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Te Whare Wānanga o Otāgo

NEW ZEALAND

Role of CaMKII and Alpha Stimulation in Heart Function

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Abstract

Cardiovascular disease is a major cause of morbidity and mortality in New Zealand. Elucidating its pathogenesis will help us better treat those who are affected by it. One protein that has been implicated in cardiovascular pathology is Calcium (Ca^{2+})/Calmodulin-dependent protein Kinase II (CaMKII). As its name suggests, it is activated by Ca^{2+}-bound calmodulin. In a healthy heart, CaMKII plays a role in regulating Ca^{2+}-handling proteins in the cardiomyocyte, such as Ryanodine Receptor and Phospholamban. CaMKII has the ability to become autonomously activated so that it can exert its effects even when the Ca^{2+} concentration decreases. However, when CaMKII is activated when it does not need to be, it can have pathological effects. It can be activated pathologically by hyperglycemia, oxidative stress and hyperadrenergic states, all of which are seen in cardiovascular pathology such as heart failure or diabetic cardiomyopathy. Alpha-adrenergic receptor signaling can also activate CaMKII, but little is known about the effects of alpha-adrenergic stimulation on the heart.

The aim of this project was the determine the functional effects of the alpha-adrenergic agonist Methoxamine on isolated wild-type mouse hearts, and in isolated CaMKII knockout mouse heart, in incremental doses to observe differences in the responses between the two groups to determine the role of CaMKII in the functional response to alpha-adrenergic stimulus in the heart.

The technique used to address these aims was the isolated heart set-up, which is also known as the Langendorff setup. The mouse hearts were surgically removed from the animal, with part of the aorta intact, and placed in an arresting buffer with a high potassium chloride concentration to ionically arrest the heart. The heart was then cannulated via the aorta, and perfused with a Krebs-Henseleit Buffer (KHB) that had all the necessary ions and nutrients to allow the heart to contract outside the body. The stability of the heart was determined via measurements such as coronary flow rate, perfusion pressure, and temperature. A fluid-filled balloon inserted into the ventricle allowed measurement of contractile and relaxation parameters. Increasing doses of alpha-adrenergic receptor agonist, Methoxamine, were added to the KHB perfusate.

I found that in response to alpha-adrenergic stimulation, there was a significant increase in the rate of contraction and the rate of relaxation in the healthy, wild-type mouse hearts, but not in the CaMKII knockout mouse hearts. There was a significant increase in the total

developed pressure in the heart during systole in the healthy mouse hearts in response to alpha-adrenergic stimulation, but this increase was not seen in the CaMKII knockout mice. There was also a significant reduction in the minimum pressure reached by the heart, and the end-diastolic pressure in the healthy wild-type mouse hearts in response to alpha-adrenergic stimulation. Again, this change was not seen in the CaMKII knockout mouse hearts.

The results show that CaMKII may be responsible for the regulation of some contractile and relaxation parameters in the heart. By phosphorylation of its target protein, Ryanodine Receptor, CaMKII can increase the amount and rate of Ca^{2+} release into the cytoplasm of the cardiomyocyte which would allow for faster and more robust contractions. Furthermore, by phosphorylating Phospholamban, CaMKII can increase the rate and amount of Ca^{2+} cleared from the cytoplasm of the cardiomyocyte which would allow for faster and better relaxation. The results also tell us that CaMKII may be party of the alpha-adrenergic signaling pathway.

Future research into the involvement of alpha-adrenergic signaling in cardiovascular pathology, and whether inhibiting CaMKII attenuates or completely ablates disease progression will allow us to target CaMKII or the alpha-adrenergic signaling pathway as a therapeutic for cardiovascular disease.

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List of Abbreviations

AR- Adrenergic Receptor

ATP- Adenosine Tri-Phosphate

Ca2+ - Calcium

CaMKII- Calcium/Calmodulin-dependent protein Kinase II

CO- Cardiac Output

DAG- Diacylglycerol

EDP- End Diastolic Pressure

EDV- End-Diastolic Volume

EF- Ejection Fraction

ESV- End-Systolic Volume

FS- Fractional Shortening

GPCR- G-Protein Coupled Receptors

HR- Heart Rate

IP3- 1, 4, 5-Inositol Triphosphate

KHB- Krebs Henseleit Buffer

KO- Knock-out

LVIDd- Left ventricular Internal Diameter in diastole

LVIDs- Left ventricular Internal Diameter in systole

MTX- Methoxamine

NCX- Sodium-Calcium Exchanger

OCT3- Organic Cation Transporter 3

O-GlcNAc- O-Linked N-acetylglucosamine

Pdev- Developed Pressure

PIP2- Phosphotidylinositol-4, 5 Bisphosphate

PKC- Protein Kinase C

PLB- Phospholamban

PLC - Phospholipase C

ROS- Reactive Oxygen Species

SERCA- Sarcoplasmic Endoplasmic Reticulum Calcium ATPase

SR- Sarcoplasmic Reticulum

SV- Stroke Volume

WT- Wild-type

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Chapter 1: Introduction

1.1 Cardiovascular Disease in NZ

Over the last decade, there have been many advances in medical research that have helped limit the epidemic of heart disease that is affecting many western societies. The development of statins and anti-hypertensive drugs have helped to reduce the morbidity of and mortality due to heart disease. However, despite such advances, cardiovascular disease remains to be one of the leading causes of death in New Zealand, with 33% of all deaths being caused by it (Ministry of Health, 2015). As of the 2015 NZ Health Survey, 186,000 kiwis are living with some form of cardiovascular disease (Ministry of Health, 2016).

1.2 Basic Cardiac Function

The function of the heart is to continually pump oxygenated blood into the aorta to be transported to other organs in the body so that they can carry out their functions effectively.

1.2.1 Cardiac Anatomy

There are four chambers that make up the heart- the right and left atria, and the right and left ventricles. The right atrium and ventricle receive deoxygenated blood from the body and pump to the pulmonary circulation so that oxygen from the lungs can oxygenate the blood. The left atrium and ventricle receive oxygenated blood from the pulmonary circulation and pump to the rest of the body. Each cycle of contraction and relaxation that the heart undergoes is known as the Cardiac Cycle.

The heart muscle, known as the myocardium, is made up of individual, excitable cells known cardiomyocytes. Each cardiomyocyte is several contractile units that are made up of actin and myosin filaments. Cardiomyocytes also have an internal store of calcium (Ca^{2+}) in the sarcoplasmic reticulum (SR)

To undergo contraction, the cardiomyocytes have to depolarize. The heart has a small group of cells in the right atrium that are known as pacemaker cells that set the intrinsic heart rate. The pacemaker cells autonomously depolarize and the electrical potential spreads to neighboring cardiomyocytes via channels known as gap junctions. The rate at which the pacemaker cells depolarize is regulated by sympathetic and parasympathetic innervation that basally maintains the heart rate around 60-100 beats per minute in humans, and 500–724 beats per minute in mice (Kaese and Verheule, 2012).

1.2.2 Cardiac Cycle

1.2.2.1 Systole

Systole is the phase of the cardiac cycle when the heart is contracting. Contraction in each cardiomyocyte occurs as a result of a process known as Excitation-Contraction Coupling, which begins when an electrical potential spreads to a cardiomyocyte. The depolarization triggers L-type Calcium Channels in the cell membrane to open. There is a subsequent small influx of Ca^{2+} ions into the cell. In close proximation to the cell membrane is the membrane of the SR. The influx of Ca^{2+} ions through L-type Calcium Channels triggers Ca^{2+}-permeable channels, known as Ryanodine Receptor (RyR), in the SR membrane to change conformation and open. RyR opening causes efflux of a large concentration of Ca^{2+} from the intracellular Ca^{2+} store and into the cytoplasm of the cardiomyocyte, causing a dramatic increase in intracellular Ca^{2+}. Myosin subunits are activated when the Ca^{2+} binds to them, however they have a low affinity for Ca^{2+} and thus, require a large concentration of Ca^{2+} in the cytoplasm to become functionally active. When the Ca^{2+} binds to myosin, the actin binding site is exposed and they bind to the actin filaments. Myosin also binds and hydrolyses Adenosine Tri-Phosphate (ATP), and the energy released from this reaction causes the myosin to flex and pull the actin filaments closer to the center of the contractile apparatus. This results in the cardiomyocyte shortening and when several cardiomyocytes undergo this process simultaneously, contraction occurs (Bers 2002).

1.2.2.2 Diastole

Diastole is the phase of the cardiac cycle when the heart is relaxing. To relax, the Ca^{2+} in the cytoplasm must be removed and this is done in three ways. A small portion of the Ca^{2+} is removed by a secondary active transporter called the Sodium-Calcium Exchanger (NCX) that pumps Ca^{2+} out of the cell in exchange for sodium. Another way in which a small amount of the Ca^{2+} is removed is via the Calcium ATPase, which uses energy from ATP hydrolysis to pump Ca^{2+} out of the cell. The primary way in which Ca^{2+} is removed from the cytosol is by being pumped back into the intracellular Ca^{2+} store, the SR, by an ATP-dependent pump known as

Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA). The basal activity of SERCA is downregulated by a protein known as Phospholamban (PLB). When the Ca^{2+} concentration in the cytoplasm decreases, myosin is no longer able to interact with actin and therefore the contractile filaments return to their basal length. This results in the cardiomyocyte relaxing and thus, so does the heart.

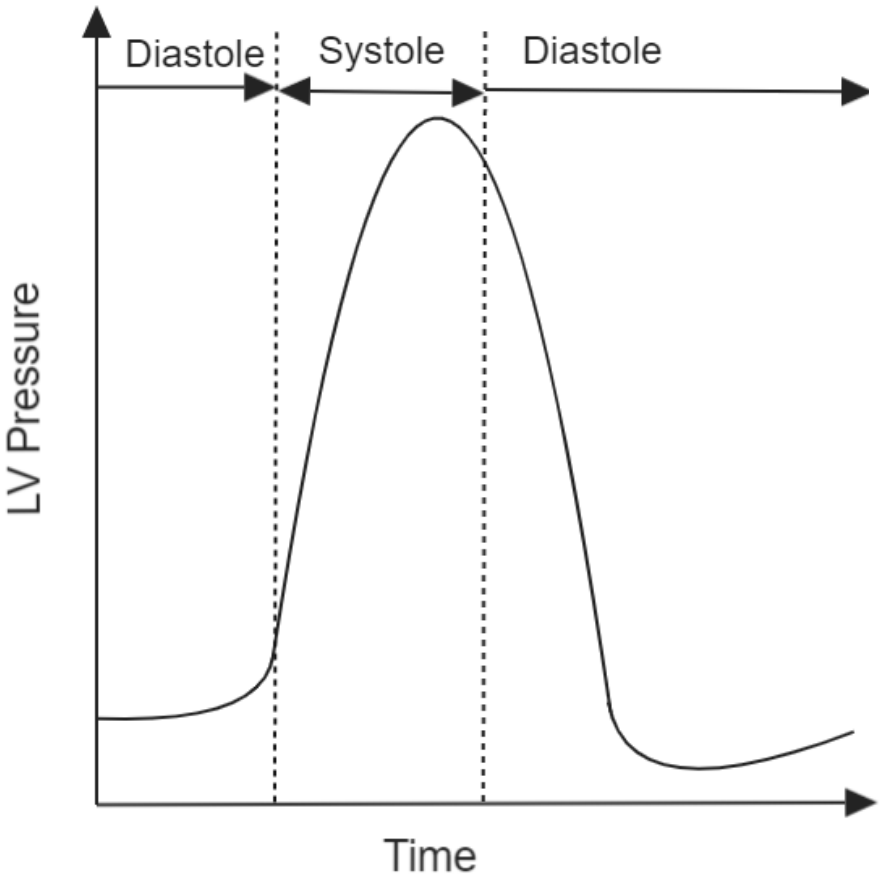

Figure 1.1- Changes in the left ventricular pressure during the cardiac cycle. Two-thirds of the cycle is spent in diastole, where the heart is relaxing and the ventricles are filling with blood. One-third of the cycle is spent in systole, where the heart is contracting to eject blood out of the ventricles.

1.2.3 Cardiac Output

The cardiac output (CO; the volume of blood pumped out of the heart per minute; $ml.min^{-1}$) can be altered by altering the heart rate (HR; the number of times the heart contracts per minute; $beats.min^{-1}$) or the stroke volume (SV; the volume of blood that is pumped out per beat; $ml.beat^{-1}$). In order to increase the CO, the body can increase either or both of the HR and the SV. An increase in HR and SV occurs in the presence of adrenaline/noradrenaline, which acts on adrenergic receptors (ARs) in the myocardium that cause an increase in the contractility, and rate of contraction and relaxation in the heart.

1.2.3.1 Heart Rate

The intrinsic rhythm of the heart beat is generated by pacemaker cells that generate an electrical potential that spreads to neighboring cells via gap junctions. The intrinsic rate at which these pacemaker cells generate electrical potentials can be altered by neuronal, endocrine or autocrine modulation. If the body requires the heart rate to be elevated, there is an increase in the sympathetic input and a decrease in the parasympathetic input to the pacemaker cells, and vice versa if the body required the heart rate to be decreased.

1.2.3.2 Stroke Volume

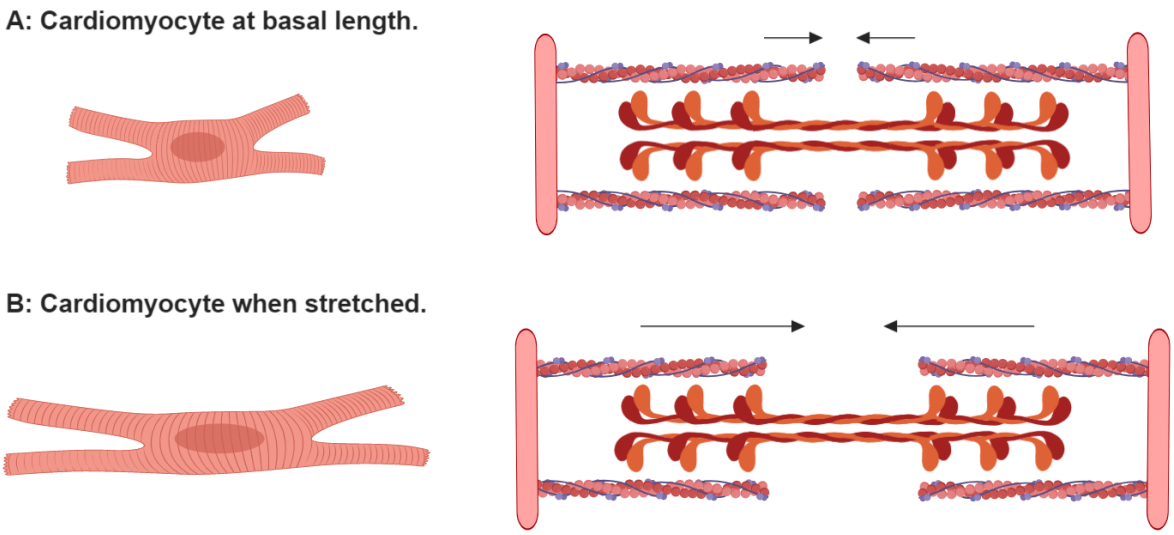

Figure 1.2- Length-Dependent Activation. A) When the cardiomyocyte is at basal length, there is more overlap between the actin and myosin filaments in the contractile apparatus. Only a few interactions between the actin and myosin are required to pull the actin filaments towards the midline, leading to a smaller contraction. **B)** When there is increased venous blood returned to the heart and the ventricles fill more, the cardiomyocytes are stretched, thus reducing the overlap between the actin and myosin filaments. The reduced overlap means that more interactions between the actin and myosin are required to pull the actin filaments towards the midline, leading to a greater contraction.

Changes in the stroke volume are caused by three factors- Preload, Afterload, and Contractility. The preload is defined by the amount of venous blood that returns to the heart from the systemic or pulmonary circulations. The higher the venous return, the higher the End-Diastolic Volume (EDV), which is the volume of blood in the ventricle at the end of diastole (Norton. 2001). Increased EDV increases the stroke volume by a mechanism known as length-dependent activation, whereby the increased blood volume causes the cardiomyocytes in the ventricular walls to stretch, in turn causing the contractile apparatus within these cardiomyocytes to also stretch. Because the actin and myosin filaments overlap each other, stretching of the cardiomyocytes reduces the overlap and allows more potential

for the actin-myosin filaments to interact. More actin-myosin interaction causes a larger contractile force by shortening the contractile apparatus more and developing more tension as a result (See Figure 1.2). The afterload is defined by the pressure in the aorta that needs to be overcome by the ventricle to open the aortic valve and eject blood (Norton 2001). If there is a higher aortic pressure, the ventricular cardiomyocytes must develop more tension in order to increase the intraventricular pressure enough so that it is higher than that in the aorta. If the ventricle is not able to sustain the higher tension for the entirety of the systolic phase, the duration of the phase is shortened, and thus less blood is ejected which means there is a lower stroke volume. Over time, the heart can undergo modifications such as hypertrophy in order to compensate for the new need for higher tension development in the ventricular wall.

1.3 Alpha1- Adrenergic Receptor

1.3.1 Alpha-receptor Characterization

There are 3 alpha receptor subtypes- alpha 1a/c, 1b, and 1d (Weinberg et al. 1994). Alpha adrenergic receptors are G-Protein Coupled Receptor (GPCRs) that mainly couple with Gaq proteins (Graham 1996). The protein structure is a single polypeptide chain made up of ~429 - 561 amino acids, similar to other members of the same GPCR family. The receptors couple mainly with Gaq -proteins that consequently activates beta-isoform of Phospholipase C (PLC-beta). PLC is an enzyme that cleaves Phosphotidylinositol-4, 5 Bisphosphate (PIP2) into Diacylglycerol (DAG) and 1, 4, 5-Inositol Triphosphate (IP3). IP3 is water soluble and can diffuse into the cytosol to the Sarcoplasmic Reticulum (SR) to activate ligand-gated Ca^{2+} channels and mediate Ca^{2+} release from SR stores into the cytosol. DAG is lipid soluble and thus stays close to the cell membrane, where it activates Protein Kinase C (PKC). Endogenously, they are activated by epinephrine and norepinephrine- catecholamines that are released in response to stress. Many agonists have been developed for alpha ARs. However, they lack specificity to any one subtype therefore making it difficult to determine which subtype the observed response can be attributed to.

1.3.2 Alpha1- AR Expression and Sensitivity in the Heart

Ligand binding studies show humans have similar expression of alpha1ARs in the heart as mice and most other species. However, rats have a 10-fold higher expression comparatively (Jensen et al 2009, Noguchi et al 1995). Messenger Ribonucleic Acid (mRNA) for all three alpha AR subtypes have been found in the human heart (Jensen et al. 2009). Expression levels of alpha AR subtypes vary between species (Cotecchia 2010). In humans, the alpha1a/c subtype is the dominant subtype whereas in rodents, it is alpha-1b that is highly expressed in the heart. While the alpha1d receptor subtype is expressed in very low levels in the myocardium itself, it is highly expressed in the coronary artery smooth muscle cells and activation causes vasoconstriction, which is when the lumen of the blood vessels reduces in diameter (Jensen et al. 2009). The alpha1b receptor subtype is also expressed in the endothelial cells of the coronary arteries and can cause vasodilation, which is when the lumen of blood vessels increases in diameter (Jensen et al. 2010).

1.3.3 Localization and Signalling Pathways of Alpha1 ARs

O'Connell et al (2014) review a novel view of GPCR signalling that occurs at the nuclear membrane and refer to it as "Inside Out" signalling. Alpha1ARs are thought to be primarily expressed on the plasma membrane because of ligand-binding studies (Fujita et al 2001). However, there is a lack of distinction between the membranes during the precipitation process which makes it hard to determine if the protein is on the plasma, sarcoplasmic or nuclear membrane. Studies have been done using a caveolar marker Caveolin-3, which is a vesicle that transports proteins from the SR to the plasma membrane and found roughly 20% of all alpha1ARs to be associated with caveolin-3 (Wright et al. 2008). When the nuclear membrane marker LAP2 is used, around 80% of all alpha1ARs were found to associate with it suggesting that a large proportion of alpha1ARs localize at the nuclear membrane (Wright et al. 2008).

Furthermore, lack of specific antibodies makes immunochemistry a less credible technique to determine alpha-AR localization as well. The Inside-Out theory is supported by the abundant presence of Organic cation transporter 3 (OCT3) in the heart which can transport catecholamines into the myocyte so that they can bind to the a1AR on the nuclear membrane. (Zwart 2001, Wright 2008). The well-known alpha antagonist prazosin is able to cross the plasma membrane freely as well.

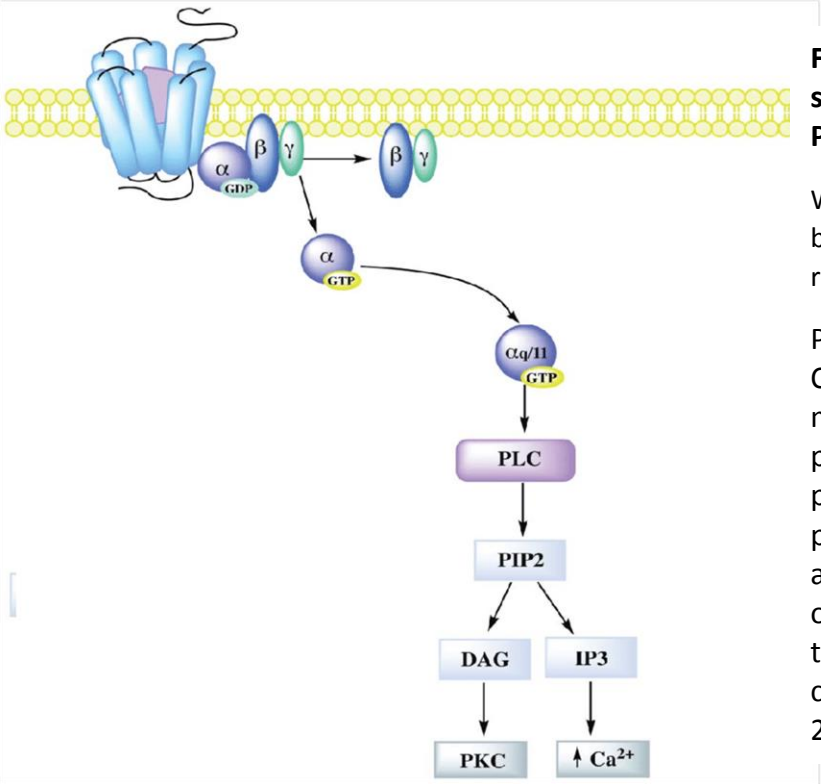

Figure 1.3- Generic signalling pathway for Gaq Protein Coupled Receptor.

When adrenalin/noradrenalin bind to the alpha adrenergic receptor,

Protein Kinase C (PKC) and Calcium (Ca^{2+}) act as second messengers for this pathway. PKC goes on to phosphorylate target proteins whereas can activate proteins and/or open channels to achieve the desired effect within the cell (González-Mariscal et al. 2017).

1.3.4 Effects of Activation in Normal Heart

Addition of alpha-adrenergic agonist, Methoxamine, increased cytosolic free Ca^{2+} in isolated cardiomyocytes, due to efflux of Ca^{2+} from intracellular stores indicating it may have a positive effect on contractility of the heart (Eckel 1991). Alpha adrenergic stimulation evokes different responses in the right and left ventricles (Wang et al. 2006, Chu et al 2013). In the right ventricle, stimulation with a non-specific alpha agonist Phenylephrine caused a “triphasic response”, where there was an initial brief rise in developed force, followed by the large dip, and then a rise and plateau (Wang et al. 2006). However, this was overall a negative ionotropic response. In contrast, the left ventricle showed a positive ionotropic response, but in the same triphasic waveform, where the force initially peaked briefly, and dipped and rose again to a plateau. The dip in the force was not as deep in the left ventricle as it was in the

right, and the rise to plateau was higher than the original baseline force, whereas in the right ventricle the plateau was lower than the baseline force. The literature conclude that the response is alpha1A specific because they saw a similar response to an alpha1A specific agonist A-61603. It is also proposed that the different subtypes represented in the heart have different effects- alpha1a AR subtype increases ionotropy (Ross et al., 2003; O-Uchi et al., 2008). Transgenic Overexpression of the alpha1a subtype in mouse hearts has a positive effect on contraction , whereas that of the alpha1b subtype has a negative effect on contraction (Lin et al 2001; Grupp et al., 1998; Wang et al., 2000; Iaccarino et al., 2001; Lemire et al., 2001) Transgenic knock-out of either of these subtypes has no effect on baseline cardiac function, suggesting that alpha1AR-mediated ionotropy is not needed in normal cardiac function (Rokosh and Simpson, 2002; Vecchione et al., 2002)

1.3.5 Alpha1-AR and Cell Death

In vitro studies involving cultured cardiac myocytes concluded that alpha1ARs are needed for cell survival signalling (Zhu et al., 2000). When these studies were translated in vivo, using transgenic mice that overexpress alpha1ARs or render them constitutively active, the results were variable (Du et al., 2004, 2006; Chaulet et al., 2006). On the contrary, studies that used transgenic mice that had loss-of function mutations in the alpha1ARs showed that these mice were worse off in conditions that simulated heart failure and ischemia, compared to the wild type counterparts, and they underwent pathological remodelling that largely involved cardiomyocyte cell death. They were able to come to the same conclusion as the in vitro studies, that alpha-1ARs are necessary for cellular survival. (O'Connell et al., 2006; Huang et al., 2007, 2008)

1.4 Ca^{2+}/Calmodulin-dependent protein Kinase II (CaMKII)

Calcium/calmodulin-dependent protein Kinase II (CaM Kinase II or CaMKII) is a protein kinase that phosphorylates proteins, and is also able to autophosphorylate, and activate itself. It is found in several parts of the body, mainly in the heart and the brain.

1.4.1 Structure of CaMKII

CaMKII is a dodecameric holoenzyme, which means that it is made up of 12 subunits that are arranged in two rings of 6 subunits. Each subunit is made up of four domains- one Ser/Thr-specific kinase domain, one regulatory segment, a linker region that varies in length between different isoforms, and one hub/association domain. The hub domains of each subunit associate together to form the two rings of six subunits. In humans, there are four isoforms of CaMKII- α, β, γ, and δ. These vary mainly in the length of the linker region and in their expression levels in certain tissues. (Bayer et al 2002, Chao et al 2011)

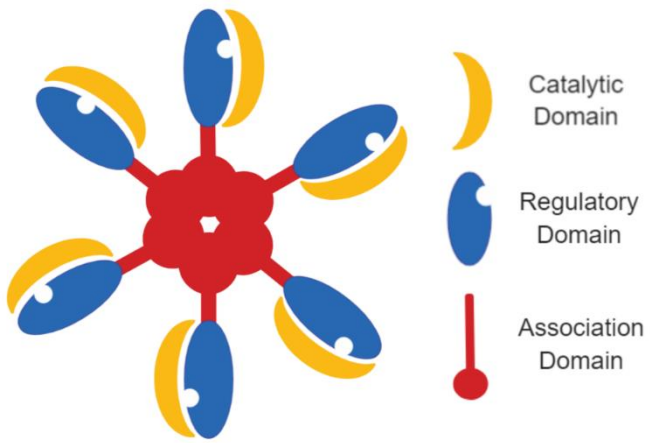

Figure 1.4- 2-D structure of CaMKII Protein.

Six subunits of CaMKII protein out of a total of twelve have been depicted in the image. Each subunit has three domains- Catalytic Domain (Yellow), Regulatory Domain (Blue) and Association Domain (Red)

1.4.2 Localization of CaMKII

The main CaMKII isoform in the heart is CaMKIIδ, of which there are two variants- $\delta_{3/B}$ and $\delta_{2/C}$. CaMKII$\delta_{3/B}$ has an 11-amino acid sequence that localizes the CaMKII variant to the nucleus, while CaMKII$\delta_{2/C}$ lacks this nuclear localizing sequence and thus is localized in the cytosol. In vitro studies where both variants are expressed in cells in varying ratios also show that increased expression of the one variant influences the localization of the other variant. Furthermore, the CaMKII$\delta_{2/C}$ is more abundantly expressed variant in the adult heart. (Schworer et al 1993; Gray and Heller Brown 2014).

1.4.3 Targets of CaMKII

CaMKII plays a role in regulating Ca^{2+}-handling proteins in the heart.

1.4.3.1 Ryanodine Receptor (RyR)

Ryanodine Receptor (RyR) is one of CaMKII's phosphorylation targets. The phosphorylation sites proposed are Serine-2809 and Serine-2815, both of which remain disputed by researchers who attribute the phosphorylation and resulting RyR regulation to Protein Kinase A (PKA). However, both sites are shown to be phosphorylated in heart failure when CaMKII has also been shown to have increased autophosphorylation, and diastolic Ca^{2+} leak from the SR in these rabbit heart failure models is reversed when CaMKII is inhibited, but not when PKA is inhibited, which supports the idea that CaMKII is a regulator of RyR (Maier et al 2007; Kohlhaas et al 2006). When CaMKII phosphorylates RyR, it increases the open probability of RyR by changing the conformation of the protein so that the pore size is greater and causes Ca^{2+} to leak out.

1.4.3.2 Phospholamban (PLB)

Another CaMKII target is Phospholamban (PLB), a protein that associates with the Sarcoplasmic/Endoplasmic Reticulum Calcium ATP-ase (SERCA) that pumps Ca^{2+} back into the SR during diastole to restore intracellular Ca^{2+} levels. PLB inhibits SERCA so that Ca^{2+} uptake into the SR is slower. However, when PLB is phosphorylated, its inhibitory effects on SERCA is reduced, and so Ca^{2+} uptake into the SR is faster. CaMKII phosphorylates PLB at Threonine-17, but PLB can also be phosphorylated by PKA at Serine-16. If Ca^{2+} is pumped back into the SR faster, the heart is able to relax faster as well (Maier et al. 2007)

1.4.4 Activation of CaMKII

When CaMKII is inactivated, the regulatory domain is bound tightly to the catalytic/kinase domain of the enzyme on each of the subunits and therefore protein activity is auto-inhibited. An increase in intracellular Ca^{2+} causes Ca^{2+} binding to calmodulin to activate it, which in turn acts as a second messenger to activate various kinases, one of which is CaMKII. Ca^{2+}/CaM bind to a site on the regulatory domain and cause a conformational change that exposes the catalytic/kinase domain. (Hudmon 2002)

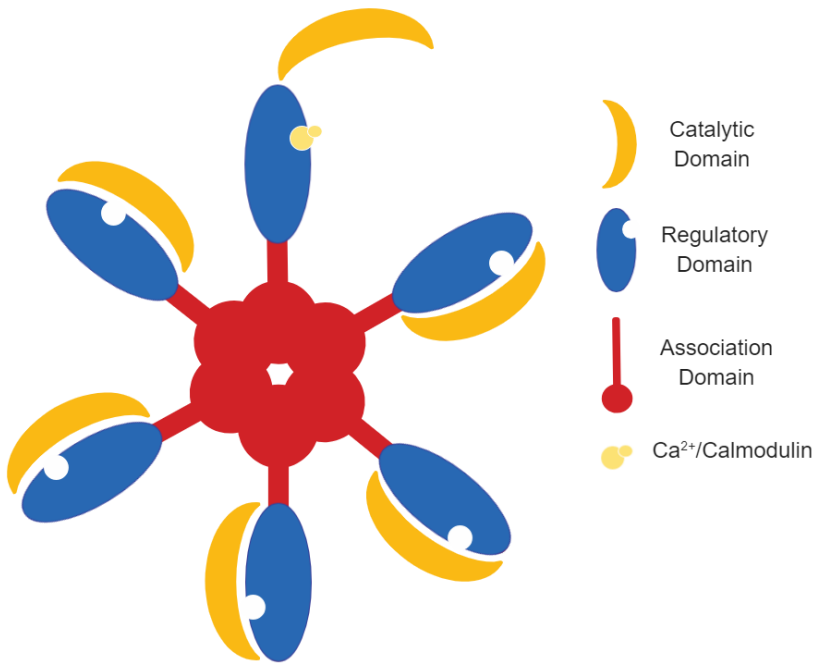

Figure 1.5- 2-dimensional depiction of the activation of CaMKII protein.

The image shows a calcium-bound calmodulin molecule binding to and acting on the regulatory domain of CaMKII.

The activation domain undergoes a subsequent conformational change and can phosphorylate target proteins.

1.4.5 Pathological Activation of CaMKII

1.4.5.1 Oxidation

CaMKII can also be activated by oxidation. In conditions where Reactive Oxygen Species (ROS) are elevated, such as inflammation or diabetes, CaMKII can be oxidised at Met 281 and 282 which not only makes it autonomously activated, it also allows for autophosphorylation of the holoenzyme so that it is pathologically active. Furthermore, ROS also reduce phosphatase activity, which makes it harder to reverse CaMKII autophosphorylation, as well as reverse phosphorylation of its targets. (Erickson et al 2008; Mollova et al 2015).

1.4.5.2 O-linked N-acetyl glucosamine

Prolonged high blood glucose (hyperglycemia) can also cause post-translational modification of CaMKII and of other proteins, altering their function. A post-translational modification where O-linked N-acetylglucosamine is added to a protein (O-GlcNAcylation; O-GlcNAc) is seen in states where glucose is high, such as in a diabetes state. In high glucose states, there is a high concentration of a sugar called Uridine Di-Phosphate-N-acetylglucosamine that is the substrate of O-GlcNAc. The addition of this sugar to proteins is catalysed by an enzyme called O-GlcNAc Transferase, and the removal is facilitated by O-GlcNAcase. CaMKII can undergo this modification on Threonine-286 and Serine-279. CaMKII has been shown to have increased activity in high glucose conditions, and the increased activity is abolished when an O-GlcNAc modification inhibitor, Diazo-5-Oxonorleucine, was added. When an O-GlcNAcase

inhibitor is added, CaMKII activity is increased, which provides strong evidence for the pathological activation of CaMKII in diabetes (Erickson et al 2013).

1.4.6 Autonomous Activity of CaMKII

The kinase domain can go on to phosphorylate and activate CaMKII's targets, or it can autophosphorylate on the Threonine-286/7 amino acid, which reduces the binding affinity of the regulatory domain for the catalytic/kinase domain. In other words, phosphorylation at the Threonine-286/7 site releases CaMKII autoinhibition, and thus the enzyme can remain active even when the intracellular Ca^{2+} concentrations are decreased, and Ca^{2+}/calmodulin is no longer bound to CaMKII (Hudmon 2002). Mutations introduced to substitute Threonine-286/7 with amino acids that cannot be phosphorylated showed that the CaMKII enzyme was still functional in the presence of Ca^{2+}/CaM but could not function autonomously. Therefore, this phosphorylation site is needed for CaMKII to function independently of Ca^{2+}/CaM (Hudmon 2002). The main benefit of autophosphorylation is to allow for potentiation of the signal when Ca^{2+} spikes occur in high frequencies. For example, when heart rate is high, depolarization occurs at a high frequency and the period between each spike of Ca^{2+} is short enough so that there isn't enough time for the activated CaMKII subunit to dephosphorylate and become inactive before the next spike occurs. Another subunit may become activated, and subsequently autophosphorylated by the first subunit. The significance of the varying length of the linker region between the different CaMKII isoforms is that if the linker region is short in length, the enzyme spends more time in a compact configuration that makes its Ca^{2+}/CaM binding site harder to access. A compact configuration means that the CaMKII needs a higher frequency of Ca^{2+} spikes to trigger autophosphorylation and activation of the enzyme. With a long linker region, the enzyme spends more time in an extended configuration that makes its Ca^{2+}/CaM binding site easier to access, so it is more likely to autophosphorylate and become activated, even at a lower frequency of Ca^{2+} spike

Furthermore, autophosphorylation has been shown to increase the affinity for Ca^{2+}/CaM by over 1000x (Hudmon 2002). This phenomenon has been referred to as CaM trapping. This sequence of Ca^{2+}/CaM binding, autophosphorylation, and CaM trapping gives CaMKII the ability to be sensitive to Ca^{2+} spike frequencies, which means that at higher frequencies it is able to tailor its enzyme activity to the frequency of spikes that occur (Hudmon 2002). This

can be beneficial in a healthy heart by allowing for potentiation of the Ca^{2+} signalling and thus, a stronger response.

1.4.7 Effects on Gene Expression

Overexpression of the CaMKII$\delta_{3/B}$ subtype develop hypertrophy but did not show an increase in phosphorylation of CaMKII's targets. It is thought that the nuclear localization of this subtype along with the lack of increased phosphorylation in the transgenic models imply that this subtype is more involved in gene expression, namely hypertrophic genes. (Zhang 2007; Gray and Heller Brown 2014).CaMKII$\delta_{3/B}$ is thought to promote cell survival through gene expression by upregulating expression of anti-apoptotic factors Bcl2 and inducible Heat Shock Protein 70 in stressful conditions (Little et al 2009; Peng et al 2010; Gray and Heller Brown 2014). The CaMKII$\delta_{2/C}$ variant has also been shown to activate the transcription factor HDAC4 (Zhang et al 2007). As opposed to CAMKII$\delta_{3/B}$, CaMKII$\delta_{2/C}$ is thought to promote cell death through gene expression, such as by upregulating p53 and NF-kB, both of which are pro-apoptotic transcription factors (Toko et al 2010; Ling et al 2013; Gray and Heller Brown 2014).

1.5 Aim and Hypothesis

The aim of this project was the determine the functional effects of the alpha-adrenergic agonist Methoxamine on isolated wild-type mouse hearts, and in isolated CaMKII knockout mouse heart, in incremental doses to observe differences in the responses between the two groups to determine the role of CaMKII in the functional response to alpha-adrenergic stimulus in the heart.

I hypothesize that in response to methoxamine, the contractility of the mouse hearts will increase because of the increased cytosolic Ca^{2+}. However, I hypothesize that the CaMKII knockout mouse hearts will show a diminished responsiveness to alpha stimulation and thus have a smaller increase in contractility compared to the wild-type mouse hearts because of the role that CaMKII plays in upregulating Ca^{2+}-handling proteins in the heart and because of CaMKII's potential to be part of the alpha-adrenergic signaling pathway in the heart.

Chapter 2: Methods

2.1 Ethics

Ethical Approval for this project was provided by the University of Otago Animal Ethics Committee under the ethics code ET09/15. The experiments were compliant with the committees Code of Ethical Conduct, and with the Animal Welfare Act 1999. Any genetically modified animals imported were approved by Environmental Protection Agency (EPA; formerly ERMA) and by the Institutional Biological Safety Committee (IBSC).

2.2 Animals

2.2.1 Animal Model

C57Bl/6 Mice species variant were used as wild-type mice, and a genetically modified strain of the C57Bl/6 mouse species where the CaMKII protein is knocked out were also used. These mice were house in the Hercus Taieri Research Unit (HTRU) in groups of up to four mice with *ad libitum* access to standard chow and water.

2.2.2 Groups

There were two groups in the experiment. The first group was the Control group, made up exclusively of Wild Type C57Bl6 mice (WT). The second group was the genetically modified group, made up exclusively of the CaMKII Knock Out mice (KO). There were 6 mice in each group.

2.3 Echocardiography

The mice were first anaesthetized with 3% Isoflurane in a gas chamber. Once the fur from the thorax was removed, a parasternal short-axis echocardiograph was performed (GE Vivid E9/EchoPAC PC system) to attain measurements for in-vivo heart function parameters.

2.3.1 In-Vivo Heart Function Parameters

2.3.1.1 LV Fractional shortening (FS; %)

Fractional shortening is a measure of how much the internal diameter of the left ventricle is reduced by when the heart contracts during systole, compared to when it is relaxed during diastole. It can be defined by the following equation (Chengode, 2016)-

$$FS\ (\%) = \frac{LVIDd - LVIDs}{LVIDd} \times 100$$

Where- FS = Fractional Shortening (%)

LVIDd = Left Ventricle Internal Diameter in diastole (cm)

LVIDs = Left Ventricle Internal Diameter in systole (cm)

2.3.1.2 Ejection Fraction (EF; %)

Ejection Fraction is a measure of how much of the volume of blood in the left ventricle at the end of diastole is pumped out of the ventricle when the heart contracts during systole. This gives us a way to measure how effectively the heart is contracting. It can be defined by the following equation (Chengode, 2016)-

$$EF\ (\%) = \frac{SV}{EDV} \times 100 = \frac{(EDV - ESV)}{EDV} \times 100$$

Where- EF = Ejection Fraction %)

SV = Stroke Volume (mL)

EDV = End Diastolic Volume (mL)

ESV = End Systolic Volume (mL)

2.3.1.3 Cardiac Output (CO; l/min)

Cardiac Output is a measure of the volume of blood that is pumped out of the left ventricle per minute. It can be defined by the following equation (Chengode, 2016)-

$$CO\ (mL.min^{-1}) = SV \times HR$$

Where- CO = Cardiac Output ($mL.min^{-1}$)

SV = Stroke Volume ($mL.beat^{-1}$)

HR = Heart Rate ($beats.min^{-1}$)

2.4 Heart Extraction

2.4.1 Pre-surgical Procedures

Prior to surgery, the mice were injected with 0.1 ml 10Units of Heparin in 0.9% Saline intraperitoneally. Heparin is an anticoagulant which minimises the coagulation of blood in the coronary arteries to allow better perfusion of the heart during the Langendorff. The tibia length was measured with digital callipers and blood glucose was measured from the tail tip using an AccuCheck glucometer after the heparin injection was given. The body weight was also recorded prior to anaesthesia.

2.4.2 Surgery to excise the heart

The mice were overdosed with 5% Isoflurane inhaled through a nose cone and their pedal withdrawal-reflex was checked to ensure successful anesthesia. The overlying skin on the thorax was removed, and a transverse incision was made just below the xiphoid process, inferior to the diaphragm. The diaphragm was then cut to gain access to the thoracic cavity. A timer was started to record the amount of time taken to cannulate the heart from when the diaphragm was cut. Two longitudinal incisions were made through the rib cage at the mid-clavicular line. The sternum and attached ribs were then reflected upwards to expose the heart. Fine surgical forceps and scissors were used to clamp the aorta at the arch and the aorta was cut. The heart was then removed with a small portion of the aorta exiting the heart still intact.

2.4.3 Arresting Solution

The heart was immediately placed in a warm, high potassium Krebs Henseleit Buffer solution. The high potassium concentration in the arresting solution would cause the heart to arrest by saturating the electrical gradient, and the other salts and glucose would allow the cells to continue being metabolically active and prevent cell death so that they can restore their function when the heart is mounted onto the Langendorff apparatus.

Table 2.1- Composition of Krebs Henseleit Buffer used in arresting solution

Components	Molecular Weight (kDa)	Concentration (mmol.L^{-1})
KCl	74.6	18.8
NaCl	58.4	118.5
MgO4S.7H2O	246.5	1.2
KH2PO4.H2O	136.1	1.2
NaHCO3	84	25.0
Glucose	198.2	11.0
CaCl	147	1.5

2.5 Langendorff Setup

The Langendorff setup was first developed by Oscar Langendorff in 1895 as a means to perfuse an isolated mammalian heart in a retrograde fashion (Bell 2011). Figure 2.1 shows a schematic representation of the Langendorff setup.

2.5.1 Perfusion Buffer

In order to keep the heart beating, the cardiac tissue must be supplied with a number of electrolytes, glucose, pH regulation, thermoregulation, and oxygen. The buffer used in these experiments was Krebs Henseleit Buffer (KHB) with the following composition-

Table 2.2- Composition of Krebs Henseleit Buffer used in perfusing solution

Components	Molecular Weight (kDa)	Concentration (mmol.L^{-1})
KCl	74.6	4.7
NaCl	58.4	118.5
MgO4S.7H2O	246.5	1.2
KH2PO4.H2O	136.1	1.2
NaHCO3	84	25.0
Glucose	198.2	11.0
CaCl	147	1.5

The buffer had a pH of 7.4 and was maintained at a temperature of 36.5°C-37.5°C by a water bath (Julabo GMBH, Seelbach, Germany). Carbogen (95% O_2 and 5% CO_2) was bubbled through the buffer to maintain the pH and to oxygenate it.

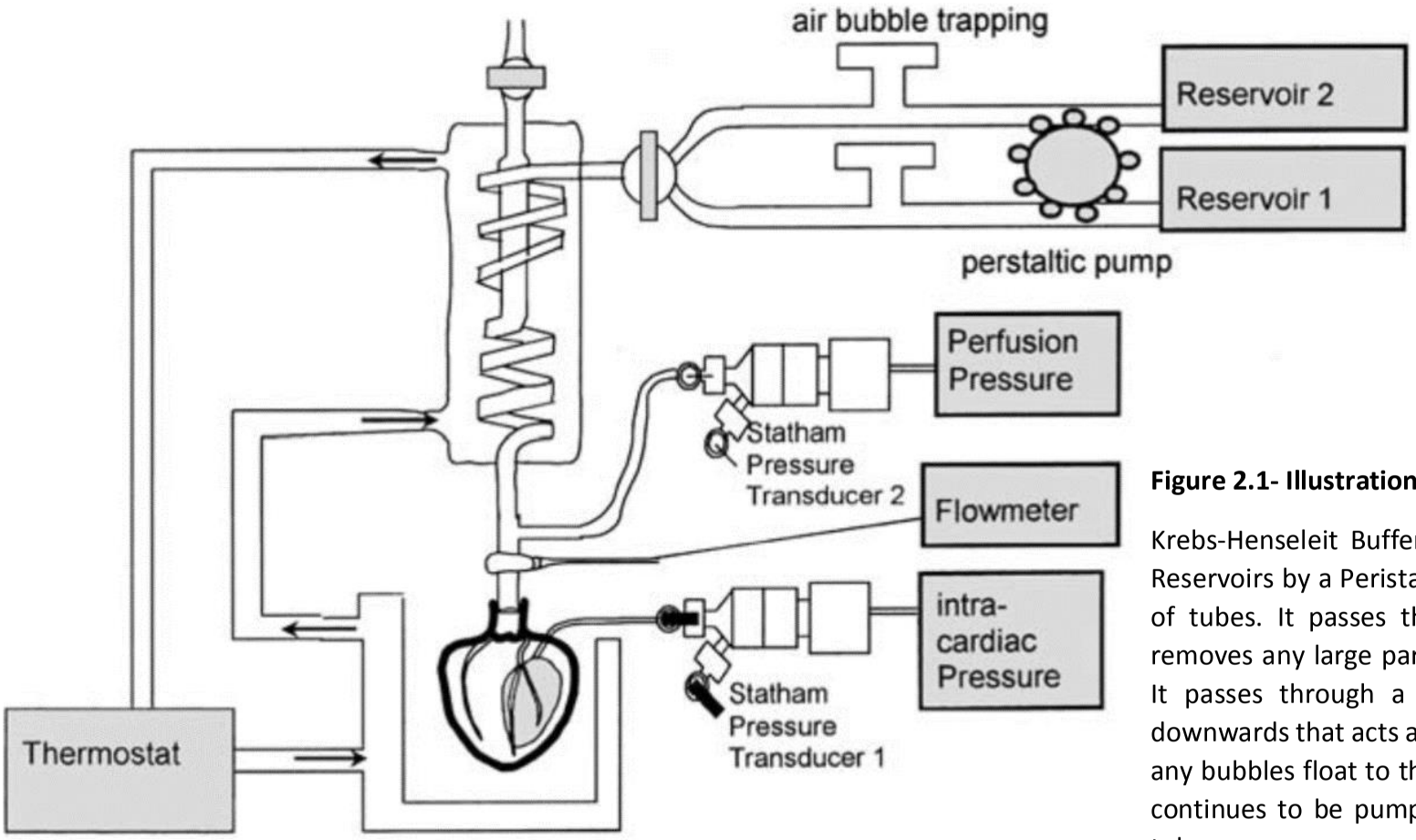

Figure 2.1- Illustration of Langendorff Setup

Krebs-Henseleit Buffer is pumped from the Reservoirs by a Peristaltic Pump into a series of tubes. It passes through a filter, which removes any large particles from the buffer. It passes through a glass tube that coils downwards that acts as a bubble-trap so that any bubbles float to the top while the buffer continues to be pumped down through the tubes.

A flowmeter measures the flow rate of the perfusate. A fluid filled tube that extends from the apparatus to a pressure transducer allows measurement of the perfusion pressure via displacement of fluid in the tube. The buffer exits via a cannula to which the aorta of the heart is tied on to. All the glass tubing in the apparatus is double jacketed, which means that it is covered by an extra glass compartment through which warm distilled water from the water bath is continuously flowing to keep the perfusate at optimal temperature.

The buffer was pumped through a series of tubes with the help of a peristaltic pump (ADInstruments, NZ). The perfusion pressure was kept constant at 75 mmHg, and flow was adjusted accordingly to maintain that perfusion pressure. The buffer was also passed through a 0.45 µM filter (Simplepure™ PES Syringe Filter, Membrane Solutions, WA, USA). A 10 mL syringe filled partly with air and partly with buffer was also incorporated into the set up, and acted as a Windkessel which helped reduce the pulsatility of flow in the same way that the complaint walls of the aorta would in-vivo. This allowed the coronary flow remained consistent during both systole and diastole so that there was consistent perfusion to the coronary arteries.

2.5.2 Basal Parameters

Two pressure transducers (Physiological Pressure Transducer, ADInstruments, NZ) allowed measurement of coronary pressure and left ventricular pressure. Information from both the pressure transducers was relayed to PowerLab and subsequently, the information was converted to digital data that is interpreted by the LabChart software.

The coronary pressure was detected by a fluid filled tube that inserted into the main perfusion system, close to the cannula that the aorta would be tied onto. Fluid in the tube was displaced when there was a change in the coronary pressure. The fluid displacement that was detected by the pressure transducer which allowed for the most direct reading of the coronary pressure.

The left ventricular pressure was detected by a fluid-filled balloon that was inserted into the left ventricle via the left atrioventricular valve upon removal of the left atrium. The balloon is empty when inserted, but gradually filled with distilled water to inflate it. 10-15 µL of distilled water was injected into the balloon, depending on the size of the balloon. When heart contracted, the balloon was squeezed, and displacement of the fluid was detected by the pressure transducer.

Coronary flow was measured by a flowmeter and transmitted via PowerLab to the LabChart software. A temperature probe was placed in a water bath that the cannulated heart was submerged in to ensure the heart was constantly at optimal temperature between 36.5°C and 37.5°C.

2.5.3. Cannulation

A 23-gauge needle was modified to have a flat tip, with circular grooves indented close to the tip was used as the cannula, as this was the correct diameter to fit a mouse aorta and the grooves allowed for the aorta to be tied on with surgical silk without sliding off the cannula. The heart was removed from the arresting solution and placed in a petri-dish with arresting buffer in it. The tip of the cannula was also submerged in the arresting buffer in the petri dish so as to minimize bubbles being introduced during cannulation. Surgical forceps were used hold the cut end of the aorta and slide it onto the cannula. Once the aorta was cannulated, it was held on the cannula with a bulldog clamp briefly before surgical silk was used to tie it on.

Once the heart was tied onto the cannula, the cannula was secured on a clamp that hung the heart into a small, double-jacketed glass water bath that had water at 37°C circulating within it, and warm, oxygenated buffer that the heart sat in. This allowed further regulation of the temperature immediately around the heart.

The coronary flow was then slowly increased until the coronary pressure was 75 mmHg, at which point the pressure was held constant. The heart was allowed to stabilize at this new pressure and flow for three minutes. The left atrium was removed and the empty pressure sensing balloon was inserted into the left ventricle, via the left atrioventricular valve. The tube attached to the balloon was taped to the cannula so that the balloon stayed in place. The balloon was filled with distilled water at 2 µL at a time, with at least one minute of stabilization time in between each fill. When the max volume was reached (~ 10 – 15 µL), the heart was allowed another stabilization period of five minutes.

2.5.4 Ex-Vivo Heart Function Parameters

LabChart software allowed for measurement and calculation of several parameters. These can either be used to determine the performance of the heart during systole (Systolic Function) or during diastole (Diastolic Function).

2.5.4.1 Systolic Function

Max Pressure is the maximum pressure developed during one ventricular cycle.

Systolic Duration is the time the ventricles spend contracting. It is calculated as the time between the End-Diastolic Pressure and the start of the Min dP/dT measurement on the ventricular cycle.

Max dP/dT is a measure of how fast the heart is able to contract, that is, the change in the pressure over time. LabChart calculates this as the steepest slope during the upstroke of one ventricular cycle.

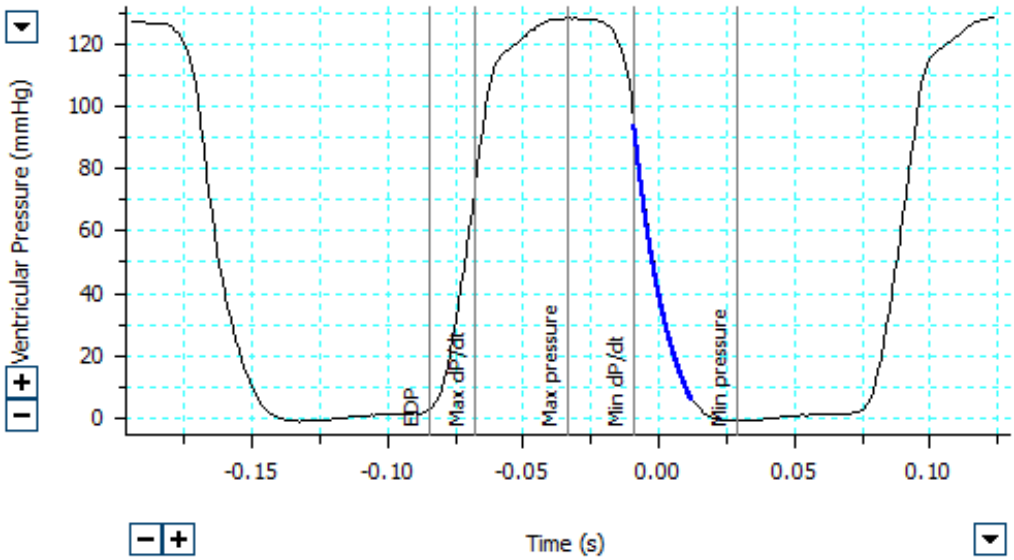

Figure 2.2- LabChart Analysis View of one ventricular pressure waveform. The measured parameters of End-Diastolic Pressure (EDP), Rate of Contraction (Max dP/dT), Maximum Pressure (Max Pressure), Rate of Relaxation (Min dP/dT), and Minimum Pressure (Min Pressure) are marked on the waveform.

Developed Pressure is how much pressure the ventricles are able to develop during contraction, calculated as the difference between the maximum pressure and the minimum pressure in one ventricular cycle.

2.5.4.2 Diastolic Function

Min Pressure is the minimum pressure reached when the ventricles relax during one ventricular cycle.

Diastolic Duration is the time the ventricles spend relaxing. It is calculated as the time between the start of the Min dP/dT measurement and the end of the ventricular cycle.

Min dP/dT is a measure of how fast the heart is able to relax. LabChart calculates this as the steepest slope during the down stroke of one ventricular cycle.

End-Diastolic Pressure (EDP) is pressure in the ventricle at the end of diastole when the heart has fully relaxed, and before the start of systole where the heart begins to contract. It is not

the lowest pressure in the cycle (which is the Min Pressure), because at the end of diastole the ventricles have filled with blood which raises the ventricular pressure slightly, even though the ventricles have not started contracting yet. LabChart calculates this geometrically to keep the EDP point consistent between all the waveforms.

2.5.4.3 Other Measures

Heart Rate is the number of times the ventricles contract in 60 seconds. LabChart calculates the heart rate as 60 divided by the duration of one ventricular cycle.

2.6 Experimental Protocol

2.6.1 Baseline Ex-Vivo Function

The heart was allowed to stabilize for 2 minutes after the balloon was inserted and filled. This was followed by a 3 minute baseline recording.

2.6.2 Methoxamine (MTX) Dose Curve

Once the heart was stabilized, a MTX dose curve was performed. Three doses were used; 10^{-8}, 10^{-6}, and 10^{-4}. The doses used were determined based on doses used in the literature. Schumann et al. (1975) used the 10^{-4} dose of MTX on rabbit papillary muscles and showed an increase in developed tension. It took approximately three minutes for a 10^{-4} dose of MTX to cause a significant increase in the developed tension in the rabbit papillary muscle, which was shown to be a slower effect compared to beta-adrenergic agonists (Schumann et al. 1975)

To perfuse the heart with MTX at 10^{-8}, 250 µL of a 10 µM stock solution was added to 250 mL of KHB solution that had already been warmed in the water bath and carbogenated. The solution was perfused through the setup and to the heart for 15 minutes, which allowed sufficient time for the solution to flow through the setup itself and reach the heart, as well as for the MTX to act on the alpha-adrenergic receptors in the heart tissue.

Similarly, when treating with MTX at 10^{-6}, 2.5 mL of a 100 µM stock solution was added to 250mL of the warmed, carbogenated KHB solution and perfused for 15 minutes, and when treating with MTX at 10^{-4}, 2.5 mL of a 10 µM stock solution was added to 250 mL of the warmed, carbogenated KHB solution and perfused for 15 minutes.

Between doses, there were no washout periods. Therefore, the doses were additive. However, the purpose of the dose curve was to elicit an incremental response to increasing doses of the drug, and the doses were increased 10-fold each time, making them an inconsequential addition to the next dose up. Thus a washout was not deemed necessary.

2.6.3 Tissue storage

At the end of the final dose perfusion period, without any washout period, the heart was removed from the cannula. The left ventricle, right atrium, and right ventricle were dissected and snap-frozen immediately in liquid nitrogen. This was done so that the MTX treated tissue could be used for future investigations into the acute effects of alpha stimulation on protein modifications in cardiac tissue. However, this was not looked at in this thesis.

2.7 Data Analysis

2.7.1 Echocardiography Data Analysis

Echocardiographs were analyzed on the GE Vivid E9/EchoPAC PC system to acquire raw values for in-vivo parameters (See Section 2.3.1). Five measurements were taken of the posterior wall and interventricular septum, in both diastole and systole. From this, the internal diameter of the left ventricle could be calculated for each of diastole and systole. Three measurements were taken from diastole of one cycle to diastole of the consecutive cycle, which allowed calculation of the heart rate. Figure 2.3 shows a cross sectional schematic of the heart from the echocardiograph. Figure 2.4 shows the points on the echocardiography trace where each of the in-vivo measurements were taken.

2.7.2 Langendorff Data Analysis

Data analysis was performed using LabChart Pro 7 to collect data points, Microsoft Excel 2016 to collate the data, and GraphPad Prism 7 to perform statistical analyses.

Data points were derived from the last 3 minutes of the 15 minute perfusion period set for each dose. LabChart produced the values for every parameter for every contraction within the selected region on the trace. The points were copied from the data pad to Microsoft Excel. The raw data averages were compiled on Microsoft Excel to calculate the change from baseline and fold change.

The raw data averages, change from baseline, and the fold change for each parameter were analyzed on GraphPad using a 2-way ANOVA to determine if there were differences between the WT and KO groups for each dose, as well as if there were changes between the increasing doses of MTX within each group. Significance was drawn at $p<0.05$.

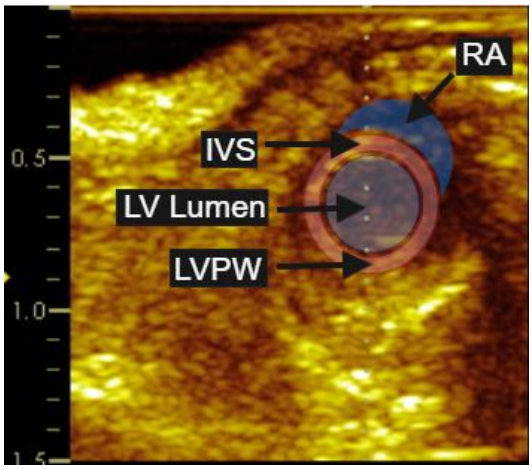

Figure 2.3- 2-dimension cross-sectional image of the mouse heart as seen on the echocardiograph.

Labelled on the image is the right atrium (RA; blue shading), the left ventricle lumen (LV Lumen; grey shading), and the myocardial wall (red shading) with the interventricular septum (IVS) and the left ventricular posterior wall (LVPW)

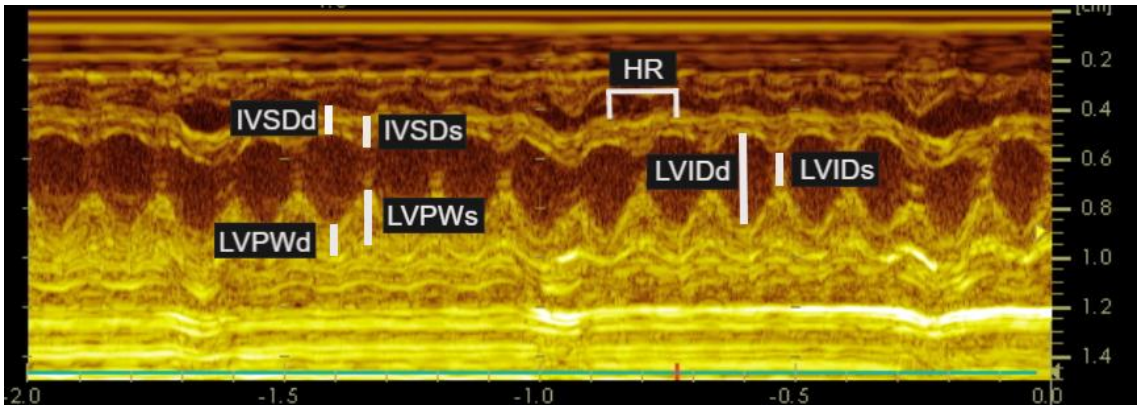

Figure 2.4- Example echocardiography trace from which in-vivo echo parameters were measured.

The following measurements were taken from the echocardiography trace- Thickness of the interventricular septum diameter during diastole (IVSDd) and during systole (IVSDs), thickness of the left ventricular posterior wall during diastole (LVPWd) and during systole (LVPWs), left ventricle internal diameter during diastole (LVIDd) and during systole (LVIDs), and heart rate (HR). The white lines represent where the measurements were taken from.

Chapter 3: Results

3.1 Basal parameters

There were 6 mice in each of the WT and KO groups. Average values, standard errors of mean, and p values have been presented in Table 3.1. Basal parameters of age, blood glucose, weight, and tibia length were measured for all mice used in the experiments. There was no difference in the age between the WT and KO groups or in the tibia length between the WT and KO groups. While there was no statistical difference in the blood glucose between the WT and KO mice, there appears to be a trend towards the KO mice having a lower blood glucose compared to the WT mice. The mice in the KO group had a greater weight on average compared to the mice in the WT group.

Table 3.2- Table showing the basal parameters of the WT and KO mice.

Parameter	Group		P Value
	WT (n=6)	KO (n=6)	
Age (days)	106.3 ± 7.233	114.8 ± 5.873	0.3831
Body Weight (g)	40.17g ± 2.203	31.2 ± 0.4066	**0.0025****
Blood Glucose (mmol/L)	12.08 ± 1.169	9.45 ± 0.3233	0.0550
Tibia Length (mm)	15.84 ± 0.4292	16.02 ± 0.4187	0.7640

WT = Wild Type Group; KO = Knock Out Group; all values displayed as mean ±SEM. Unpaired t-test used to compare groups for all parameters. * = $p<0.05$; ** = $p<0.01$.

3.2 In-vivo echocardiography parameters

The average values, standard errors of mean, and p values have been presented in Table 3.2. In-vivo parameters of FS, EF, CO, SV, HR, LVIDs and LVIDd were measured for mice from both groups. There was no difference between the mice in the WT and KO groups for any of the parameters measured during the echo.

Table 3.2- Table showing the in-vivo echocardiography parameters of the WT and KO mice.

Parameter	Group		P Value
	WT (n=3)	KO (n=4)	
FS (%)	41.48 ± 4.563	40.2 ± 3.125	0.8203
EF (%)	78.12 ± 4.651	76.96 ± 3.362	0.8424
SV (L)	0.08793 ± 0.00448	0.08337 ± 0.007655	0.6610
CO (L/min)	0.03673 ± 0.00444	0.0367 ± 0.003606	0.9959
HR (beats/min)	416.2 ± 40.35	443.2 ± 37.02	0.6455
LVIDd (mm)	2.072 ± 0.1688	2.096 ± 0.1832	0.9290
LVIDs (mm)	3.539 ± 0.01909	3.489 ± 0.1465	0.7839

WT = Wild Type Group; KO = Knock Out Group; all values displayed as mean ±SEM. Unpaired t-test used to compare groups for all parameters. * = $p<0.05$; ** = $p<0.01$.

3.3 Baseline parameters

During the baseline protocol, there was not difference between the WT and KO groups in any of the systolic parameters. The WT and KO groups showed no statistical difference in the max pressure (WT= 44.37 ± 7.124; KO= 35.02 ± 11.77; p= 0.5120), systolic duration (WT= 0.08522 ± 0.01302; KO= 0.1026 ± 0.01302; p= 0.3683), max dP/dT (WT= 1383 ± 222.4; KO= 994 ± 280.4; p= 0.3025), and developed pressure (WT= 37.54 ± 6.245; KO= 28.82 ± 10.95; p= 0.5045).

The diastolic parameters also showed no difference during the baseline protocol between the two groups. The WT and KO groups showed no statistical difference in the min pressure (WT= 6.83 ± 1.201; KO= 6.201 ± 1.056; p= 0.7025), EDP (WT= 8.132 ± 1.325; KO= 7.378 ± 1.152; p= 0.6767), diastolic duration (WT= 0.2434 ± 0.04489; KO= 0.303 ± 0.04328; p= 0.3614), and min

dP/dT (WT= -701.9 ± 90.8; KO= -556.4 ± 99.16; p= 0.3047). Heart rate also showed no difference between the two groups.

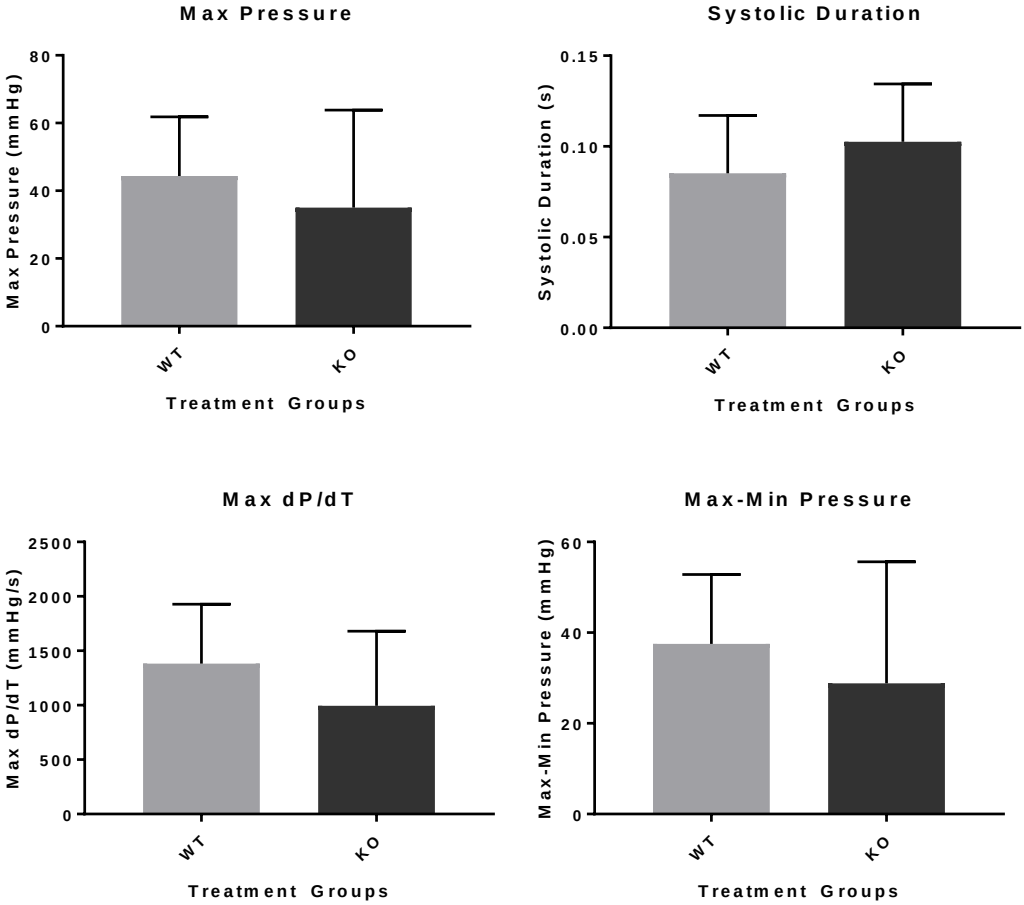

Figure 3.1- Graphs showing baseline comparisons of systolic function parameters. A) Max Pressure B) Systolic Duration C) Max dP/dT D) Max-Min Pressure. WT = Wild Type Group; KO = Knock Out Group; all values displayed as mean ±SEM. Unpaired t-test used to compare groups for all parameters. * = p<0.05; ** = p<0.01. n=6 for each group.

3.4 Ex-vivo heart function parameters

3.4.1 Systolic function

3.4.1.1 Maximum pressure

There was no statistically significant increase in the max pressure with increasing doses of methoxamine in both, the WT and the KO groups. In the WT group, compared to baseline, there is no significant change in the max pressure in response to MTX doses 10^{-8} (p= 0.9937), 10^{-6} (p= 0.9572), 10^{-4} (p= 0.2704). In the KO group, compared to baseline, there is no

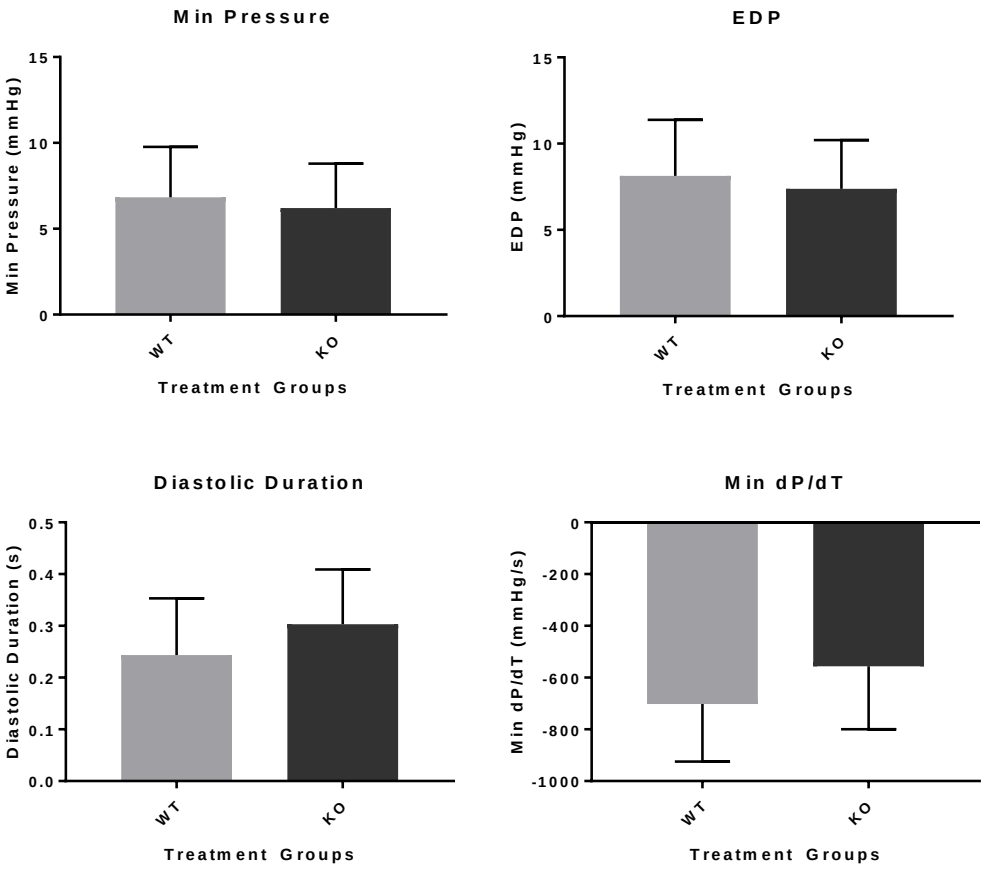

Figure 3.2- Graphs showing baseline comparisons of diastolic function parameters. A) Min Pressure B) EDP C) Diastolic Duration D) Min dP/dT. WT = Wild Type Group; KO = Knock Out Group; all values displayed as mean ±SEM. Unpaired t-test used to compare groups for all parameters. * = $p<0.05$; ** = $p<0.01$. n=6 for each group.

significant change in the max pressure in response to MTX doses 10^{-8} (p= 0.9987), 10^{-6} (p= 0.9991) and 10^{-4} (p >0.9999). There was also no difference between the WT and KO groups at the MTX 10^{-8} dose (0.9669 vs. 1.019; p= 0.9969), MTX 10^{-6} dose (0.9355 vs. 0.9826; p= 0.9980), and the MTX 10^{-4} dose (1.236 vs. 1.006; p= 0.5766).

3.4.1.2 Systolic Duration

In the WT group, there was no statistically significant change in the systolic duration in response to MTX 10^{-8} (p= 0.8621), MTX 10^{-6} (p= 0.5929), and MTX 10^{-4} (p= 0.9939). In the KO group, there was no statistically significant increase in the systolic duration in response to MTX 10^{-8} (p= 0.9900), MTX 10^{-6} (p= 0.9976), and MTX 10^{-4} (p= 0.9426). There was also no difference between the WT and KO groups at the MTX 10^{-8} dose (0.9315 vs. 1.027; p= 0.7694), MTX 10^{-6} dose (0.8894 vs. 0.9835; p= 0.7768), and MTX 10^{-4} dose (1.023 vs. 1.049; p= 0.9974).

3.4.1.3 Max dP/dT

There was a statistically significant increase in the max dP/dT in the WT group at the MTX 10^{-4} dose where there is an average 1.384-fold increase (p= 0.0114), which means the rate of contraction increased in response to the highest methoxamine dose in the WT mice. However, there was no significant change in the max dP/dT at the MTX 10^{-8} (p= 0.9103) and 10^{-6} doses (p= 0.8094). The KO group showed no change in the max dP/dT at the MTX 10^{-8} (p= 0.3105), MTX 10^{-6} (p= 0.2583), and MTX 10^{-4} (p= 0.5080) dose. There was also no statistically significant difference between the WT and KO groups at the MTX 10^{-8} dose (1.079 vs. 1.21; p= 0.9065), MTX 10^{-6} dose (1.106 vs. 1.224; p= 0.9348), and MTX 10^{-4} (1.398 vs. 1.167; p= 0.5561).

3.4.1.4 Developed Pressure

There was a statistically significant 1.407-fold increase in the developed pressure in the WT group in response to the MTX 10^{-4} dose (p= 0.0206), but not in response to the MTX 10^{-8} dose (p= 0.9908) or the MTX 10^{-6} dose (p= 0.9921). There was no significant increase in the developed pressure in the KO group in response to the MTX 10^{-8} dose (p= 0.8631), the MTX 10^{-6} dose (p= 0.9435), or the MTX 10^{-4} dose (p= 0.8750). There is also no statistically significant difference between the WT and KO groups at the MTX 10^{-8} dose (1.039 vs. 1.102; p= 0.9941), MTX 10^{-6} dose (1.037 vs. 1.073; p= 0.9993), or MTX 10^{-4} dose (1.407 vs. 1.099; p= 0.3174).

3.4.2 Diastolic Function

3.4.2.1 Min Pressure

The mice in the WT group showed a statistically significant decrease in min pressure with increasing methoxamine dose. At the MTX 10^{-8} dose, the min pressure decreases to 0.5418 of the baseline min pressure (p= 0.0299), at the MTX 10^{-6} dose, it decreases further to 0.3412 of the baseline value (p= 0.0011), and at the MTX 10^{-4} dose, it decreases more so to 0.2523 of the baseline value (p= 0.0002). However, in the KO group there is no significant decrease in the min pressure at any of the doses of methoxamine. At MTX 10^{-8}, the min pressure in the KO mice is 0.7438 of baseline (p= 0.3690), at MTX 10^{-6}, it is 0.6928 of baseline (p= 0.2204), and at MTX 10^{-4} it is 0.7811 of baseline (p= 0.5051). There is no difference in the change in min pressure between the WT and KO groups at any of the three doses.

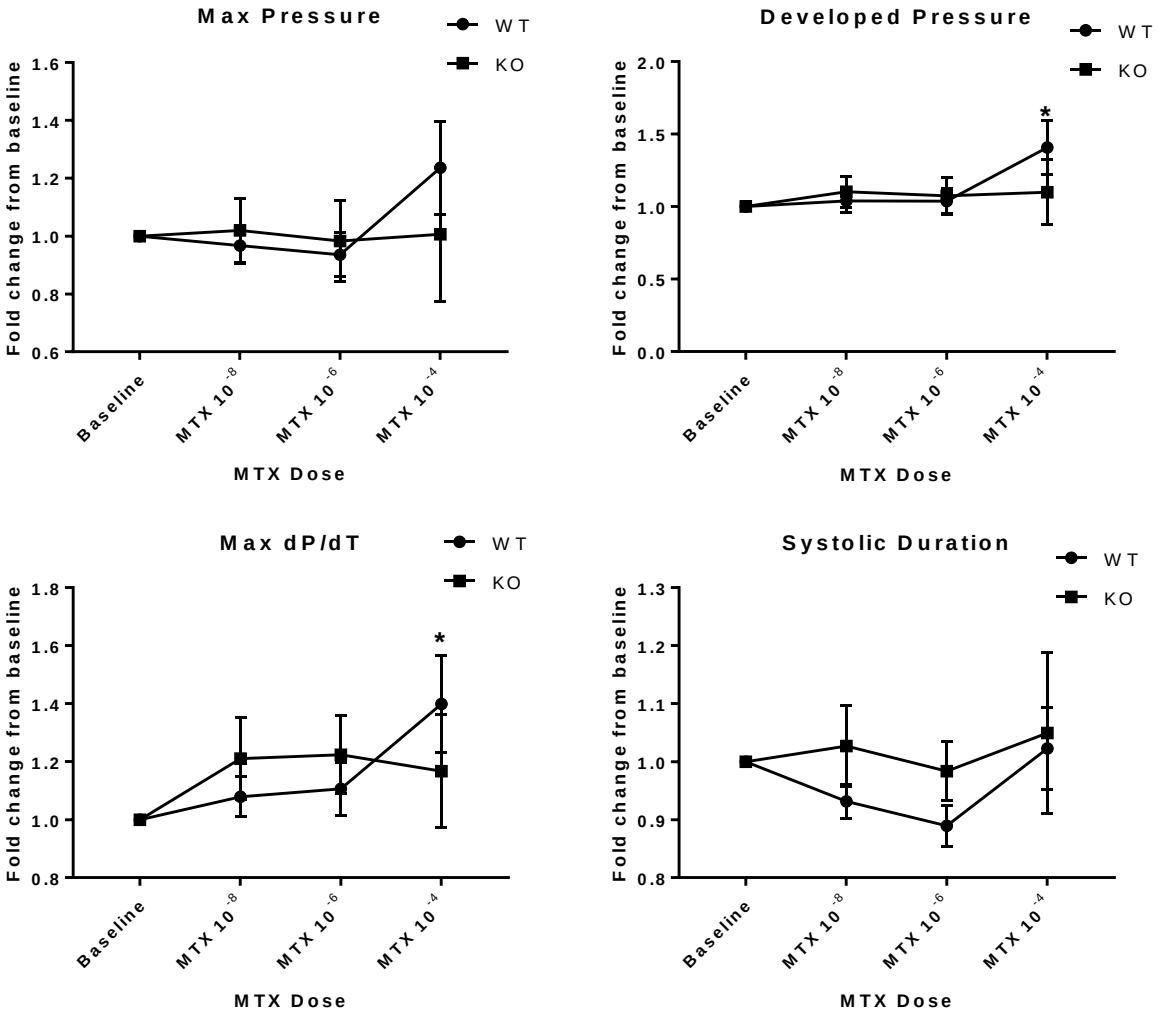

Figure 3.3- Graphs showing fold change in systolic function parameters in WT and KO mice in response to increasing MTX doses A) Max Pressure B) Developed Pressure C) Max dP/dT D) Systolic Duration. WT = Wild Type Group; KO = Knock Out Group; MTX= Methoxamine all values displayed as mean fold change from baseline ±SEM. Two-way Anova used to compare changes between WT and KO groups, and to compare changes between doses within each group. * = $p<0.05$ for WT group only compared to baseline; #= $p<0.05$ for KO groups only compared to baseline. + = $p<0.05$ n=6 for each group.

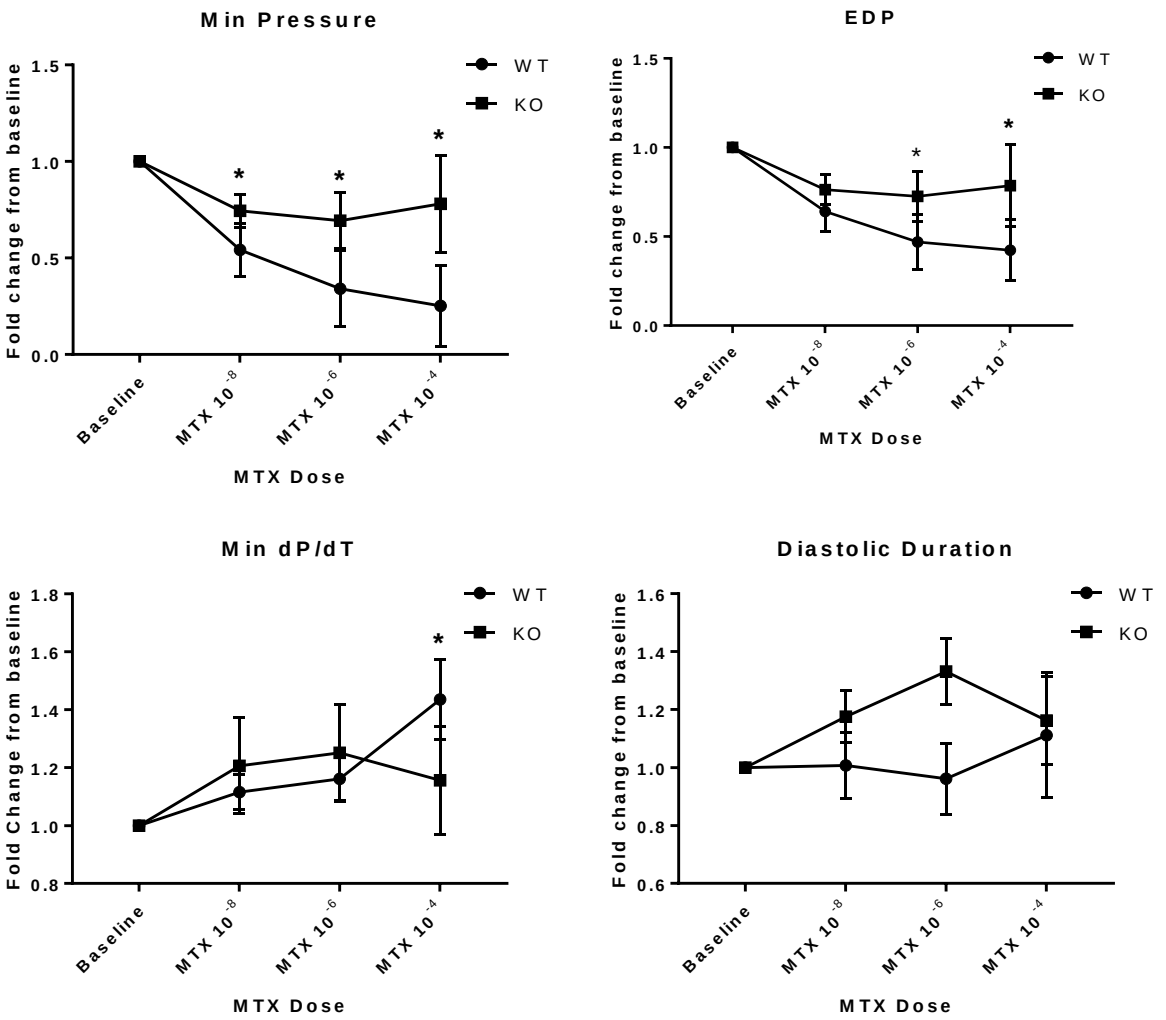

Figure 3.4- Graphs showing fold change in diastolic function parameters in WT and KO mice in response to increasing MTX doses A) Min Pressure B) End-Diastolic Pressure C) Min dP/dT D) Diastolic Duration. WT = Wild Type Group; KO = Knock Out Group; MTX= Methoxamine all values displayed as mean fold change from baseline ±SEM. Two-way Anova used to compare changes between WT and KO groups, and to compare changes between doses within each group. * = $p<0.05$ for WT group only compared to baseline; #= $p<0.05$ for KO groups only compared to baseline. + = $p<0.05$ n=6 for each group.

3.4.2.2 End Diastolic Pressure

The EDP showed a similar trend to the Min Pressure in that there was a significant reduction in the EDP in the WT group at the MTX 10^{-6} dose (0.4689-fold change; p= 0.0021) and MTX 10^{-4} dose (0.4237-fold change; p= 0.0008) compared to the baseline. However, there was no significant change at the MTX 10^{-8} dose (0.7634-fold change; p=0.0522). However, in the KO group, there was no significant change in the EDP at the MTX 10^{-8} dose (0.7634-fold change;

p=0.3030), MTX 10^{-6} dose (0.7423-fold change; p=0.1877), or the MTX 10^{-4} dose (0.786-fold change; p=0.3891). There was also no significant difference between the WT and KO groups at any of the three doses.

3.4.2.3 Min dP/dT

The min dP/dT showed a significant fold change of 1.435 at the MTX 10^{-4} dose in the WT group (p= 0.0066), which means the rate of relaxation was faster in the WT mice at the highest methoxamine dose. However, there was no significant change at the MTX 10^{-8} (1.115-fold change; p=0.7826) and MTX 10^{-6} (1.162-fold change; p= 0.5573) in the WT group. In the KO group, there was no significant change in the min dP/dT at the MTX 10^{-8} dose (1.206-fold change; p= 0.3483), MTX 10^{-6} dose (1.252-fold change; p=0.1909), or the MTX 10^{-4} dose (1.156-fold change; p= 0.5862). There was also no significant difference between the WT and KO groups at any of the three doses.

3.4.2.4 Diastolic Duration

In the WT group, there was no statistically significant change in the diastolic duration at the MTX 10^{-8} dose (1.007-fold change; p> 0.9999), or the MTX 10^{-6} dose (0.9611-fold change; p=0.9144), or at the MTX 10^{-4} dose (1.112-fold change; p= 0.8889). In the KO group, there was no statistically significant change in the diastolic duration at the MTX 10^{-8} dose (1.175-fold change; p=0.6751), or the MTX 10^{-6} dose (1.331-fold change; p= 0.1658), or at the MTX 10^{-4} dose (1.162-fold change; p=0.7250). There was also no statistical difference between the WT and KO groups at any of the MTX doses.

Chapter 4: Discussion

This thesis is concerned with the role of alpha-adrenergic receptor activity and the role of CaMKII in the heart. My project used an isolated heart perfusion system, where the alpha1-AR agonist, methoxamine, was used to address the first aim of this project- to determine the response of alpha-AR stimulation in the isolated heart of WT mice. The second aim of this project was to determine if CaMKII is involved in mediating the response to alpha-AR stimulation in the isolated heart, and CaMKII knockout mice were used.

I had hypothesized that there would be a positive contractile response, that is, increased max pressure, increased max dP/dT, and increased developed pressure in the WT mouse hearts when the alpha-AR agonist is applied. I also hypothesized that there would be a faster cardiac cycle, that is a shorter systolic and diastolic duration, and thus an increased in the heart rate in the WT mouse hearts. With respect to CaMKII involvement, I had hypothesized that CaMKII is involved in the mediating the effects of alpha-AR signalling in the heart, and thus the functional changes seen in the WT hearts in response to alpha-AR stimulation would be lost in the KO mouse hearts when methoxamine was added.

4.1 Does alpha-AR stimulation have functional effects in the isolated heart and is CaMKII involved in producing these effects?

4.1.1 Systolic Function

The WT mouse hearts were responsive to the highest dose of MTX, as seen in the increase in Max dP/dT and Pdev.

The rate of contraction increased in the isolated WT mouse hearts when 10-4 MTX was added to the perfusate. With this, we can conclude that alpha-AR stimulation increases the rate of contraction in the normal heart. However, this increase is not seen in the KO mouse hearts. This implicates CaMKII as a mediator for the increased rate of contraction in response to alpha-AR stimulation. This can be explained through the downstream proteins activated by CaMKII- namely, RyR and PLB. CaMKII phosphorylates RyR specifically on the site. By phosphorylating RyR, CaMKII increases its activity and thus Ca^{2+} release from the SR (Grimm and Brown, 2010). Therefore, in the knockout mouse hearts CaMKII is unable to carry out this function so the resulting increase in contractility is not seen.

The Pdev was higher in the presence of alpha-AR stimulation in the isolated WT mouse hearts when the 10-4 MTX dose was added to the perfusate. Therefore, not only does alpha-AR stimulation increase the rate of contraction, it also increases the pressure that the heart can develop with each contraction. Again, this was a phenomenon that was not seen in the KO mouse hearts, implicating CaMKII as a mediator for the increased developed pressure in response to alpha-AR stimulation. The positive effect of CaMKII on contractility of the heart was also shown by Daniels et al. (2018), whereby inhibition of CaMKII using KN-93 reduced the developed pressure in trabeculae from rat hearts (Daniels et al. 2018). It is unlikely that the increase in Pdev is due to an increase in the max pressure reached by the heart since we don't see a significant increase in max pressure in the WT mouse hearts. There is, however, a significant change in the min pressure at the highest dose of MTX in the WT hearts.

4.1.2 Diastolic Function

The WT mouse hearts were also responsive to the MTX doses when examining functional parameters such as Min Pressure, EDP, and Min dP/dT.

Min pressure reduces significantly when the lowest dose of MTX is added to the perfusate, and the min pressure continues to drop with increasing doses of MTX. This shows that the min pressure in isolated WT mouse hearts is sensitive to alpha-AR stimulation and that alpha-adrenergic signalling in the heart may be more involved in the regulation of diastolic function. This is reinforced by a similar trend seen in the EDP which shows a statistically significant reduction in the WT mouse hearts in response to increasing doses of MTX.

The reduction in both min pressure and EDP in the WT mouse hearts in response to alpha-AR stimulation is a phenomenon that is not seen in the KO mouse hearts. In the absence of CaMKII, alpha-AR stimulation does not cause an enhanced relaxation in the heart, implying that CaMKII may be causing the reduction in EDP and min pressure in response to alpha-AR stimulation. One of the targets of CaMKII that has not been mentioned in this thesis so far is the myofilament protein known as Titin. Passive tension in the cardiomyocyte is determined by the elastic protein titin, which acts as a spring that recoils during diastole. Phosphorylation of titin can alter its stiffness, and thus affect the passive tension in the cardiomyocyte. The higher the passive tension, the less relaxed the cardiomyocyte is during diastole. CaMKII has been shown to phosphorylate titin and reduce the passive tension in the cardiomyocyte, allowing for better relaxation (Hamdani et al. 2013). CaMKII KO models were shown to have

a higher passive tension at rest, whereas CaMKII overexpression models had lower passive tension compared to their WT counterparts (Hamdani et al 2013).

The rate of relaxation was significantly faster in the presence of alpha-AR stimulation but only at the highest MTX dose in the WT mouse hearts. Thus, alpha-AR stimulation not only increases the rate of contraction but also the rate of relaxation. This increase in rate of relaxation was not seen in the KO mouse hearts, even at the highest dose of MTX. This implies that CaMKII needs to be present for the response to occur, and thus may be a mediator for the increased rate of relaxation in a healthy heart. Again, considering the targets of CaMKII in the cardiomyocyte, this increased rate of relaxation can be explained by CaMKII phosphorylating PLB, which regulates the pump SERCA that is responsible for pumping Ca^{2+} back into the SR after contraction has occurred so that the cardiomyocyte can relax. PLB inhibits SERCA and acts as a break on its enzymatic activity. When CaMKII phosphorylates PLB, the break is released and SERCA is upregulated, which means that Ca^{2+} uptake into the SR after a contraction is faster. Thus, the cardiomyocyte relaxes faster (Maier et al. 2007).

4.2 CaMKII KO Animal Model

4.2.1 Generation of Knockout Model

The cardiac specific CaMKII-delta knockout mouse model was generated using Cre-LoxP to delete the catalytic domain of the CaMKII-delta protein. Embryonic Cells with the gene construct were injected into blastocysts that were implanted into mice. The offspring were crossed with Pro-Cre mice to produce offspring that had the CaMKII-delta allele with LoxP as well as the Pro-Cre allele. The Cre enzyme splices out the segment of DNA that is flanked by the LoxP segments. Thus, the catalytic domain of CaMKII delta would be spliced out rendering the kinase enzyme non-functional. Heterozygous offspring were crossed with one another until homozygous offspring were produced. These were genotyped using tail-tip samples and western blots were performed to confirm CaMKII knockout was successful. The western blots also showed that there was no compensatory increase in other CaMKII isoforms and that there was reduced basal phosphorylation of the CaMKII targets, PLB and RyR at their respective CaMKII phosphorylation sites (Ling et al. 2009). All these results establish that the knockout was successful.

4.2.2 Basal Parameters

The CaMKII KO model used in these experiments had been developed relatively recently, and little is known about their basal function. The only significant difference I found was between the body weights of the two groups. The WT mice weighed significantly more than then KO mice. There were no differences expected between the WT and KO mice. Researchers who have previously used the KO model have not noticed any significant differences between the KO and WT groups with regards to mortality or the presence of physical abnormalities. They did not compare the body weight between the two groups but did look at the heart weight normalized to the body weight and saw no differences between the two groups in control conditions (Ling et al. 2009).

4.2.3 In-vivo Echocardiography

There were also no significant structural or functional changes observed on echocardiography between the two groups which is consistent with what was previously shown in the literature (Ling et al. 2009). This tells us that the knockout of CaMKII did not cause any structural or functional abnormalities in the mouse that were detectable, and we can assume that the outcomes of the experiments could be attributed directly to the CaMKII protein and not any secondary effects caused by the knockout itself.

4.3 Langendorff Experiment Model

The Langendorff setup is a complex technique that demands prompt surgical excision and cannulation of the heart before excessive deterioration compromises the functionality of the heart.

4.3.1 Optimizing the setup

The optimal coronary flow and coronary perfusion pressure were obtained from current literature, as well as from past mouse Langendorff experiments performed by Jess Calverly (PGDipSci, Dept. of Physiology, University of Otago, 2017) and Dr. Rosalind Cook (PhD, Dept. of Physiology, University of Otago; previous Research Officer at Baker Heart and Diabetes Institute; current Product Specialist at Culpan Medical Pty Ltd.).

The use of Heparin, an anti-coagulant, was recommended by Dr. Cook prior to opening up the thorax for excision of the heart. The heparin preventing micro-clots from blocking the coronary circulation when the aorta was cut. Sodium Nitroprusside, a vasodilator, was added to the arresting solution so as to maximize exposure of the heart tissue to the arresting

solution and, once the heart was cannulated, allowed the perfusing solution to thoroughly perfuse all the coronary vessels to wash out and remaining blood. Despite measures taken to prevent clots from forming, it is likely that clots would have formed and prevented small areas of the myocardium from being perfused, thus compromising cardiac function. The sodium nitroprusside was allowed to wash out before alpha-AR stimulation with MTX was carried out, thus ensuring that it did not affect the results.

While the isolated heart setup aims to supply the heart with all the ions, metabolic requirements, oxygenation, and pH regulation that is needed for the heart to continue beating outside the animal model, it is an artificial setup and deterioration of the heart over time is inevitable. Thus, it was important to keep each protocol short enough so that the heart was not overly deteriorated by the end of the experiment. But, literature suggests that there is a temporal delay before the alpha-AR stimulation showed effects, and the time taken for the drug to pass through the Langendorff tubing to reach the heart had to also be accounted for. Thus, it was a balance between giving the drug enough time to take effect and also ensuring the heart wasn't hung up for so long that it functionally deteriorated.

4.3.2 Procedural and technical limitations.

While I have significant experience in this technique with rat models, adapting it to the much smaller mouse model was a challenge. The smaller and more fragile aorta was more difficult to identify during the surgical excision of the heart, and differentiating it from the other large vessels entering or exiting the heart was a challenge. This meant that a lot of trial and error was involved in not only perfecting the technique, but also cannulating the heart within the recommended time frame of <5 minutes (Bell 2011). Despite several tries, it was not always feasible to cannulate the heart within 5minutes, which means the hearts may have endured a small degree of ischemic preconditioning or hypoxic damage, which has the potential to affect the function of the heart when it was perfused.

Another challenge was being able to get the developed pressure to optimal values. The pressure developed by the left ventricle was detected with the fluid-filled balloon that was inserted into the ventricle through the left atrio-ventricular valve. Dr. Cook also provided, via personal communication, the technique used by her to make the smaller balloons. The balloons had to be frequently replaced due to their fragile nature, and thus the same balloon was not used for all the mouse heart Langendorff experiments. Different balloons of different

sizes were used, but the results were still valid as the response to alpha was compared to the respective baseline measure of the same heart. However, despite several attempts at optimizing the Langendorff, the developed pressure did not reach the expected value of 100mmHg. It is likely that this was a technical limitation of the mouse Langendorff setup due to the small size of the heart. While a balloon with a larger volume may have better detected the left ventricular developed pressure, the larger size would have made it harder to insert the balloon into the ventricular chamber without significantly damaging the cardiac tissue or applying a significant force which would in turn cause the aorta to rip, compromising the coronary flow.

4.3.3 Advantages and disadvantages of Langendorff setup

The Langendorff setup is a technically complex procedure but it also allows us to determine the effect that a particular protocol or treatment had on the heart itself, without any input from endocrine and neuronal systems. It gives a snapshot of the intrinsic function or dysfunction of the heart tissue itself, allowing us to better understand the organ in isolation.

This does not give us a realistic picture of how the organ functions in concert with the rest of the body. Mechanisms extrinsic to the heart may compensate for intrinsic dysfunction, such as by an increase in the sympathetic “fight or flight” input to the heart to upregulate its function. Thus, isolated heart experiments only provide a piece of a much larger puzzle.

4.4 Clinical Implications and Future Directions

Understanding CaMKII and its effects in the heart is important because it has been implicated in the pathogenesis of many cardiovascular diseases. By better understanding the functional effects and its associated signalling pathways, CaMKII can be targeted as a therapy for cardiovascular diseases.

4.4.1 CaMKII and alpha-adrenergic stimulation in diabetic cardiomyopathy

CaMKII has been shown to be activated by oxidative stress and by hyperglycemia, both of which are conditions seen in diabetes. Current literature has shown that there is an increased amount of phosphorylated and glycated, and therefore activated, CaMKII in diabetic hearts compared to non-diabetic hearts (Daniels et al. 2018). It was also shown that the impaired

contractility in diabetic hearts was partially restored when CaMKII was inhibited (Daniels et al. 2018).

Diabetes is also a hyperadrenergic state, whereby there is a reduction in the parasympathetic activity and an increase in the sympathetic input to the heart (Jia et al. 2018). Increased sensitivity to alpha-AR stimulation in diabetic rats was shown by Bussey et al. 2014. While diabetic mice were not used in these experiments, it could be beneficial to look at the determine whether CaMKII is playing a role in the pathology seen in diabetic cardiomyopathy by replicating the experiment in a diabetic mouse model and inhibiting CaMKII, or producing a diabetic CaMKII KO mouse and stimulating it with alpha-AR stimulation.

4.4.2 CaMKII and alpha-adrenergic stimulation in heart failure

Mouse models that were genetically modified to overexpress CaMKII developed heart failure, implicating its role in the pathogenesis of HF. The targets of CaMKII, PLB and RyR, also showed an increase in phosphorylation and therefore activation at CaMKII specific phosphorylation sites (Zhang et al. 2003). This was further supported by knock-out models that were protected from heart failure (Back et al. 2009). Increased CaMKII activity was also shown in failing human hearts (Hoch B et al. 1999). Further understanding how CaMKII plays a role in the pathogenesis of heart failure can allow us to target it to treat or slow the progression of heart failure.

4.5 Conclusion

In this thesis, I aimed to determine whether alpha-adrenergic receptor stimulation had any functional effects in the heart and found that it increase systolic parameters such as the rate of contraction and the developed pressure, and improved diastolic parameters such as by reducing the min pressure and EDP, and increasing the rate of relaxation. All of these functional changes were only seen in the healthy wild-type mouse hearts, and were lost in the CaMKII knockout mouse hearts, thus implicating CaMKII's role in mediating these functional changes in response to alpha-adrenergic stimulation. CaMKII has been shown to be upregulated in diabetic cardiomyopathy and heart failure, and implicated in their pathogenesis. Further research into the response to alpha-adrenergic stimulation in these disease state, and inhibition or knockout of CaMKII can provide stronger links between the alpha adrenergic signalling pathway and the CaMKII activation and signalling pathways, and potentially allow us to target CaMKII as therapy for cardiovascular disease.

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