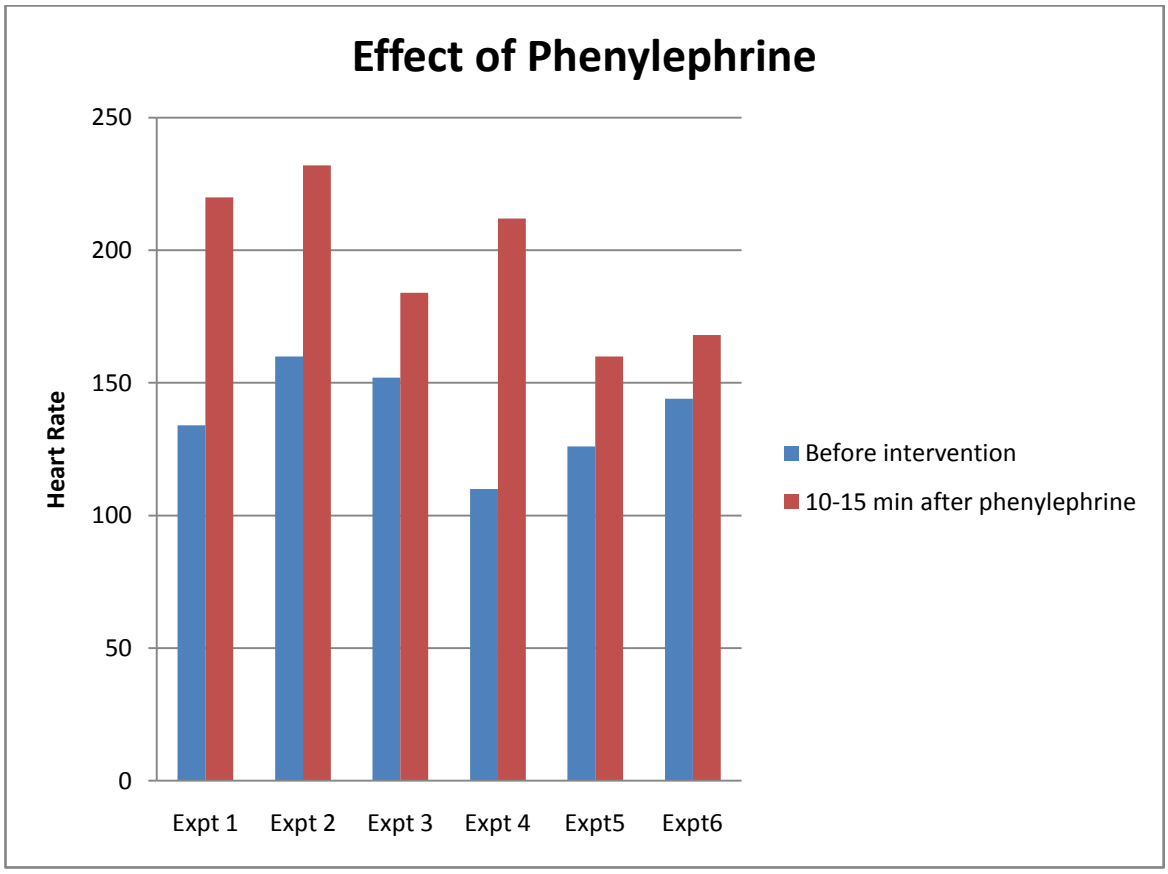


Figure 24: A category plot showing the effect of Phenylephrine on heart rate on six experiments

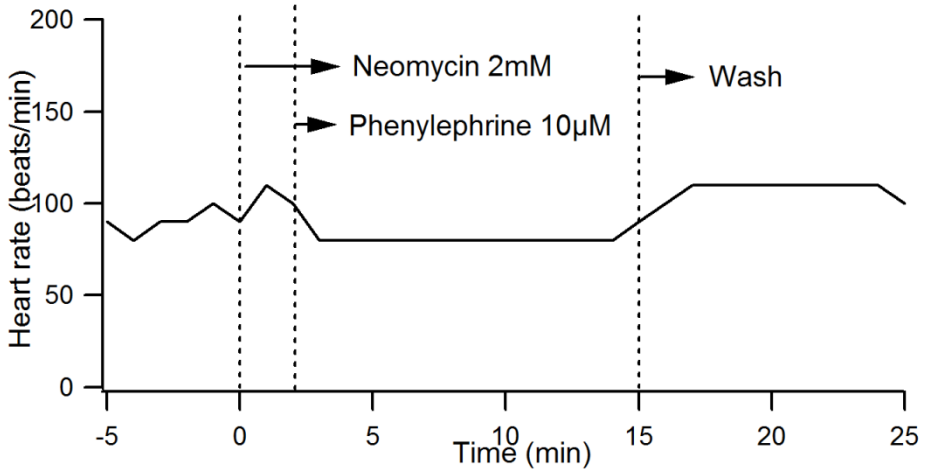
Statistical Analysis:

The basal heart rates at initial 5 minutes were compared with the heart rates at 10 to 15 minutes to see the effect of Phenylephrine. Wilcoxon Signed Rank Test was used for this and SPSS software was used for analysing. The *P* value was 0.028. This shows that the increase in heart rate with Phenylephrine was significant.

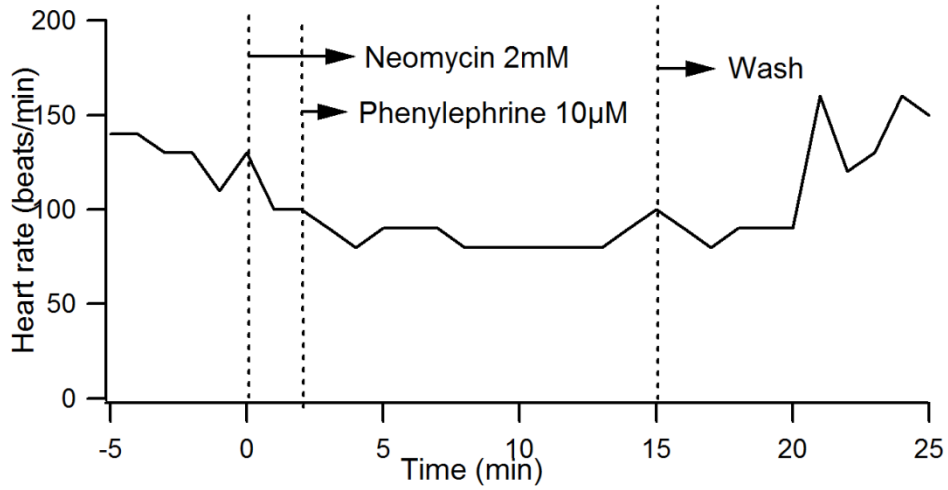
INTERVENTION GROUP 2 (Neomycin 2mM + Phenylephrine 10µM)

The heart rates calculated and tabulated from the surface ECG were plotted against time for all the six experiments in the intervention group 2. IGORPro software was used to make these graphs.

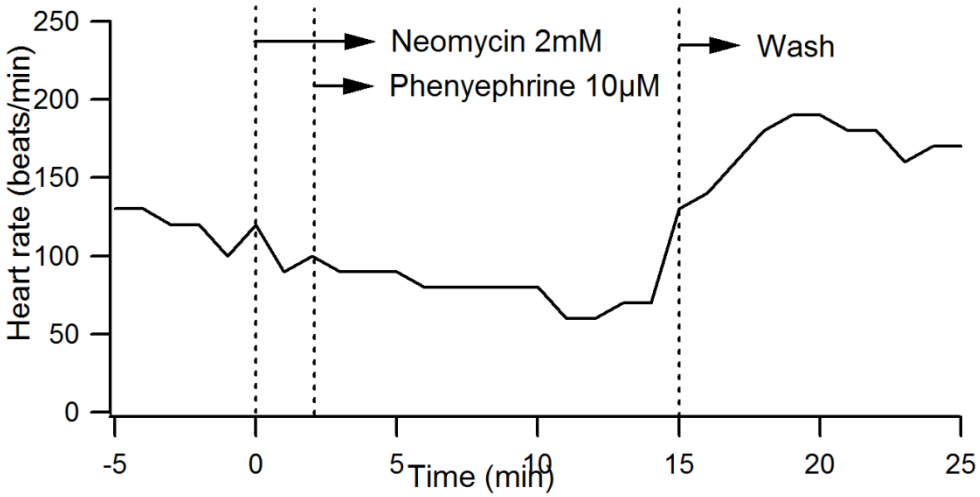
EXPERIMENT 1:



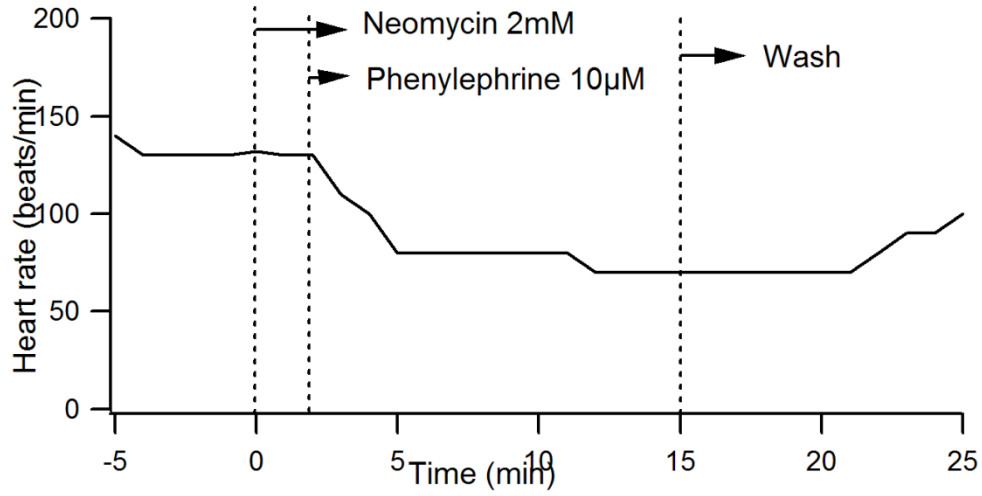
EXPERIMENT 2:



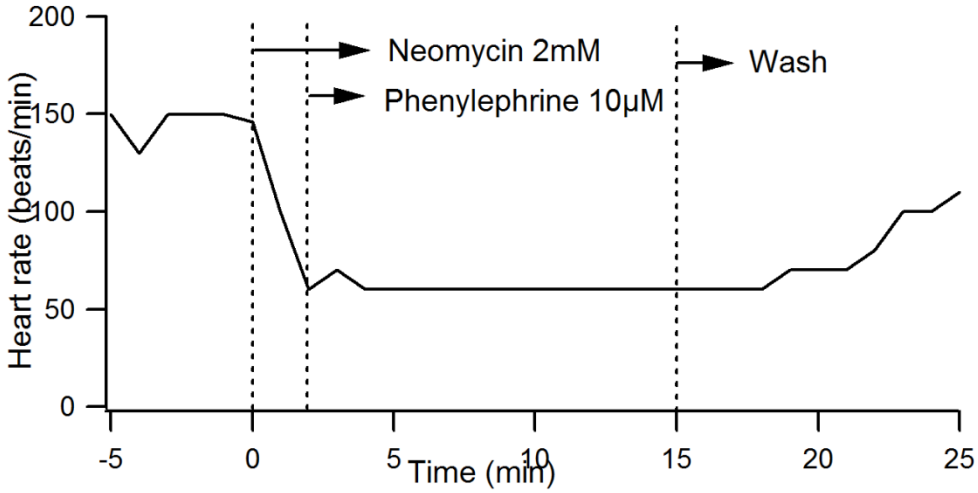
EXPERIMENT 3:



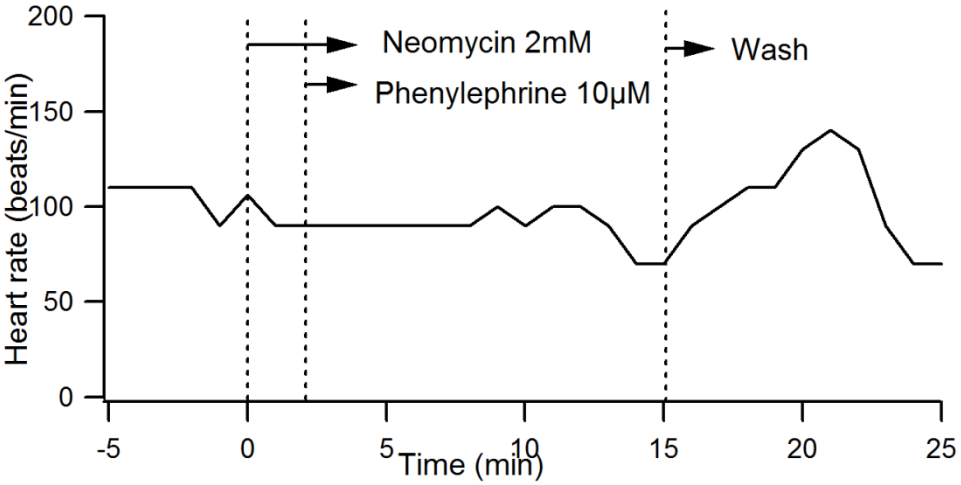
EXPERIMENT 4:



EXPERIMENT 5:



EXPERIMENT 6:



EFFECT OF NEOMYCIN + PHENYLEPHRINE:

Since the basal heart rates were different in all the rats, the heart rates were normalized to the basal heart rate and then they were compared. The mean and standard deviation was calculated and plotted against time using the IGORPro software.

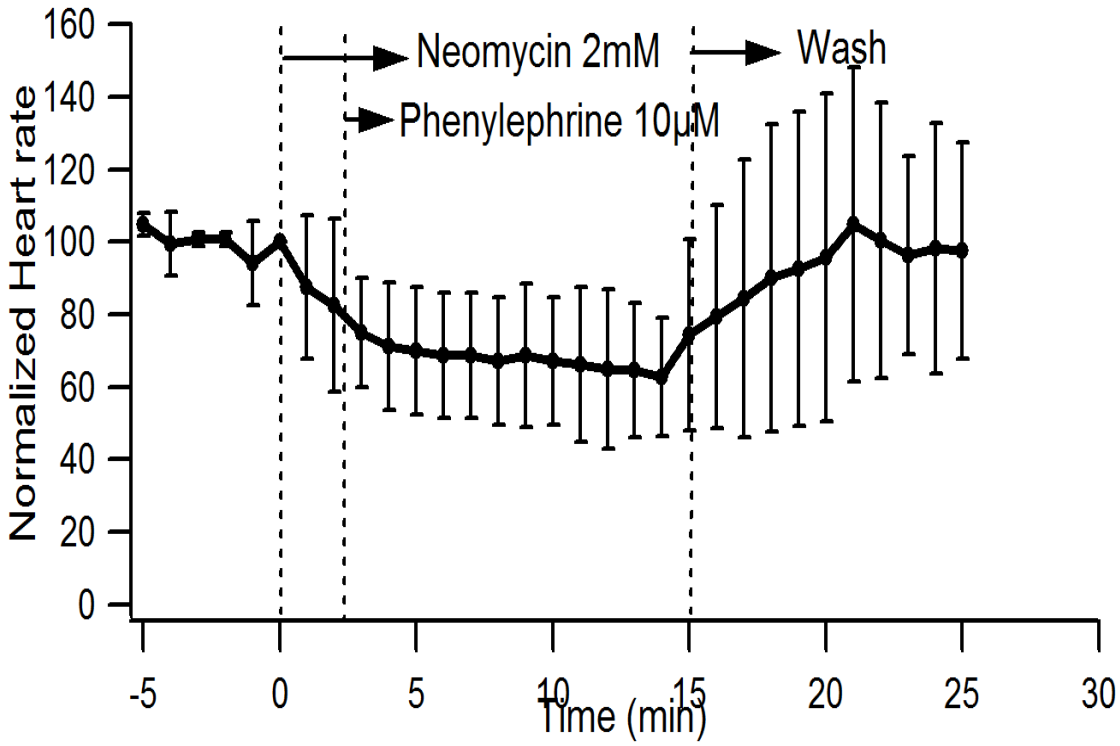


Figure 25 : Neomycin 2mM blocked the phenylephrine induced increase in heart rate in isolated rat heart perfused in Langendorff mode

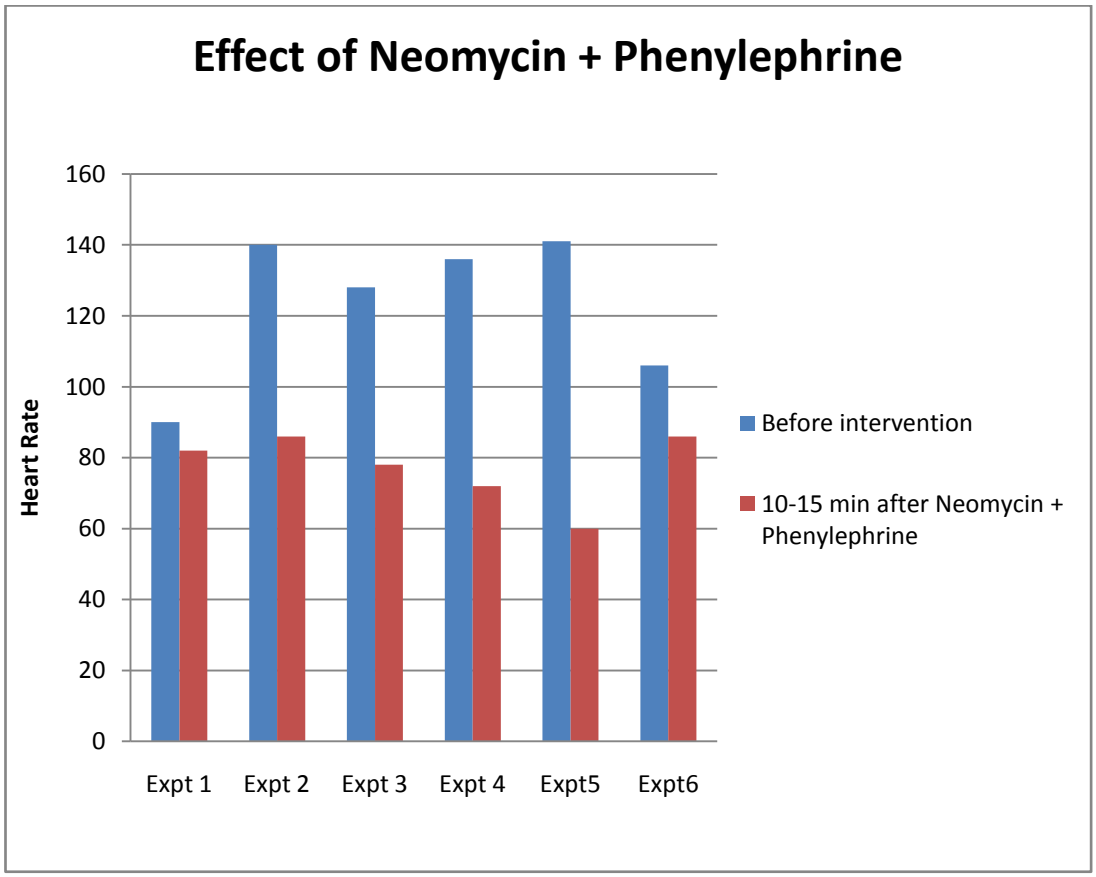


Figure 26: A category plot showing the effect of Neomycin+Phenylephrine on heart rate in six experiments

Statistical Analysis:

The basal heart rates and the heart rates after intervention i.e., at 10 to 15 minutes were compared to see the effect of Neomycin and Phenylephrine. This was analysed by Wilcoxon Signed Rank Test using SPSS software. The *P* value obtained was 0.028. This shows that there was no increase in heart rate with Phenylephrine in the presence of Neomycin. Also the decrease in heart rate by Neomycin was significant.

COMPARING THE THREE GROUPS:

The mean and standard deviations of the heart rates (normalized) of all the six experiments of three groups were plotted in the same graph for comparison.

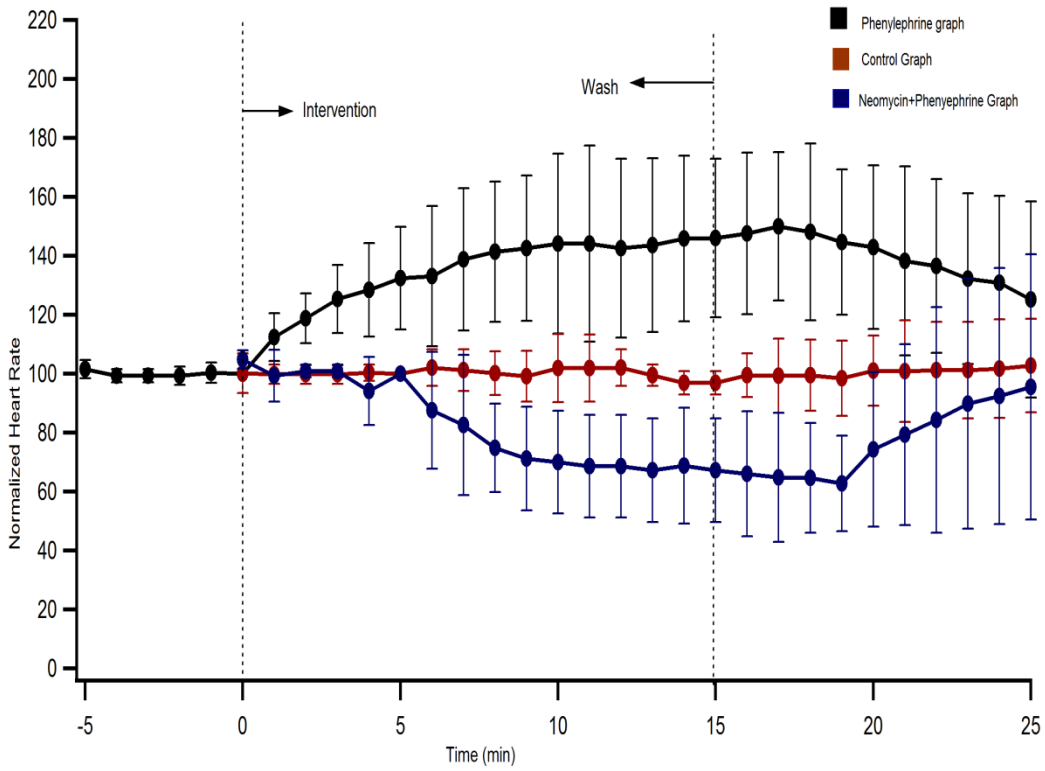


Figure 27: The mean and standard deviation of all the three groups (Control group- red colour, Phenylephrine group- black colour, Neomycin+Phenylephrine group- blue colour) are shown in a single graph

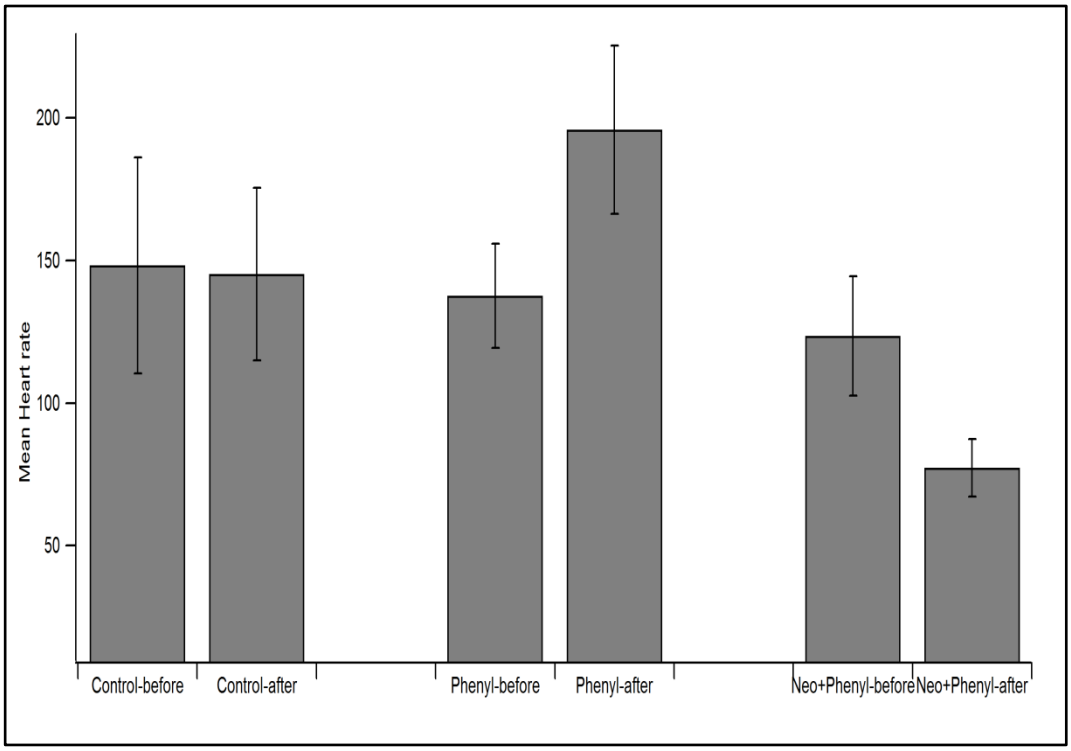


Figure 28: A category plot showing the mean heart rate with standard deviation before and after intervention in all three groups

The heart rates at 10 to 15 minutes of all the three groups were compared using Kruskal Wallis Test using SPSS software. The *P* value was 0.001. This shows that the inter-group variations in heart rates are significant.

DISCUSSION

DISCUSSION:

One of the electrophysiological properties of heart is Chronotropy. It is the ability of the heart to produce electrical impulses on its own. This property is also called as automaticity or excitability. Many studies and research have been done to unravel the molecular mechanism behind this pacemaker property of heart.

The Sinoatrial nodal is the pacemaker of the heart. It is a group of spontaneously depolarizing cells located in the right atrium near the opening of superior vena cava. The action potential of the Sinoatrial nodal cells is different from those of the working cardiomyocytes.

Sinoatrial node action potential:

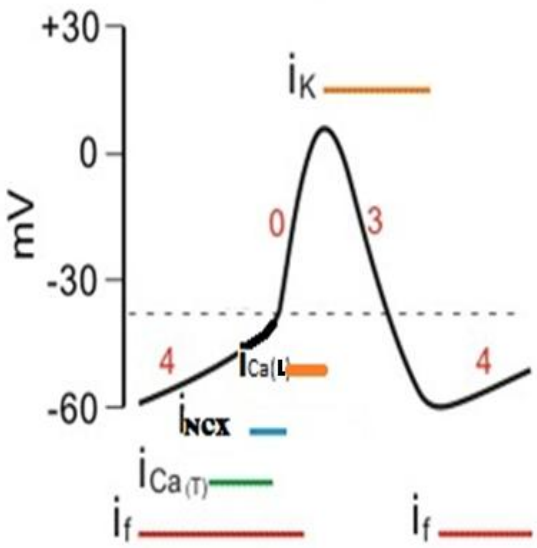


Figure 29: Sinoatrial node action potential and the ion channels involved

The Sinoatrial node action potential has three phases: Phase 4 of Diastolic depolarisation, Phase 0 of depolarisation and Phase 3 of repolarisation.

The cells in Sinoatrial node do not have a true resting membrane potential like the other cells in heart, instead they undergo a slow depolarisation called the Diastolic depolarisation in phase 4. At the end of repolarisation the membrane potential reaches a value of -60 mV. The spontaneous diastolic depolarisation begins with the opening of HCN (Hyperpolarisation activated cyclic nucleotide gated) channels which carry a hyperpolarisation activated current called funny currents (I_f). This is an inward current creating depolarisation of the membrane. As the membrane depolarises and reaches a voltage around -50 mV, the T-type calcium channels get activated and produce more depolarisation. The calcium ions that entered the cell through open T-type calcium channels activate calcium-induced calcium release from the sarcoplasmic reticular stores through Ryanodine receptors and IP_3 receptors. This increase in intracellular calcium drives the sodium calcium exchanger in forward mode, resulting in an inward current which depolarises the membrane further. When the membrane potential reaches -40 mV, it activates the L-type calcium channels. Opening of L-type calcium channels causes calcium to enter into the cell and it depolarises the cell till the threshold potential is reached (Grant, 2009).

Phase 0 of Sinoatrial action potential is due to the activated L-type calcium channels and increased conductance of calcium through these channels. Since the conductance of calcium is not as fast as sodium conductance, the rate of depolarisation is slow compared to that of working myocardial cells. Hence the action potential is called ‘Slow response’ action potential (de Carvalho et al., 1969).

Phase 3 is the repolarisation phase in Sinoatrial node action potential. It is due to the potassium conductance through the voltage gated potassium channels producing an outward hyperpolarising current. At this time the calcium channels become inactivated and closed. Once the cell is completely repolarised and reaches -60 mV, the cycle is repeated spontaneously.

Diastolic depolarisation phase is the essence of pacemaker cell automaticity. Because of this slow depolarisation that occurs in the phase 4 of action potential, the pacemaker cells are able to produce spontaneous and rhythmic electrical impulses. Regarding the molecular mechanism behind the diastolic depolarisation, two important theories are being debated namely the membrane clock theory and the calcium clock theory.

The membrane clock theory states that the funny currents produced by the HCN channels are responsible for diastolic depolarisation. These currents get activated on hyperpolarisation and they produce an inward

current. And these funny current producing HCN channels are expressed only in the Sinoatrial node and Purkinje fibres but not in the surrounding working myocardiocytes. Hence the funny currents are called as pacemaker currents (Brown et al., 1979; DiFRANCESCO and Zaza, 1992).

The other theory is the calcium clock theory. It states that the endoplasmic reticulum inside the cells has spontaneous and rhythmic local calcium release during diastole. This local calcium release drives sodium calcium exchanger in forward mode which extrudes one calcium ion for three sodium ions. This results in an inward current producing depolarisation (Bogdanov et al., 2001; Maltsev and Lakatta, 2007) . Thus as a local calcium release by an intracellular clock produces depolarisation, this theory is called as ‘Calcium clock’ theory.

The sodium calcium exchanger is emerging as the pacemaker current in heart. This is driven by the local calcium release from the endoplasmic reticulum. Endoplasmic reticulum is the major internal store of calcium. The endoplasmic reticulum has two calcium release channels present on its membrane. They are Ryanodine receptor and Inositol 1,4,5-triphosphate receptor (IP_3R). So the local calcium release responsible for driving the sodium calcium exchanger in forward mode and thereby diastolic depolarisation can be through one of these two receptors.

Ryanodine receptor is a large calcium release channel present in the sarcoplasmic reticulum. . In skeletal muscles the Ryanodine receptor is voltage gated. It is regulated by calcium in cardiac cells. Calcium produced a calcium induced calcium release through the Ryanodine receptors in cardiac myocytes during systole (Griffiths and MacLeod, 2003).

IP_3 receptor is also a calcium release channel present in the sarcoplasmic reticulum. This receptor needs both calcium and IP_3 for its activation(Bootman et al., 1995). When the receptor is bound to IP_3, even very little calcium as seen in diastolic phase can activate the receptor to release calcium (Gilbert et al., 1991). Hence the role of IP_3 receptor on diastolic calcium release can be more significant.

The aim of this study was to assess if IP_3 has a role in diastolic calcium release and thereby in rhythm generation of heart. Hence we used pharmacological modulators of IP_3 and saw the effect of them on heart rate. We used Phenylephrine which is an α-adrenergic agonist. Phenylephrine binds with its receptor which is a G protein coupled receptor. Gq gets activated and this activated the membrane bound enzyme Phospholipase C (PLC). PLC hydrolysis PIP2 into IP_3 and DAG. Thus Phenylephrine increases the concentration of IP_3 (Scholz et al., 1988).

With the addition of Phenylephrine to the perfusate in the isolated rat heart preparation the heart rate increased to nearly 44% and this was also

statistically significant. (p value = 0.028) . On washing of the drug by perfusing with normal extracellular solution decreased the heart rate but it did not come back to the basal heart rate.

We also used Neomycin which binds with the membrane phospholipids and blocks the action of PLC on them. Thus Neomycin blocks the production of IP_3 and decreased its concentration (Walz et al., 2000). On using Neomycin along with Phenylephrine in the perfusate of the isolated perfused rat heart, the increase in heart rate seen with Phenylephrine alone was not seen. Also a decrease in heart rate was seen with Neomycin and Phenylephrine in the perfusate and this was also statistically significant (p value = 0.028). On wash off of the drugs by perfusing with normal extracellular solution, the heart rate increased initially followed by a decrease. This shows that Neomycin is washed away faster than Phenylephrine which exerts its effect and then weans off.

Our results show that Phenylephrine which acts by increasing IP_3 concentration increases the heart rate in an isolated heart preparation. Since this is an isolated heart preparation, this effect of Phenylephrine is the direct effect on heart. To confirm that Phenylephrine acts through IP_3 pathway, we have used Neomycin which blocks the production of IP_3. In the presence of Neomycin the increase in heart rate by Phenylephrine was not seen. This confirms that Phenylephrine acts by increasing IP_3 concentration. The result

allows us to conclude that calcium release through IP_3 receptors may be an important rhythm-generating mechanism.

The results in our control group show that the heart rate of the isolated perfused rat hearts did not change with time. Since the hearts were perfused and ECG was recorded for 45 minutes any change in heart rate with time can act as a confounder in this study. Hence a control group was done without any intervention for the same 45 minutes and it was found that the heart rate did not change with time.

The results from this study show that when the concentration of IP_3 is increased with Phenylephrine the heart rate increases and the heart rate does not increase when Neomycin is added along with phenylephrine which blocks the production of IP_3. Thus IP_3 definitely has a role in rhythm generation of heart.

This role of IP_3 on rhythm generation or Chronotropy has not been shown in adult cardiomyocytes in any studies before. The positive chronotropic effect of IP_3 has been shown in neo-natal rat cardiomyocytes and in embryonic stem cell derived cardiomyocytes (Hadad et al., 2013; Kapur and Banach, 2007; Méry et al., 2005). In these two conditions the IP_3 receptor expression is more than Ryanodine receptor expression in these cardiomyocytes and the major calcium handling is done by IP_3 receptors (Rosemblit et al., 1999). As the cardiomyocytes differentiate , there is

increased expression of Ryanodine receptors and the calcium handling mechanism is taken over by other calcium channels (Fu et al., 2006). But the IP_3 receptor expression is not completely lost. It is also expressed in adult cardiomyocytes but in less number compared to Ryanodine receptors. And it has a definitive role in diastolic calcium release which is shown in this experiment.

SUMMARY

SUMMARY:

Diastolic depolarisation is the essence of pacemaker automaticity. Two important theories have been proposed regarding the molecular mechanism behind the diastolic depolarisation. They are the 'Membrane clock theory' and the 'Calcium clock theory'

According to the calcium clock theory, the endoplasmic reticulum which is the main internal store of calcium has spontaneous and rhythmic local calcium release events. This local calcium release drives the sodium calcium exchanger in forward mode which extrudes one calcium ion for three sodium ions resulting in an inward current. This inward current produces depolarisation of the membrane during diastolic phase. Since a local calcium release by an intracellular clock produces depolarisation, this is called as 'calcium clock'. Thus the sodium calcium exchanger is emerging as the pacemaker current.

The local calcium release that drives the sodium calcium exchanger in forward mode is from the endoplasmic reticulum. The endoplasmic reticulum has two calcium release channels: the Ryanodine receptor and the IP_3 receptor. This study was aimed to assess if IP_3 has a role in this diastolic calcium release and thereby in rhythm generation.

The results of this study show that Phenylephrine increases the heart rate in an isolated rat heart preparation perfused with normal extracellular solution at 37 degree Celsius and oxygenated constantly. Phenylephrine is known to act by increasing IP_3 concentration. To confirm that the increase in heart rate by Phenylephrine was because of increased IP_3 concentration, we used Neomycin which blocks the production of IP_3. In the presence of Neomycin the increase in heart rate by Phenylephrine was not seen. This confirms that Phenylephrine acts by increasing IP_3 concentration. Thus the results show that calcium release through IP_3 is an important mechanism in rhythm generation.

CONCLUSION

CONCLUSION:

In an isolated rat heart preparation perfused with normal extracellular solution in Langendorff mode, addition of Phenylephrine increased the heart rate. In the presence of Neomycin, the increase in heart rate by Phenylephrine was not seen. These results show that Phenylephrine can increase heart rate and it acts by increasing the concentration of IP_3. Thus calcium release through IP_3 receptor in diastolic phase has a role in rhythm generation.

LIMITATIONS

LIMITATIONS:

Limitations of our study:

The pharmacological manipulator used in our study: Neomycin does not have specific actions only on $IP_{3.}$ They can also act on other channels and give a non-specific effect. Neomycin has been found to block L-type calcium channels and Ryanodine receptors also. Even though many studies have used Neomycin as an IP_3 antagonist, this is a limitation in this study.

FUTURE COURSE

OF THE STUDY

FUTURE COURSE OF THIS STUDY:

- See the effect of IP_3 manipulation on heart rate by using more specific pharmacological modulators or using knockout animals.
- To do experiments to see if Ryanodine receptor has any role in diastolic calcium release.
- Compare the role of IP_3 receptor to Ryanodine receptor in diastolic calcium release and thereby rhythm generation.

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

October 4, 2013

Dr. Jesi.W
PG Demonstrator
Department of Physiology
Christian Medical College
Vellore 632 002

Sub: **Fluid Research grant project: (Animal Study)**
Study on the effect of Inositol-1, 4, 5-triphosphate on heart rate in Wistar rats.
Dr. Jesi.W, PG Demonstrator, Dr. Solomon Sathishkumar, Dr..Sathya Subramani, Physiology.

Ref: IRB Min. No. 8373 dated 18.07.2013

Dear Dr. Jesi.W,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Study on the effect of Inositol-1, 4, 5-triphosphate on heart rate in Wistar rats." on July 18, 2013.

The Committees reviewed the following documents:

1. IRB Application Format
2. CV's of Drs Jesi.W, Solomon Sathishkumar, Sathya Subramani
3. No of documents 1-2

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on July 18, 2013 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

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

Name	Qualification	Designation	Other Affiliations
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Surgery (Colorectal), CMC.	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC.	Internal, Clinician
Dr. Chandrasingh	MS, MCH, DMB	Urology, CMC	Internal, Clinician
Dr. Ellen Ebenezer Benjamin	M.Sc, Ph.D	Maternity Nursing, CMC	Internal, Nurse
Dr. Vathsala Sadan	M.Sc, Ph.D	Addl. Deputy Dean, College of Nursing, CMC.	Internal, Nurse
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerlogy & Leprosy, CMC.	Internal, Clinician
Dr. Anand Zachariah	MBBS, MD, DNB	Professor, Medicine, CMC	Internal, Clinician
Mrs. Pattabiraman	B Sc, DSSA	Social Worker, Vellore	External, Lay Person
Mr. Sampath	B Sc, BL	Advocate	External, Legal Expert
Dr. Rajesh Kannangai	MD, PhD.	Professor & In-charge Retrovirus Laboratory (NRL under NACO), Clinical Virology, CMC	Internal, Clinician
Dr. B. J. Prashantham (Chairperson), IRB Blue - Internal	MA (Counseling), MA (Theology), Dr Min (Clinical)	Chairperson(IRB)& Director, Christian Counselling Centre	External, Scientist
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist & Epidemiologist

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

Dr. Anuradha Rose	MBBS, MD	Assistant Professor, Community Health, CMC	Internal, Clinician
Dr. Nihal Thomas	MD MNAMS DNB(Endo) FRACP(Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology& Addl. Vice Principal (Research), CMC.	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.

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)

A sum of Rs. 80,000/- (Rupees Eighty Thousand only) for 2 years. A sum of Rs 40,000/- will be sanctioned for 12 months after receipt of the revised proposal, subsequent installment of 40,000/- each will be released at the end of the first year following the receipt of the progress report.

Yours sincerely

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

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

CC: Dr. Solomon Sathishkumar, Physiology, CMC

MINISTERED UNTO BUT TO MINISTER
CHRISTIAN MEDICAL COLLEGE
VELLORE
INDIA

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

Institutional Animal Ethics Committee Approval Letter

INSTITUTIONAL ANIMAL ETHICS COMMITTEE (IAEC)

CHRISTIAN MEDICAL COLLEGE

VELLORE, INDIA

Dr. Alfred Job Daniel
Principal & Chairman

Dr. Solomon Sathishkumar
Secretary

9th October 2013

Dr. Jesi.W,
PG Demonstrator,
Department of Physiology.

Dear Dr. Jesi,

Your research proposal titled "Study on the effect of Inositol-1,4,5-triphosphate on heart rate and contractility in Wistar rats" has been approved by the Institutional Animal Ethics Committee (IAEC).

After discussion, 22 wistar rats have been approved for the study.

The IAEC approval number for the study is 16/2013.

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. Solomon Sathishkumar
Secretary, IAEC