CARDIOPROTECTIVE EFFECT OF Na^+/Ca^{2+} EXCHANGE INHIBITION IN CARDIOPLEGIC ARREST BY SEA0400

by

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Dedication

This thesis is dedicated to my parents, David and Shelagh Egar.

Thank you for showing me what it really is to keep fighting and persevere for the good when the odds are against you!

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ABSTRACT

This study investigated the effects of SEA0400, a Na^{+}/Ca^{2+} exchange inhibitor, in cardioplegia on rat myocyte contractile function. SEA0400 significantly reduced the accumulation of diastolic Ca^{2+} throughout cardioplegic arrest compared to ischemic control and cardioplegia. Cells treated with SEA0400 during cardioplegic arrest showed significantly larger Ca^{2+} transient amplitudes and contractions throughout reperfusion compared to cells treated with cardiopelgia alone. Intracellular Ca^{2+} stores were similar in both cardioplegic groups at baseline and during reperfusion. Together these results suggest that SEA0400 has beneficial effects at protecting ventricular myocytes during cardioplegic arrest and that SEA0400 in cardioplegia may affect myofilament Ca^{2+} sensitivity.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$[Ca^{2+}]_i$	Intracellular calcium concentration
$[Na^{+}]_i$	Intracellular sodium concentration
%	Percentage
°C	Degree Celsius
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
CABG	Coronary artery bypass grafting
CICR	Calcium induced calcium release
cm	Centimeter
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
EC-coupling	Excitation contraction-coupling
EC_{50}	Half maximal effective concentration
EGTA	Ethylene glycol tetraacetic acid
F	Fluorescence intensity
F0	Background
g	Gram
H^{+}	Hydrogen ion
Hz	Hertz
IP	Intraperitoneal
K^{+}	Potassium ion
KB-R7943	2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate
Kd	Dissociation constant
kg	Kilogram
L	Liter
Li^{+}	Lithium
M	Molar
mA	Milliamp
min	Minute
ml	Milliliter
mM	Milimolar
mm	Millimeter
mmHg	Millimeters of mercury
mos	Months
MPTP	Mitochondrial permeability transition pore
msec	Millisecond
mV	Millivolts
nA	Nano amp

N_2	Nitrogen gas
Na^+	Sodium
$NaHCO_3$	Sodium bicarbonate
$Na_2S_2O_4$	Sodium dithionite
NCX	Sodium-calcium exchanger
NHE	Sodium-hydrogen exchanger
nm	Nanometer
O_2	Oxygen
PCI	Percutaneous catheter intervention
pH_i	Intracellular pH
P_i	Inorganic phosphate
PLB	Phospholamban
pO_2	Partial pressure of oxygen
PTI	Photon Technology International
RyR	Ryanodine receptor
RM ANOVA	Repeated measures analysis of variance
SEA0400	2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline
sec	Second
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SR	Sarcoplasmic reticulum
t-tubule	Transverse tubule
µg	Microgram
µm	Micrometer
µM	Micromolar

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CHAPTER 1. INTRODUCTION

1.1 Overview

It is well established that cardiovascular disease is one of the leading causes of death among Canadians. Although the cardiovascular death rate in Canada has decreased by approximately 40% in the last decade, more than 69, 500 people die each year because of cardiovascular disease. As well, 275,652 people were hospitalized with ischemic heart disease, heart attack or congestive heart failure between 2005 and 2006. These numbers demonstrate that heart disease is still the leading cause of hospitalization in Canada and more than $20 billion is spent each year on hospitalizations for heart disease in Canada. Of the nearly 70 000 deaths per year, 54% are due to ischemic heart disease alone (Heart and Stroke Foundation of Canada, 2011).

Ischemic heart disease (myocardial ischemia) is a disease in which there is a partial or complete blockage of the coronary arteries reducing blood supply to an area of myocardium. This reduction in blood flow results in hypoxia and nutrient deprivation of the affected heart tissue as well as accumulation of metabolic byproducts such as lactate, hydrogen ions (H^+) and carbon dioxide (CO_2) (Neely and Feuvray, 1981). This reduction in blood flow can arise from coronary artery disease caused by atherosclerosis. The build up of cholesterol and triglycerides forms plaques that narrow the blood vessels, limits the blood flow to the heart and can ultimately become a clot which completely blocks blood flow to a certain area of myocardium. The most common manifestation of myocardial ischemia is angina pectoris. Patients who suffer from angina pectoris experience chest discomfort or pain. In advanced atherosclerosis the plaque can rupture and form a clot. When a sudden and severe block of the coronary arteries occurs, a person may experience

myocardial infarction (heart attack). Ischemic heart disease can damage the heart muscle and reduce its ability to efficiently pump blood, with the possible outcome of myocardial infarction and even death.

Treatment options for patients suffering from myocardial ischemia are directed at improving blood flow to the heart. Most commonly this includes percutaneous catheter intervention (PCI) to perform a balloon coronary angioplasty and stenting. For patients whose coronary artery syndromes are not amenable to PCI, surgeons will perform coronary artery bypass grafting (CABG) which requires cardiopulmonary bypass (open-heart surgery). Open-heart surgery is also performed on many patients with valve disease in which the valves need to be repaired or replaced as well on patients with atrial fibrillation and on young patients with congenital heart defects. As a result significant numbers of patients undergo open-heart surgery every year. In the United States, more than 500,000 heart surgeries are performed annually (U.S. Department of Health & Human Services 2012).

"Traditional" open-heart surgery requires the interruption of blood flow to the heart for a period of time. During this time the patient is put on cardiopulmonary bypass and the surgeon administers a cardioplegic solution that rapidly arrest the heart. Cardioplegic solutions provide the surgeon with a quiescent and visually optimal (blood free) heart on which to operate. During this period of cardioplegic, arrest the heart is also ischemic. Interestingly, upon reperfusion when blood flow is returned to the heart, undesirable consequences such as hypercontracture and cell death can occur (Piper et al., 1998; Carden and Granger, 2000). These consequences are termed ischemia-reperfusion injury. Therefore, complications with recovery of the myocardium after surgery are not

due necessarily to the cardioplegic and ischemic arrest, but caused in by the reintroduction of normoxic blood to the heart.

Ischemia-reperfusion injury is thought to be mediated by intracellular Ca^{2+} overload during ischemia and reperfusion (Nayler, 1981). Ca^{2+} homeostasis is critical in the heart. It is the direct interaction of Ca^{2+} with the myofilaments that causes contraction (Bers, 2002). Therefore a heart without Ca^{2+} does not function. However, too much Ca^{2+} can be just as detrimental, as a surplus of Ca^{2+} can cause ischemia-reperfusion injury, arrhythmias and contractile dysfunction (Murphy and Steenbergen, 2008). As such research is focused on developing strategies to regulate intracellular Ca^{2+} during ischemic arrest and prevent the damaging Ca^{2+} overload still present at the onset of reperfusion. One method is to control the Ca^{2+} influx via reverse mode Na^{+}/Ca^{2+} exchange (NCX) during ischemic arrest with a drug developed by Taisho Pharmaceutical Co., Ltd. (Saitama, Japan), 2-[4-[92,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400). SEA0400 has been shown to block reverse mode NCX and provide improved protection against ischemia-reperfusion injury in ventricular cardiomyocytes (Takahashi et al., 2003) and whole hearts (Motegi et al., 2007).

The following introduction will discuss the mechanisms of ventricular myocyte Ca^{2+} homeostasis and the role it plays in cardiac excitation-contraction (EC) coupling. As well, cardioplegic arrest and the effects of ischemia-reperfusion injury on myocyte calcium handling and contractile function will be covered. Strategies to improve myocardial function during reperfusion following cardioplegic arrest will also be discussed, with emphasis on drugs that inhibit Ca^{2+} influx during ischemia and reperfusion.

1.2 Cardiac EC-coupling

The conversion of the electrical signal of membrane excitation, in this case an action potential, into the mechanical response of myocardial contraction is termed cardiac excitation-contraction (EC) coupling (Bers, 2002). At rest cardiomyocytes have a membrane potential of approximately -80 mV driven primarily by potassium (K^+) concentrations inside (135 mM) and outside (4 mM) the cell (Levy, 2007). When a cardiomyocyte is depolarized to threshold (approximately -65 mV), Na^+ channels are opened and a large rapid influx of Na^+ causes the membrane potential to reach around +40 mV (Levy, 2007). After this rapid depolarization the Na^+ channels close and the cell repolarizes slightly with the opening of K^+ channels (Knollmann et al., 2007). Ca^{2+} channels, predominantly L-type Ca^{2+} channels, also open and allow Ca^{2+} entry into the cell. The Ca^{2+} entering the cell is called the inward Ca^{2+} current (Bers, 2002). After a delay, more K^+ channels open allowing K^+ to leave the cell resulting in repolarization. The Na^+-K^+ ATPase pumps 3 Na^+ out of the cell in exchange for 2 K^+ entering the cell to restore the proper Na^+ and K^+ gradients inside and outside the cell (Apell and Karlish, 2001).

Action potentials are initiated at pacemaker cardiomyocytes in the sinoatrial node and they propagate throughout the heart resulting in unified contractions. As previously stated contractions arise from a process termed cardiac EC-coupling. Figure 1.1 provides an overview of the EC coupling pathway. The action potential propagates along the sarcolemma membrane of heart cells depolarizing the membrane and opening voltage-gated L-type Ca^{2+} channels. These channels are found predominately within T-tubules, which are invaginations of the sarcolemma membrane. When the L-type Ca^{2+} channels

are opened, Ca^{2+} enters the cell and is referred to as inward Ca^{2+} current (Bers, 2002). The sarcoplasmic reticulum (SR) and sarcolemmal membrane form dyadic junctions so that the inward Ca^{2+} current easily interacts with ryanodine receptors (RyR) located on the SR. Ca^{2+} interaction with RyR causes these receptors to open and release Ca^{2+} stored within the SR. This is termed Ca^{2+} -induced Ca^{2+} -release (CICR) (Bers, 2002). The large influx of Ca^{2+} into the cytosol from the SR is referred to as the Ca^{2+} transient. The Ca^{2+} from the Ca^{2+} transient then binds to the myofilament protein troponin C, causing a contraction (Bers, 2002). Relaxation of the myofilaments occurs when the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) decreases, allowing Ca^{2+} to dissociate from troponin C. The decay of the Ca^{2+} transient occurs when Ca^{2+} is taken back up into the SR by the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) or when it is extruded from the cell via the NCX and Ca^{2+}-ATPase. A minimal amount of Ca^{2+} is also taken up by the mitochondria via the uniporter (Bers, 2002).

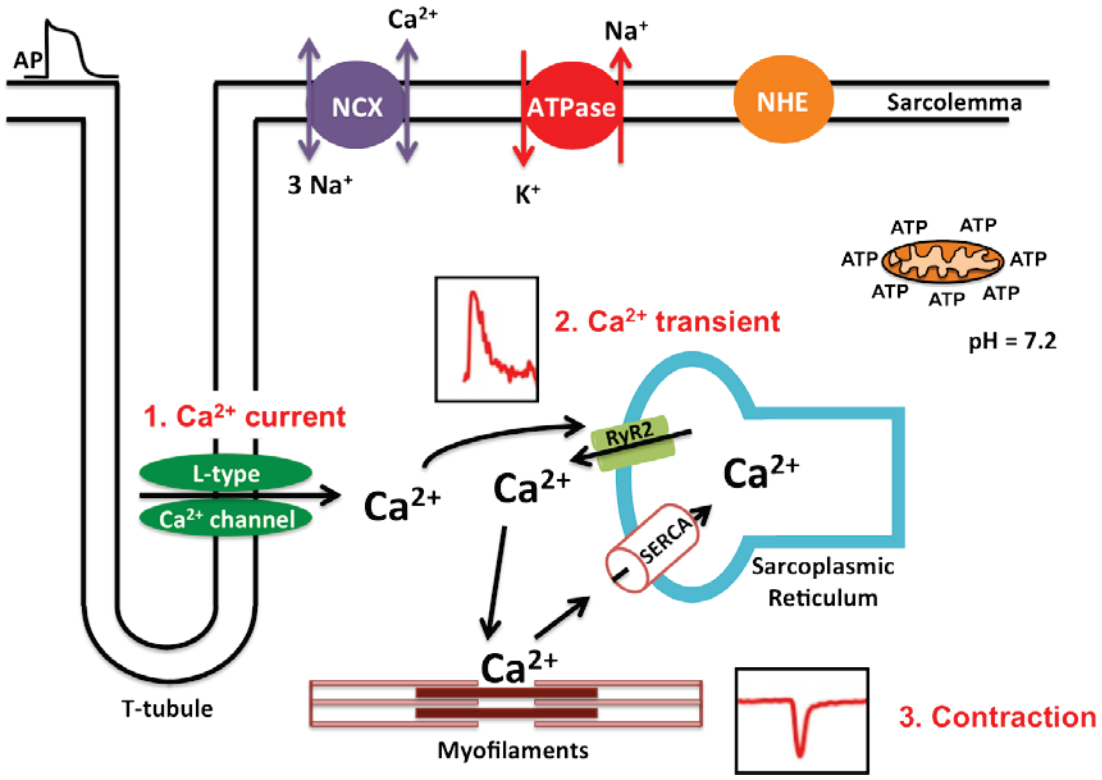

Figure 1.1 Schematic of the excitation-contraction coupling pathway in a ventricular myocyte. Propagation of an action potential along the membrane depolarizes the membrane and opens the voltage-gated L-type Ca^{2+} channel. This allows (1.) Ca^{2+} to enter the cell. The Ca^{2+} entering the cell interacts with the ryanodine receptors (RyR2) on the sarcoplasmic reticulum (SR) resulting in the release of Ca^{2+} from the SR through the RyR2. This Ca^{2+} -induced Ca^{2+} -release (CICR) is the (2.) Ca^{2+} transient. The increased $[Ca^{2+}]_i$ interacts with the myofilaments and causes (3.) contraction. Relaxation occurs when Ca^{2+} is taken back up into the SR by the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and is extruded from the cell via the Na^{+}-Ca^{2+} exchanger (NCX).

The force of contraction of the heart and the individual cardiomyocytes depends on the following factors: Ca^{2+} transient amplitude, Ca^{2+} transient duration and myofilament Ca^{2+} sensitivity (Bers, 2001). Myofilament Ca^{2+} sensitivity is reduced by acidosis and by high concentrations of phosphate and magnesium ions (Bers, 2002; Ferrari, 2002; Pinnell et al., 2007). All three of these factors are present during ischemia. As such, one would expect myofilament Ca^{2+} sensitivity to decrease as a result of cardioplegic arrest as the heart undergoes a period of ischemia.

1.3 Ischemia-reperfusion injury

1.3.1 Biochemical and mechanical changes during ischemia

As previously stated, during open-heart surgery, the blood flow to the heart is disrupted for a period of time resulting in hypoxia and ischemia. Figure 1.2 shows a schematic of the intracellular changes that occur during ischemia. When myocardium is subjected to an ischemic episode, metabolism shifts from aerobic to anaerobic glycolysis and lactic acid is produced as a byproduct. Since the blood flow to the heart is stopped, metabolites and waste cannot be washed away and there is a build up of lactic acid as a result. The resulting low intracellular pH stimulates proton extrusion through the Na^+/H^+ exchanger (NHE) during ischemia (Mentzer et al., 2003). The exchange of Na^+ entering the cell for H^+ leaving the cell leads to high intracellular Na^+ concentration ($[Na^+]_i$). During ischemia, the normally active Na^+/K^+-ATPase cannot restore the proper gradient of Na^+, as inadequate amounts of ATP are synthesized (Javadoc et al., 2009). As well, Sperelakis and Lee (1971) demonstrated that reducing intracellular pH to below 7.5

inhibits the Na^+/K^+-ATPase pump. The increasing $[Na^+]_i$ leads to activation of reverse mode NCX (Karmazyn et al., 2001; Allen and Xiao, 2003). Instead of removing one Ca^{2+} in exchange for three Na^+, the NCX will work to remove the build up of Na^+. Therefore, the increase in $[Na^+]_i$ that occurs during ischemia is responsible for the increase in diastolic $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ is exacerbated by the fact that the reduction in ATP synthesis also prevents the sarcolemma Ca^{2+} ATPase and SERCA from removing excess Ca^{2+} and sequestering Ca^{2+} into the SR (Javadoc et al., 2009). Eventually the pH of the cell is reduced to a point that both the NHE and the NCX are inhibited (Bers, 2001).

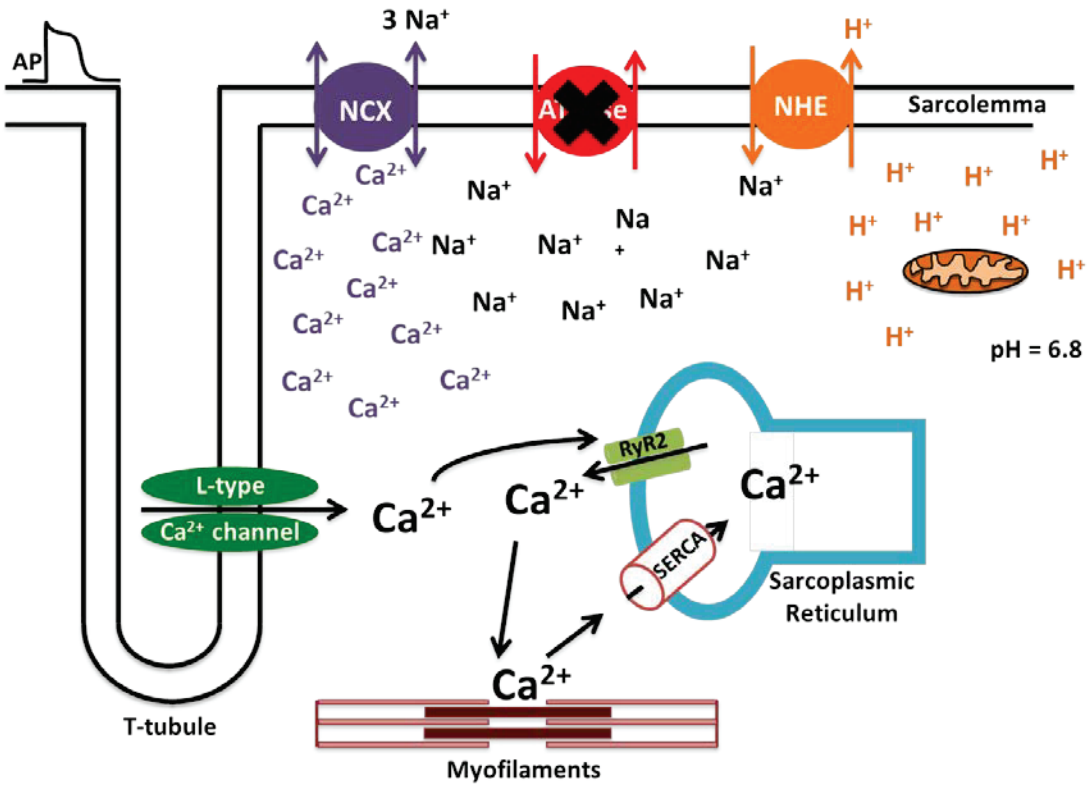

Figure 1.2. Schematic of intracellular changes during ischemia-reperfusion in ventricular myocytes. During ischemia metabolism shifts from aerobic to anaerobic resulting in an accumulation of H^+ leading to a decrease in pH to approximately 6.8. The Na^+-H^+ exchanger (NHE) will remove the excess H^+ in exchange for Na^+ entering the cell. This leads to Na^+ build up within the cell as the Na^+-K^+ ATPase is not fully functional and is unable to maintain the proper $[Na^+]_i$. The Na^+-Ca^{2+} exchanger (NCX) will begin working in reverse mode to remove 3 Na^+ in exchange for 1 Ca^{2+} entering the cell. This results in an increase in $[Ca^{2+}]_i$ that can lead to Ca^{2+} overload during ischemia and reperfusion.

As such, the contractile machinery of cardiomyocytes is also changed during ischemia, as contractions are terminated early in the ischemic period. Although the reduction in ATP synthesis is an obvious potential reason for the decline in contractile function, the time it takes for contractions to diminish is much quicker than the time it takes for the depletion of ATP (Lee and Allen, 1991). Research has indicated that myofilament Ca^{2+} sensitivity is reduced during ischemia, as peak contractions are reduced while peak Ca^{2+} transients remain unchanged (Marban et al., 1990; Bers, 2001; Louch at al., 2002). The accumulation of lactic acid and resulting low pH described above does have an inhibitory effect of on the contractility of the myocytes (Lee and Allen, 1991; Piper et al., 1998). As well, the accumulation of inorganic phosphate (P_i) that occurs during early ischemia as a result of ATP and creatine phosphate breakdown has been shown to reduce myofilament Ca^{2+} sensitivity (Kentish, 1986; Bers, 2001).

1.3.2 Ca^{2+} overload during reperfusion

Upon reperfusion, when blood flow to the myocardium is restored, there is a rapid normalization of extracellular fluid. As the pH is rapidly returned to 7.4, the NHE and NCX are reactivated. The NHE will work to return intracellular pH as quickly as possible, driving even more Na^+ into the cell and exacerbating the $[Ca^{2+}]_i$ overload that was initiated during early ischemia by reverse mode NCX activity. Ca^{2+} overload during reperfusion plays a critical role in myocardial dysfunction observed during reperfusion (Hendrikx et al., 1994). It is widely understood that this high $[Ca^{2+}]_i$ in early reperfusion is one of the causes of reperfusion injury after an ischemic episode (Tani &Neely, 1989; Bers, 2001; Murphy and Steenbergen, 2008). Elevated $[Ca^{2+}]_i$ can lead to arrhythmias,

hypercontracture, cell damage and cell death (Kloner and Jennings, 2001; Pogwizd et al., 2001).

When blood flow is reintroduced to the heart, ATP levels are quickly restored and contraction of the myofilaments can resume before Ca^{2+} homeostasis is restored. If contractile function is restored in the presence of elevated $[Ca^{2+}]_i$, arrhythmias can occur as a result of delayed afterdepolarization (Pogwizd et al., 2001). When ATP is available, SERCA can sequester the high $[Ca^{2+}]_i$ into the SR. However if the SR becomes overloaded with Ca^{2+}, it will spontaneously release Ca^{2+}. This spontaneous Ca^{2+} release can trigger the NCX to extrude Ca^{2+} in exchange for Na^+. If enough Na^+ enters the cell, the cell will depolarize and this will trigger a contraction (Levi et al., 1994). If arrhythmias do not occur, $[Ca^{2+}]_i$ can return to normal levels. However, in some myocytes, uncontrolled oscillations of $[Ca^{2+}]_i$ can lead to hypercontracture (Piper et al., 2004). Hypercontracture is the excessive contractile activation that occurs early in reperfusion as a result of normal ATP levels in the presence of elevated $[Ca^{2+}]_i$.

During ischemia and reperfusion when $[Ca^{2+}]_i$ is elevated, the mitochondria will help reduce Ca^{2+} overload by sequestering Ca^{2+} via the mitochondrial Ca^{2+} uniporter (Murphy and Steenbergen, 2008; Consolini *et al.*, 2011). The mitochondrial permeability transition pore (MPTP) is a non-selective voltage-gated channel, which, when opened, can allow the efflux of Ca^{2+} into the cytosol (Rizzuto *et al.,* 2000). Mitochondria also have NCX, which brings Ca^{2+} into the mitochondria when Na^+ concentrations are high such as during episodes of ischemia (Philipson and Nicoll, 2000). The release of Ca^{2+} through the MPTP leads to mitochondrial and cellular damage in reperfusion (Di Lisa and Bernardi, 2006; Petrosillo et al, 2009; Murphy and Steenbergen, 2008). As well,

mitochondria are known to release proapoptotic proteins when mitochondrial damage occurs (Petrosillo *et al,* 2009; McCord 1988).

1.4 NCX

There are three mammalian isoforms of the NCX; NCX1, NCX2, and NCX3 (Shigekawa & Iwamoto, 2001). All three isoforms are responsible for the countertransport of three Na^+ for one Ca^{2+}. The NCX1 isoform is found predominantly in the heart and to a limited extent in the brain. In contrast, the NCX2 and NCX3 isoforms are found predominantly in the brain. As such NCX1 will be the focus and referred to simply as NCX from here, as it is especially important in mammalian cardiomyocytes where Ca^{2+} homeostasis is crucial for maintaining cardiac EC coupling.

NCX is a transmembrane protein made up of 938 amino acids with 9 transmembrane segments present in the sarcolemma of cardiomyocytes (Blaustein & Lederer, 1999; Philipson & Nicoll, 2000). There is an extracellular N-terminus that is glycosylated and an intracellular COOH terminus. Between transmembrane segments five and six there is a long 550 amino acid intracellular loop that is conserved between isoforms.

The mechanism by which the NCX brings Ca^{2+} both into and out of the cell is not fully understood. It has been determined that the NCX is electrogenic and therefore can work in both forward mode, in which Ca^{2+} is extruded from the cell, and reverse mode, in which Ca^{2+} is taken into the cell. Which mode the NCX will operate in at a given time is dependent on membrane potential and the transmembrane gradients for Na^+ and Ca^{2+} (Cross *et al*, 1998; Maack *et al*, 2005). During systole, when the membrane is

depolarized to more positive membrane potentials the NCX functions in the reverse mode and bring Ca^{2+} into the cell (Cross *et al*, 1998). During diastole, when the membrane repolarizes to -80 mV, the NCX functions in the forward mode, moving Ca^{2+} out of the cell.

As previously stated, mitochondria also have NCX, although these mitochondrial NCX are different from sarcolemmal NCX (Bers, 2008). Mitochondrial NCX are very slow at removing Ca^{2+}, as this exchanger is extruding Ca^{2+} against a large electrochemical gradient because $[Ca^{2+}]_i$ is similar to mitochondrial Ca^{2+} concentrations (Bers, 2008; Murphy and Eisner, 2009). There is some controversy as to whether the mitochondrial NCX exchanges two or three Na^+ for one Ca^{2+} (Bers, 2008). As well, it has been found that mitochondrial NCX can exchange Na^+ or Li^+ for Ca^{2+}. The mitochondrial NCX can be blocked by the drug 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepine-2(3H)-one (CGP-37157). Although CGP-37157 does not block sarcolemmal NCX, it has been shown to block L-type Ca^{2+} channels (Thu le et al., 2006).

1.5 Cardioplegia

Although open-heart surgery is performed to help a patient lead a healthier and more comfortable life, it is clear that the period of time the blood flow is disrupted to the heart has the potential to cause problems. The ischemic episode is critical to provide the surgeon with a still heart on which to operate, as well as to provide a clear visual field. Unfortunately, a leading cause of mortality and poor outcome after heart surgery is inadequate protection of the myocardium throughout the ischemic period leading to reperfusion injury (Piper et al., 1998; Weman et al., 2000). For decades now, surgeons

help protect the myocardium during surgery by infusing a cardioplegic solution into the heart (Vinten-Johansen et al., 2000). Once the aorta is cross-clamped and the blood flow is redirected to the heart-lung machine, a catheter is inserted into the aorta, coronary sinus or coronary ostia, depending on the surgery that is to be performed. The cardioplegic solution is then delivered throughout the coronary arteries through the catheter. The purpose of cardioplegic solutions is to quickly minimize the metabolic demands of the myocardium, thereby decreasing anaerobic metabolism and ultimately limiting the ischemia-reperfusion injury while arresting the heart (Buckberg et al., 1977). Since the electro-mechanical activity accounts for the majority of myocyte metabolic activity, rapidly stopping the heart from beating decreases the severity of acidosis. Acidosis is the main driving force for Ca^{2+} accumulation inside the cardiomyocyte leading to Ca^{2+} overload, hypercontracture and cell death. The most common cardioplegic solutions contain high concentrations of K^{+}. A high K^{+} solution outside the cell depolarizes the cell membrane, inactivating the majority of the Na^{+} channels and thereby preventing the initiation and propagation of action potentials (Gay, 1975). Without action potential initiation, contractions are prevented and the heart is arrested in diastole (Vinten-Johansen et al., 2000). As a result, no contractions occur and the surgeon has a still heart to operate on while at the same time minimizing the injury caused by lack of blood flow and oxygen.

There are several different kinds of cardioplegic solutions. For instance, some cardioplegic solutions are administered as a crystalloid solution while others are mixed with blood. As well some cardioplegic solutions are administered as a hyperthermic or tepid solution (10°C or 24°C respectively) while others are administered warm at body

temperature (Fremes et al., 1985; Chocron et al., 2000). The benefit of administering cardioplegia as a hypothermic solution is that hypothermia further decreases the metabolic demands of the heart and allow for discontinuous administration of the cardioplegic solution. Warm blood cardioplegia, on the other hand, has the disadvantage of requiring either continuous perfusion or more frequent administration of the solution, which can cause a technical challenge.

A prospective study done by Mallidi et al. (2003) in which data were collected from patients who underwent isolated CABG surgery between November 1989 and February 2000 was performed to determine the effects of cold, warm and tepid cardioplegic solutions. The study concluded that perioperative death and myocardial infarction were more common in patients that had received cold blood cardioplegia in comparison to those who received warm blood. It is important to note that the majority of patients who received cold blood cardioplegia had been treated earlier in the 11 year time period and those treated with warm blood cardioplegia had been treated more recently. As well the solutions used were either chosen randomly or were surgeon's preference.

Another a study compared warm and cold blood cardioplegia also administered to patients undergoing CABG procedures in which the levels of cardiac troponin-I were measured. It was found that patients who underwent cold cardioplegic arrest had higher amounts of troponin-I release, which is indicative of cell damage (Skarysz et al., 2006). As such, warm blood cardioplegia might provide better cardioprotection if the surgeon can work around the frequent or continuous administration of solution.

There are also differences between crystalloid and blood cardioplegic solutions. Crystalloid cardioplegic solutions typically have high K^+ concentrations like most

cardioplegic solutions (approximately 20-30 mmol/L), while the Na^+ and Ca^{2+} concentrations are markedly lower than physiological extracellular fluid (Cohn, 2008). Crystalloid cardioplegic solutions must contain a buffer such as bicarbonate. An advantage to crystalloid cardioplegia is the bloodless operating field. In contrast, blood cardioplegic solutions mix the patient's blood (when available) with the cardioplegic solution. Blood has hemoglobin that carries oxygen and therefore helps the solutions remain oxygenated and better oxygenates the myocardium. As well, blood contains buffers that better maintain pH, as well as electrolytes and endogenous antioxidants and free radical scavengers (Cohn, 2008; Follette et al., 1978). Studies have shown that both crystalloid and blood cardioplegic solutions are successful at protecting the myocardium (Guyton et al., 1985; Ovrum et al., 2010).

1.6 Cardioplegia additives

The high K^+ cardioplegic solutions used today are effective at protecting the myocardium during ischemic arrest brought on during open-heart surgery. However, as previously stated, some patients suffer from arrhythmias, cell damage, contractile dysfunction and even death after surgery. As such, research has focused on improving the protective strategies during surgery to further protect patients from ischemia-reperfusion injury. Among these strategies are the inclusion of cardioplegic additives that block channels and exchangers in the myocytes, which contribute to the detrimental $[Ca^{2+}]_i$ overload. This section will focus on NHE and NCX inhibitors, as these exchangers are the main players in $[Ca^{2+}]_i$ overload.

1.6.1 NHE Inhibition

The sarcolemmal NHE is a membrane bound glycoprotein that transports one Na^+ into the cell in exchange for one H^+ out of the cell. Therefore the NHE is an electroneutral exchanger and is responsible for regulating intracellular pH (pH_i), $[Na^+]_i$ and cell volume (Haworth et al., 2003; Cingolani and Ennis, 2007; Vila-Petroff et al., 2010). There are ten isoforms of the NHE (NHE-1 to 10) (Karmazyn, 2011). NHE-1 is ubiquitously expressed in mammalian cells and is also the principal NHE isoform expressed in the heart. It is also important in cardiac cell growth. For the remainder of this thesis NHE-1 will be referred to simply as NHE, as the other nine isoforms have not been found in the mammalian heart. NHE has 12 transmembrane spanning domains made up of 500 amino acids, as well as a long cytoplasmic carboxy-terminal domain consisting of 315 amino acids. The 12 transmembrane domains are responsible for proton extrusion and contain the H^+ sensor site, while the hydrophilic cytoplasmic carboxy-terminal domain contains regulatory sites. When pH_i is around 7.4 (normal physiological pH), the NHE activity is not significant. However, when the pH_i drops to below physiological values, the proton sensor on the intracellular loop activates the NHE so as to bring the pH_i back to around 7.4. The sensitivity of this proton sensor can be enhanced by endothelin-1 (ET-1), angiotensin II (AngII) and α 1-adrenergic agonists (Karmazyn et al., 2001; Cingolani and Ennis, 2007; Karmazyn, 2011). NHE also acts as an anchor for other proteins including the actin cytoskeleton (Meima et al., 2007).

The NHE is constitutively phosphorylated and if phosphorylation increases, so does its activity. If NHE is stimulated, there will be an increase in $[Na^+]_i$ which will result in the NCX functioning in reverse mode. The elevated Na^+ levels not only bring

Ca^{2+} into the cell and can result in Ca^{2+} overload, but can also cause edema of the cell leading to myocardial dysfunction and cellular apoptosis (Meima et al., 2007).

Studies have shown that inhibition of NHE may reduce myocardial edema and Ca^{2+} overload by inhibiting the increase in Na^{+} that ultimately leads to the increase in fluid and $[Ca^{2+}]_i$. Amiloride was one of the first inhibitors developed to block NHE. Amiloride is a K^{+} sparing diuretic that blocks NHE-1, NHE-2, NCX and Na^{+} channels (Meima et al., 2007). Therefore, other derivatives of this compound were developed to be more specific inhibitors of NHE-1.

HOE694 (cariporide) is one such compound, whose main target is NHE-1 (Scholz et al., 1995). Studies showed that, when NHE was blocked with cariporide (10 μM) in the well-oxygenated guinea-pig heart, there was no change in $[Na^{+}]_i$. Once hearts were subjected to ischemia plus cariporide there was a marked decrease in $[Na^{+}]_i$. During reperfusion, cariporide significantly delayed pH recovery time. As well, left ventricular pressure, end-diastolic pressure and phosphocreatine levels fully recovered in hearts that had been treated with cariporide, whereas control hearts only partially recovered (Hartmann and Decking, 1999). Further studies administered cariporide intravenously and orally in anesthetized rats undergoing coronary artery ligation (Scholz et al., 1995; Kusumoto et al., 2001). The results showed that there were no negative effects systemically, and once again confirmed the protective effect of cariporide. This led researchers to develop a trial of cariporide for patients undergoing cardiac surgery.

The GUARDIAN trial (Chaitman, 2003) demonstrated that cariporide reduced mortality in high-risk patients undergoing CABG surgery. This led to the EXPEDITION study where 5770 high-risk patients undergoing CABG were treated with cariporide or

placebo. The patients were randomly assigned to either the placebo or the treatment group. The cariporide group received 180mg/h one hour before surgery, followed by 40 mg/h for 24 hours and 20 mg/h over the subsequent 24 hours. The placebo group received placebo infusions at the same frequency. Mentzer et al. (2008) assessed death or myocardial infarction at 5 days, 30 days and 6 months. Overall, the data showed that cariporide significantly reduced death or myocardial infarction by 3.7% (20.3% placebo treated group vs 16.6% cariporide treated group) at 5 days post-surgery. However, upon further analysis it was determined that mortality in the placebo group was only 1.5% in comparison to 2.2% ($p=0.02$) in the cariporide-treated group. The increase in death was not due to myocardial infarction; rather it was caused by cerebro-vascular strokes. Thus, although cariporide was shown to be cardioprotective, this unfavourable side effect profile resulted in the termination of its use. As a result research has shifted to look further downstream at NCX as a target.

1.6.2. NCX Inhibition

NCX is another pharmacological target for reducing ischemia-reperfusion injury, as it plays a central role in the $[Ca^{2+}]_i$ overload that leads to ischemia-reperfusion injury (Marban et al., 1994; Cordeiro et al., 1994; Cross et al., 1995; Piper at al., 1998). As well NCX has been shown to play an important role in contractile dysfunction and the formation of arrhythmias during reperfusion (Studer et al., 1994; Pogwizd et al., 1999; Murphy and Steenbergen, 1999). As previously discussed, Na^+ accumulation in the cell during ischemia and reperfusion results in reverse mode NCX, exchanging 3 Na^+ for 1 Ca^{2+}. As such, blocking the NCX would help prevent the detrimental Ca^{2+} buildup seen

during ischemia-reperfusion. The NCX blocker 2-(2-(4-(4-nitrobenzyloxy)phenyl)ethyl)-isothioureamesylate (KB-R7943) was developed by Watano et al. (1996), who reported that KB-R7943 was approximately 30 times more selective for reverse mode NCX than forward mode NCX. Further research, however, has shown that the initial experiments were performed under conditions favouring one mode of NCX activity. When conditions favouring both modes equally were examined, it was determined that KB-R7943 most likely blocked forward and reverse mode equally (Kimura et al., 1999; Lu et al., 2002). Although extensive research using KB-R7943 has shown that blocking the NCX is protective against ischemia-reperfusion injury when used in low concentrations (Barrientos et al., 2009; Macdonald and Howlett, 2008), Iwamoto et al. (2007) has shown that it is not highly selective for NCX as it also blocks RyR on the SR, sarcolemma L-type Ca^{2+} current and K^{+} currents (Tanaka et al., 2002).

A more recent NCX blocker derived from aniline 2-[4-[(2,5-difluorophenyl)-methoxy]phenoxy]-5-ethoxy-aniline (SEA0400) has been developed by Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). SEA0400 has been investigated as a potentially more selective inhibitor, as it selectively blocks NCX without altering various K^{+} and L-type Ca^{2+} currents (Matsuda et al., 2001; Tanaka et al., 2002). As well, it has been shown that SEA0400 is a potent blocker of NCX, and concentrations as low as 1 μM improved postischemic recovery and blocked more than 80% of sarcolemma NCX. In comparison, KB-R7943 required a higher concentration (eg.10 μM) to elicit the same percentage of NCX blockade (Tanaka et al., 2002). What is more, concentrations of 5 to 20 μM KB-R793 caused rapid and marked depressions of heart rate and left ventricular developed pressure (Magee et al., 2003). The EC_{50} value for SEA0400 was determined to

be 31 nM for inward NCX current (Ca^{2+} extrusion) and 28 nM for outward NCX current (Ca^{2+} influx) (Wang et al., 2006). Although SEA0400 is markedly more selective and potent when compared to KB-R7943, it has been shown that, at concentrations higher than 1 μM, SEA0400 does inhibit L-type Ca^{2+} current (Birinyi et al., 2005).

Studies have shown that SEA0400 protects against ischemia reperfusion injury in cellular and whole heart animal models. For example, SEA0400 protects mouse cardiomyocytes from Ca^{2+} overload during ischemi-reperfusion (Wang et al., 2006). As well, SEA0400 reduced infarct size in rat and rabbit hearts after ischemia-reperfusion (Magee et al., 2002). NCX also is known to contribute to arrhythmia development during reperfusion, when NCX extrudes excess Ca^{2+} from the cell, resulting in large Na^{+} influx. This can cause afterdepolarizations while the cell still has high $[Ca^{2+}]_i$, resulting in arrhythmias. Interestingly, when NCX is blocked with SEA0400 in dogs, researchers saw a marked decrease in arrhythmias (Nagy et al., 2004). In another study in an ischemia-reperfusion model of anesthetized rats, 1 mg/kg SEA0400 administered 1 minute before reperfusion significantly reduced the incidence of ventricular fibrillation from 80% in control hearts to 30% in treated hearts. As well, mortality declined from 70% in control to 20% in SEA0400-treated animals (Takahashi et al., 2003).

These results demonstrate that SEA0400 has beneficial effects on ischemia-reperfusion injury in both whole heart and isolated myocyte models. As such, SEA0400 is a good candidate to propose as a cardioplegic additive. In the initial study, Matsuda et al. (2001) reported that SEA0400 was rapidly cleared from plasma, but that it could cross the blood brain barrier. Therefore, adding SEA0400 to the cardiopelgic solution, rather

than administering the drug intravenously as they did with cariporide, could help prevent negative systemic side effects.

1.7. Objectives and Hypothesis

It is clear the NCX plays a central role in mediating the Ca^{2+} overload that occurs during ischemia-reperfusion (Xiao et al., 2003; Murphy and Steenbergen, 2008). Studies using SEA0400 to block the reverse mode of the exchanger during ischemia-reperfusion have shown that it is cardioprotective (Matsuda et al., 2001; Tanaka et al., 2002; Magee et al., 2003; Wang et al., 2006). A previous student in the lab conducted experiments using a whole rat heart model and showed that adding SEA0400 to cardiopelgic solution increased recovery of left ventricular developed pressure upon reperfusion when compared to control hearts. As well, the study showed that hearts treated with SEA0400 had reduced troponin release, indicating less cellular damage throughout the ischemic episode compared to control hearts (unpublished Ali, 2011). **The overall objectives of this study were to further evaluate the NCX blocker SEA0400 as a cardioplegic additive and determine the mechanism of protection in an isolated cell model. It is hypothesized that SEA0400 will limit Ca^{2+} accumulation during ischemia and reperfusion, resulting in improved contractile recovery and cell survival during reperfusion.**

The specific objectives were:

1) Develop an isolated cell model that reflects the ischemic conditions that occur during cardioplegic arrest.
2) Determine if $[Ca^{2+}]_i$ differs when SEA0400 is added to cardioplegia.

3) Determine if contractile function differs when SEA0400 is added to cardioplegia.
4) Determine the effect of SEA0400 on $[Ca^{2+}]_i$ and contractile function under normoxic conditions.
5) Determine if SR Ca^{2+} stores change during reperfusion in cells subjected to SEA0400 during cardioplegic arrest.

CHAPTER 2. METHODS

2.1. Animals

All experimental protocols were approved by the Dalhousie University Committee on Laboratory Animals and conformed to the guidelines published by the Canadian Council on Animal Care (CCAC; Ottawa, ON; Vol. 1, 2nd edtition, 1993; Vol. 2, 1994). Young adult male Fischer 344 rats (≈ 3 mos, ≈ 300 g) were obtained from Charles River Laboratories (St. Constant, QC). Animals were housed 2 per cage with a micro-isolator lid at the Carleton Campus Animal Care facility at Dalhousie University. All rats were allowed a minimum of 24 hours to acclimatize to animal care facility before experimental use. Rats were maintained on a 12-hour light/dark cycle and had free access to food and water. No supplemental treats were given to animals used. Rats were randomly selected on experiment day and placed in a clean cage to be transported to the laboratory.

2.2. Chemicals

HEPES buffer, EGTA, $MgCl_2$, anhydrous DMSO, and caffeine were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Fura-2 AM was obtained from Invitrogen Inc. (Burlington, ON, Canada). SEA0400 was obtained as a gift from Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). All other chemicals were purchased from BDH Inc (Toronto, ON, Canada). A stock solution of fura-2 AM was prepared by adding 20 µl of anhydrous DMSO to 50 µg of fura-2 AM to obtain a final concentration of 0.2% DMSO which was then stored at -5°C until use. A stock of SEA0400 was prepared by

dissolving 3.714 mg in 10 ml of DMSO to obtain a final concentration of 0.1% DMSO which was then stored at 4°C until use. All other chemicals were dissolved in deionized water.

2.3. Ventricular Myocyte Isolation

To anaesthetize the rats and prevent blood coagulation during myocyte isolation, sodium pentobarbital (IP, 200 mg/kg CDMV; Saint Hyacinthe, QC) was injected immediately followed by heparin (IP, 1000 U/300 g; Pharmaceutical Partners of Canada, Richmond, ON). Once unconscious (≈ 5 min) rats were weighed and then placed in the supine position on the surgical table. Once anaesthesia was confirmed by the absence of the pedal withdrawal and corneal reflexes, the forelimbs were secured using masking tape. The thoracic cavity was opened with an incision below the diaphragm followed by lateral cuts to the rib cage on the right and left side. The rib cage was folded back to expose the heart and the pericardium was removed. A silk suture (A-55, Ethicon Inc., Somerville, NJ) was placed around the aortic trunk and the aorta was cut, cannulated *in situ* and secured using the silk suture. The cannulated heart was removed from the thoracic cavity and attached to the perfusion apparatus.

The hearts were perfused retrogradely at 16 ml/min with a Ca^{2+}-containing perfusion buffer (Table 2.1) for 5 minutes to maintain the heart beating as a means to ensure proper cannulation. The heart was then perfused with a Ca^{2+}-free buffer (Table 2.2) for 5 minutes, at which point the heart stopped beating. Next, the hearts were enzymatically digested by perfusing with the same Ca^{2+}-free buffer supplemented with 50 μM Ca^{2+}, protease dispase II (0.07 mg/ml; Roche Diagnostic, Laval, QC), collagenase

type 2 (0.29 mg/ml; Worthington, Lakewood, NJ) and trypsin (0.014 mg/ml; Sigma Aldrich, Oakville, ON) for 15-18 minutes. A low pressure gauge was attached to the perfusion apparatus to monitor perfusion pressure. Perfusion was controlled using a peristaltic pump (Cole-Parmer Canada Inc., Montreal, QC). The isolation solutions were oxygenated with 100% O_2 (Praxair, Halifax, NS) and warmed to 37°C using a water-jacketed heating coil (Radnoti Glass Technology Inc., Monrovia, CA), which also served as a bubble trap to remove bubbles within the solutions. The heating coil was warmed using a Heater Circulating pump 170051A (Radnoti LLC, Monrovia, CA).

Following enzymatic digestion, the ventricles were removed from the atria, placed in a high K^+-buffer solution (Table 2.3) and minced into small tissue fragments. These fragments were washed 3 times using the K^+-buffer to ensure all enzyme was removed. The minced ventricles were stored in the K^+-buffer at room temperature until use, for a maximum of 12 hours. Ventricular myocytes were isolated/dissociated from the tissue fragments by gently agitating the beaker. The resulting cell suspension was passed through a 225 μm polyethylene filter to remove large fragments of undissociated tissue.

Table 2.1. Composition of Ca^{2+}-containing heart isolation solution.

Chemical	Concentration (mM)
NaCl	135.00
KCl	4.00
KH_2PO_4	1.2
$MgSO_4$	1.2
Hepes	10.0
Glucose	12.0
$CaCl_2$	2
pH to 7.4 with NaOH	

Table 2.2. Composition of Ca^{2+}-free heart isolation solution.

Chemical	Concentration (mM)
NaCl	135.00
KCl	4.00
KH_2PO_4	1.2
$MgSO_4$	1.2
Hepes	10.0
Glucose	12.0
pH to 7.4 NaOH	

Table 2.3. Composition of high K^+-buffer solution.

Chemical	Concentration (mM)
KCl	45
$MgSO_4$	3
KH_2PO_4	30
L-Glutamic Acid	50
Taurine	20
EGTA	0.5
Hepes	10
Glucose	10
pH to 7.4 with KOH	

2.4. Experimental Setup

The isolated myocytes (1 mL suspension in K^+-buffer) were incubated in the dark with fura-2 AM (acetoxymethyl ester; 5 μM with 0.2% anhydrous DMSO; Invitrogen, Burlington, ON) for 20 minutes at room temperature in a custom plexiglass chamber with a glass cover slip bottom. Fura-2 AM can cross the sarcolemmal membrane and enter the myocytes because of the acetoxymethyl ester functional group. Once inside, the functional group is cleaved by endogenous esterases rendering the fura-2 impermeable to the cell membrane. Therefore, the fura-2 remains in the cytosol and does not enter other organelles. Fura-2 is a ratiometric dye that absorbs light at 340 nm when it is bound to Ca^{2+} and at 380 nm when it is unbound from Ca^{2+}. Fura-2 emits light at 510 nm regardless of the excitation wavelength. As such, changes in $[Ca^{2+}]_i$ can be calculated from the ratio of emissions in the Ca^{2+}-bound and unbound state (340/380 nm).

Figure 2.1. shows a schematic of the experimental setup. The custom plexiglass chamber was mounted on the stage of an inverted microscope (Nikon Eclipse TE200; Nikon Canada, Mississauga, ON). Solutions were gravity-fed at a rate of 6 ml/min. The flow rate was controlled by an adjustable pinch clamp. On either side of the inflow opening, custom channels were mounted to allow 90% N_2/10% CO_2 gas to flow over solutions during arrest periods. An outflow channel allowed solutions to flow across the myocytes at a constant rate. All solutions were warmed to 37°C by a heat exchanger positioned at the inflow of the bath. The water within the heat exchanger was warmed and circulated by an immersion circulator (Polystat, Model #12112-10, Cole Parmer, Vernon Hills, IL).

The inverted microscope was placed on a custom anti-vibration table within a Faraday cage to minimize electrical noise. All electrical components were grounded to a ground panel. The Faraday cage was covered in black vinyl in order to block outside light and minimize interference with the fluorescence recordings. The myocytes were visualized under oil immersion (Immersol 518F, Carl Zeiss Canada Ltd. Toronto, ON) using a 40x objective (Nikon S-Fluor, numerical aperture 1.30, Nikon Canada Inc.). A closed circuit video camera (Philips, Markham, Ontario) was attached to the base of the inverted microscope and connected to a television monitor (PMM 1201; Pelco by Schneider Eletric, Clovis, CA) to display two images of the myocyte in the field of view. A video edge detector (Model # 105; Crescent Electronics, Sandy, UT) coupled to the camera was use to measure myocyte shortening using rastor lines positioned at the outer edges of the cell.

The cells were stimulated using custom-made platinum electrodes. Bipolar pulses (3 ms; 20-70 mA – 2.0 x threshold) were generated by a stimulus isolation unit (Model #SIU- 102; Warner Instruments, Hamden, CT) and the rate of myocyte stimulation (1Hz) was controlled using pClamp 8.2 sofware (Molecular Devices, Sunnyvale, CA). The electrodes were attached to a micromanipulator mounted to the microscope stage to ensure the electrodes were always on either side of the myocyte.

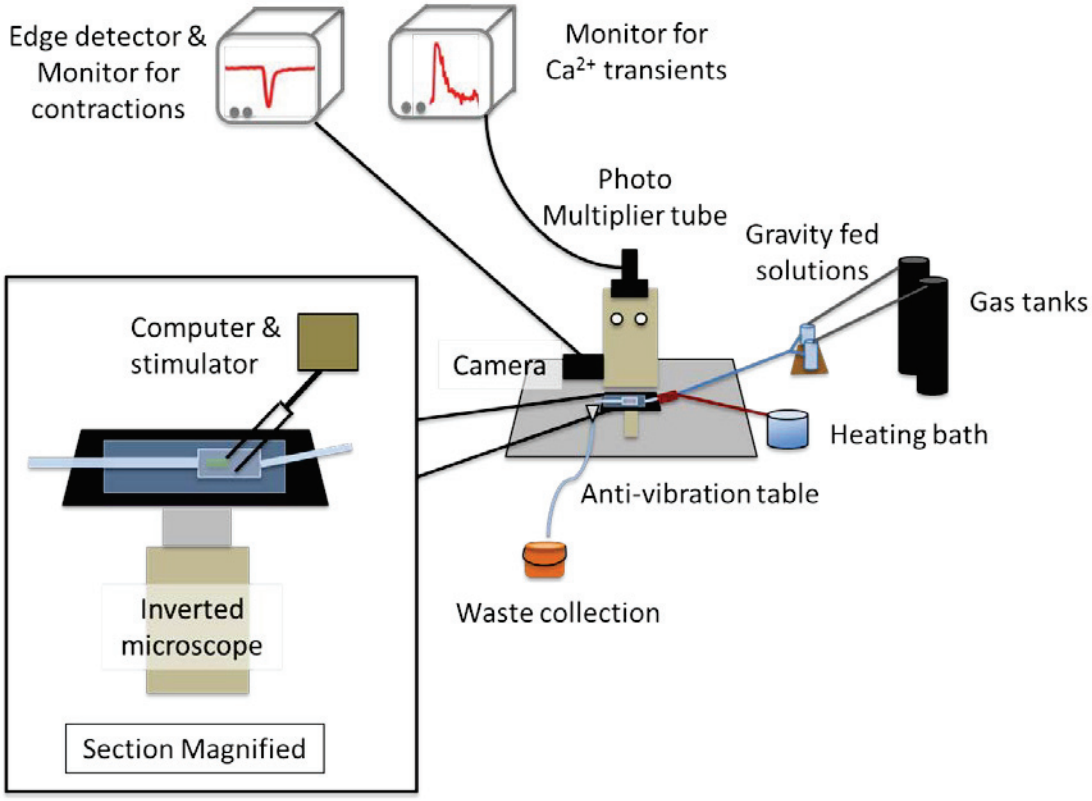

Figure 2.1. Schematic of the experimental setup. Each component is described in detail in the methods section.

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were visualized simultaneously with cell shortening by dividing the microscope light between the video camera and the photomultiplier tube (Photon Technologies International (PTI), Birmingham, NJ). A dichroic cube (Chroma Technology Corp., Rockingham VT) was used to divide the light. Light with wavelengths longer than 600 nm were directed to the video camera. An aperture of the photomultiplier tube was closed to surround the precise size of the myocyte through the viewing window to ensure emissions (510 nm) were recorded from the cell alone. The cell was alternately excited with 340 nm and 380 nm light using a DeltaRam high speed wavelength illuminator (PTI). The UV light was delivered using a liquid light guide (PTI). Fura-2 emissions were recorded using Felix sofrware (PTI) at a rate of 200 samples/second.

2.5. Experimental Protocol

2.5.1. Development of isolated cellular model

To develop an isolated cellular model that represented the conditions seen during cardiac surgery (pH, pO_2), the cardioplegic solution used was altered to mimic conditions in ischemia. To reduce the pH of the solution to 6.8, the concentration of bicarbonate buffer was reduced from 30 mM to 20 mM. Although the solution was bubbled with 90%N_2/10% CO_2 and this gas mixture was blown over the chamber during ischemic and cardioplegic arrest, the pO_2 needed to be between 5-18 mmHg to represent conditions during ischemic arrest (Carrier et al., 1998). An oxygen scavenger, sodium dithionite ($Na_2S_2O_4$; Sigma Aldrich, Oakville, ON) was added to the cardioplegic solution (Table 2.4). Concentrations of 0.5 mM to 10 mM had been reported to reduce pO_2 to ischemic

values in previous literature (Seki and Macleod, 1995; Ho et al., 2002; Koyama et al., 2003; Coutinho et al., 2013). Using iStat cardridges (Abaxis Inc.; Union City, CA) the pO_2 levels were measured in solutions containing different concentrations of $Na_2S_2O_4$. Experiments described below (*2.5.2. Simultaneous measurements of intracellular Ca^{2+} and cellular shortening*) were carried out to determine a concentration of $Na_2S_2O_4$ that resulted in a pO_2 between 5-18 mmHg, which would result in a level of hypoxia similar to that seen in cardioplegia (Carrier et al., 1998) but which would allow cells to survive.

2.5.2. Simultaneous measurements of intracellular Ca^{2+} and cellular shortening

Following incubation for 20 minutes, the cells were superfused with oxygenated Tyrode's solutions (Table 2.5) at a rate of 6 mL/min. Solutions were gravity-fed and flow rate was controlled using an adjustable pinch clamp. A quiescent rod-shaped ventricular myocyte with clear striations and no visible damage to the membrane was randomly selected as an experimental subject. The selected cell was then stimulated at a frequency of 1 Hz using custom-made platinum electrodes. Calcium and cell shortening recordings were taken simultaneously for 10 seconds every 5 minutes. The specific protocols for each experiment are described below and can be seen in Figure 2.2.

Briefly, cells were superfused with oxygenated Tyrode's solutions and paced for 20 minutes to ensure the cells were in a stable state. The superfusion was then changed to 1 of 5 different experimental solutions (Table 2.5-2.9; Figure 2.2) for 45 minutes. During the 45 minutes of ischemic or cardioplegic arrest, a 90% N_2/10% CO_2 gas mixture was blown over the chamber to ensure the pO_2 levels of the solution remained at

approximately 16 mmHg. Following the ischemic, cardioplegic, or control arrest the cells were reperfused with oxygenated Tyrode's solution for 20 minutes.

At the end of each experiment a background recording was collected by selecting an area adjacent to the cell that did not contain a cell or cell fragments. This background recording was used to correct for any auto-fluorescence in the light path.

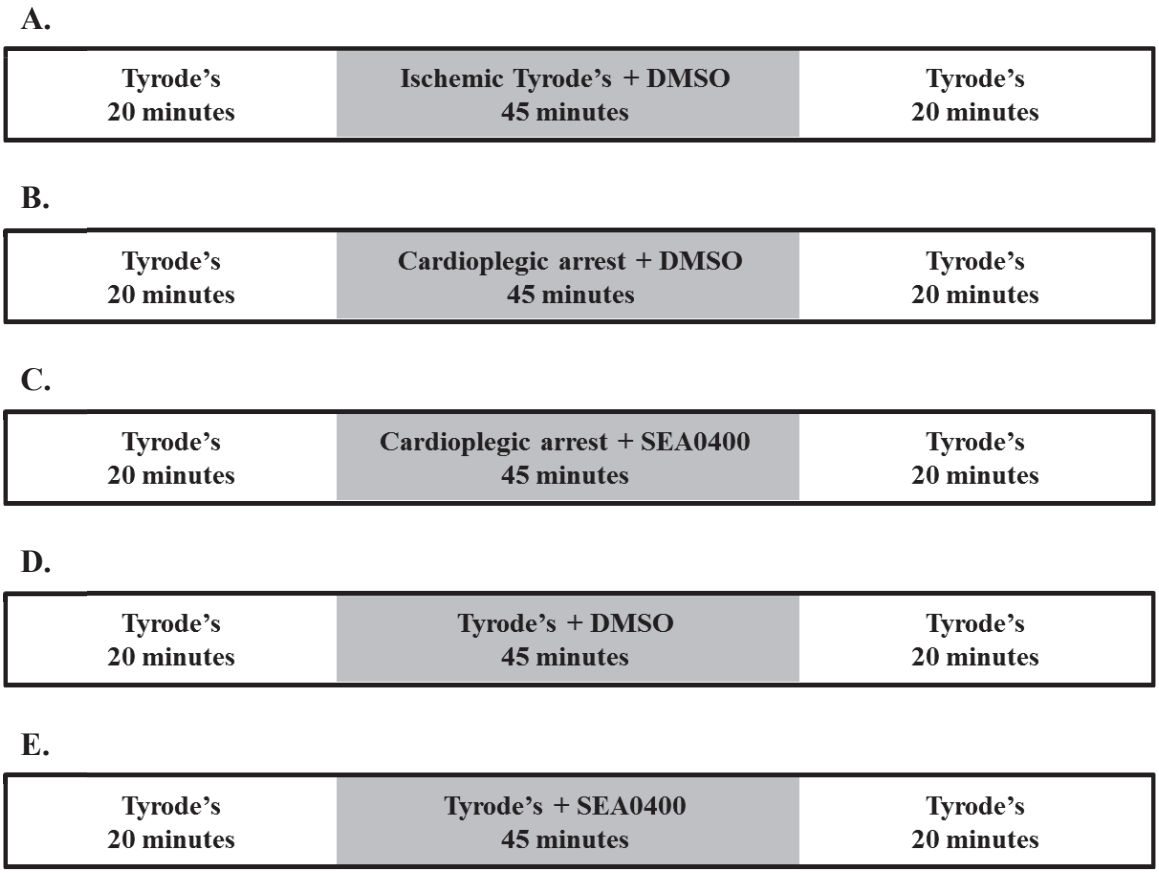

Figure 2.2. Schematic summarizing the experimental protocols for isolated ventricular myocytes. A. Isolated ventricular myocytes were subjected to global ischemia; Global ischemia group. **B.** Myocytes were subjected to cardioplegia without SEA0400; Cardioplegia group. **C.** Myocytes were subjected to cardioplegia containing 1 μM SEA0400; Cardioplegia + SEA0400 group. **D.** Myocytes were subjected to oxygenated Tyrode's; Tyrode's + DMSO group. **E.** Myocytes were subjected to oxygenated Tyrode's with 1 μM SEA0400; Tyrode's + SEA0400 group.

2.5.3. Measurement of sarcoplasmic reticulum Ca^{2+} stores

In experiments where sarcoplasmic reticulum (SR) Ca^{2+} stores were recorded the cells were exposed to the protocol described above (Fig. 2.3 B & C). A rapid switcher was placed adjacent to the cell of interest. Cells were exposed to a control HEPES buffer (Table 2.10) for 5 beats followed by a 1-second application of 10 mM caffeine (Table 2.11). Recordings of SR Ca^{2+} stores were done at baseline (20 mins) and during reperfusion (67 mins and 75 mins). Recordings were taken from one cell per experiment and different cells were used for each time point.

Table 2.4. Composition of oxygenated Tyrode's solution.

Chemical	Concentration (mM)
NaCl	126.00
$NaHCO_3$	20.00
NaH_2PO_4	0.9
KCl	4.0
$MgSO_2$	0.5
Glucose	5.5
$CaCl_2$	1.8
pH = 7.3	
Bubble with 95%O_2/5%CO_2	

Table 2.5. Composition of ischemic Tyrode's solution.

Chemical	Concentration (mM)
NaCl	126.00
$NaHCO_3$	20.00
NaH_2PO_4	0.9
KCl	4.0
$MgSO_2$	0.5
Glucose	5.5
$CaCl_2$	1.8
$Na_2S_2O_4$	5.0
pH=7.3	
0.1% DMSO; Bubble with 90%N_2/10%CO_2	

Table 2.6. Composition of simulated high K^+ cardioplegic solution.

Chemical	Concentration (mM)
NaCl	73.82
KCl	18.42
Na gluconate	18.46
$NaHCO_3$	20.00
Glucose	4.40
NaH_2PO_4	0.72
$MgSO_4$	4.61
$Na_2S_2O_4$	5.00
$CaCl_2$	1.0
pH=6.8	
0.1% DMSO; Bubble with 90%N_2/10%CO_2	

Table 2.7. Composition of simulated high K^+ cardioplegic solution with SEA0400.

Chemical	Concentration (mM)
NaCl	73.82
KCl	18.42
Na gluconate	18.46
$NaHCO_3$	20.00
Glucose	4.40
NaH_2PO_4	0.72
$MgSO_4$	4.61
$Na_2S_2O_4$	5.00
$CaCl_2$	1.0
SEA0400	0.005
pH=6.8	
0.1% DMSO; Bubble with 90%N_2/10%CO_2	

Table 2.8. Composition of oxygenated Tyrode's solution without SEA0400.

Chemical	Concentration (mM)
NaCl	126.00
$NaHCO_3$	20.00
NaH_2PO_4	0.9
KCl	4.0
$MgSO_2$	0.5
Glucose	5.5
$CaCl_2$	1.8
pH=7.3 **0.1% DMSO; Bubble with 95%O_2/5%CO_2**	

Table 2.9. Composition of oxygenated Tyrode's solution with SEA0400.

Chemical	Concentration (mM)
NaCl	126.00
$NaHCO_3$	20.00
NaH_2PO_4	0.9
KCl	4.0
$MgSO_2$	0.5
Glucose	5.5
$CaCl_2$	1.8
SEA0400	0.005
pH=7.3 **0.1% DMSO; Bubble with 95%O_2/5%CO_2**	

Table 2.10. Composition of HEPES solution.

Chemical	Concentration (mM)
NaCl	135.0
KCl	4.0
Glucose	10.0
HEPES	10.0
$MgCl_2$	1.0
$CaCl_2$	1.8
pH to 7.4 with NaOH	

Table 2.11. Composition of 0 Na^+ - 0 Ca^{2+} caffeine solution.

Chemical	Concentration (mM)
LiCl	140
KCl	4.0
Glucose	10.0
HEPES	5.0
$MgCl_2$	4.0
Caffeine	10.0
pH to 7.4 with LiOH	

2.5.4. Data and Statistical Analysis

Data analysis was performed using Clampfit 8.2 (Molecular Devices, Sunnyvale, CA). All graphs were constructed using Sigma Plot software (version 12.0; Systat Software, Inc., Point Richmond, CA). The background fluorescence recorded at the end of each experiment was subtracted from the fluorescence measurements at each wavelength. Felix software (PTI) was used to convert the calculated measurements to an emission ratio (340/380 nm). Ratios were converted to Ca^{2+} concentrations using a Ca^{2+} calibration curve previously determined to fit the linear equation: y=334.8x – 116.3. The calibration curve was determined *in vitro* by measuring known concentrations of Ca^{2+} in solutions warmed to 37°C. Ca^{2+} transients were measured as the difference between diastolic and peak systolic Ca^{2+}. Contraction size was measured as the difference between resting cell length (diastolic cell length) and peak contraction (peak systolic cell length). Velocities to peak, 50% and 90% decay were calculated using Sigma Plot software. Velocity accounts for the amplitude of the transient, whereas time to peak could be misleading if the transient amplitudes are significantly larger. For statistical analysis, time points containing a sample size lower than 3 were discarded (45-85 minute time points for ischemic control). Statistical analyses were also performed using Sigma Plot software. Differences between means were determined using *t*-tests and two-way repeated-measures analysis of variance (ANOVA). Cell survival analysis was conducted using the Kaplan Meier log Rank Survival Curve. Multiple comparisons were performed using a Holm-Sidak post hoc test. The statistical test used in each case is noted in the related section. Data are presented as means ± SEM. Differences were considered significant when $p<0.05$.

Chapter 3. Results

3.1. Development of the Ischemic Isolated Ventricular Cell Model.

To develop an isolated cell model that represents the conditions typically encountered during a period of ischemic arrest, the pH and the pO_2 were modified from simulated cardioplegic solutions used previously (unpublished Ali, 2011). A pH of 6.8 was achieved by reducing the concentration of $NaHCO_3$ from 30 mM to 20 mM. The pO_2 of an oxygenated Tyrode's solution was determined to be approximately 580 mmHg using CG8+ iStat cartridges described in chapter 2. The simulated cardioplegic solution was bubbled with 90%N_2/10%CO_2 to reduce the pO_2 from 580 mmHg to approximately 38 mmHg. The oxygen scavenger sodium dithionite ($Na_2S_2O_4$) was then added in increasing concentrations until a pO_2 of approximately 10 mmHg was achieved. Figure 3.1 shows the measured pO_2 levels achieved at various concentrations of $Na_2S_2O_4$ from 0.5 mM to 6 mM. A concentration of 5 mM $Na_2S_2O_4$ resulted in a pO_2 of 10 mmHg and was selected for use in additional studies.

3.2. Survival of Isolated Ventricular Myocytes from Fischer-344 Rats Subjected to Ischemic or Simulated Cardioplegic Arrest.

Cell survival was followed throughout the 85-minute protocol and Kaplan-Meier analysis was used to assess the relationship between cell survival and the ischemic or cardioplegic solution used during the arrest period. In total, 25 cells were used in the cardioplegia plus SEA0400 group, 24 cells were used in the cardioplegia group, 8 cells were used in the ischemic control group, 5 cells were used in the Tyrode`s control group

and 4 cells were used in the Tyrode`s plus SEA0400 group. Cells that remained rod-shaped and contracted throughout the protocol were deemed viable. Cells that rounded and stopped contracting were considered not viable and did not survive the protocol. Figure 3.2 shows that survival was significantly improved in cells treated with the cardioplegia plus SEA0400 compared to cells treated with ischemic control. Only one cell in the ischemic control group survived the entire protocol. Survival was not significantly improved in cells treated with the cardioplegia in comparison to cells treated with the ischemic control. All cells treated with Tyrode`s and Tyrode`s plus SEA0400 survived the entire protocol.

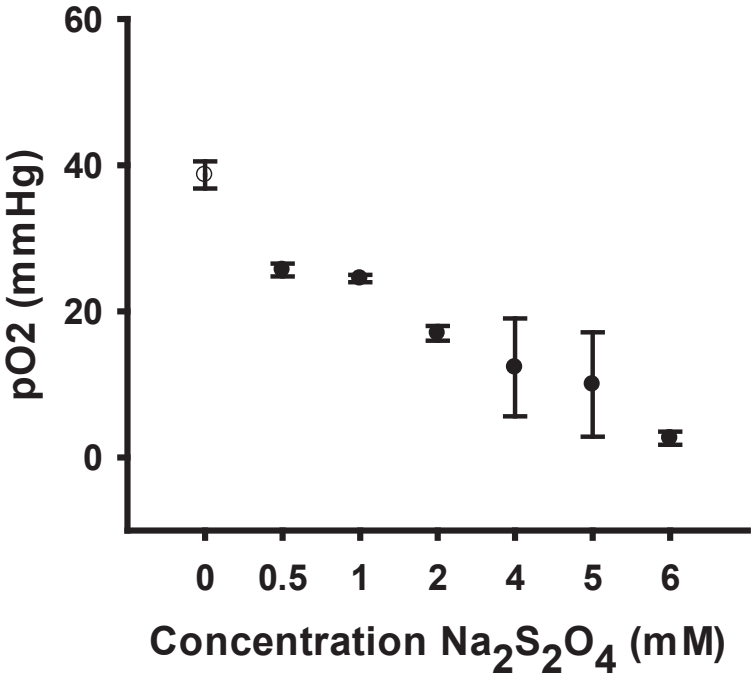

Figure 3.1. Partial pressure of oxygen (mmHg) decreased in the cardioplegic solutions as the concentration of sodium dithionite ($Na_2S_2O_4$) increased. Cardioplegic solutions (23°C & 37°C) containing concentrations of $Na_2S_2O_4$ ranging from 0.5 to 6.0 mM were analyzed using CG8+ iStat cartridges to determine the pO_2 of the solutions. A concentration of 5.0 mM resulted in a pO_2 of approximately 11 mmHg (0.5 mM n=3, 1.0 mM n=2, 2.0 mM n=2, 4.0 mM n=3, 5.0 mM n=7, 6.0 mM n=2).

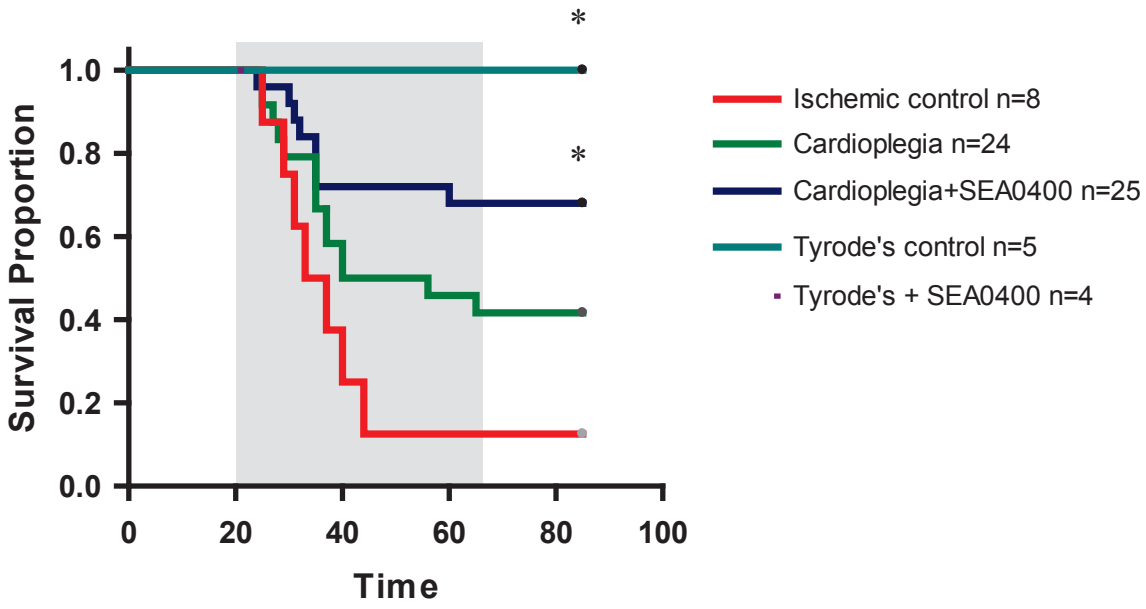

Figure 3.2. Cell survival increased in cells subjected to cardioplegia containing SEA0400. Myocytes isolated from rats were field stimulated at 1 Hz (37ºC). Cell survival was significantly increased in cells subjected to cardioplegia with SEA0400 (blue) when compared to ischemic control (red). Survival was not significantly improved in cells subjected to cardiolegia alone (green). All myocyte subjected to Tyrode`s control and Tyrode`s + SEA0400 survived the entire protocol. (n=25 cardioplegia+SEA0400, n=24 cardioplegia, n=8 ischemic control, n=5 Tyrode`s control, n=4 Tyrode`s+SEA0400; * denotes $p<0.05$ vs ischemic control, Kaplan Meier log Rank Survival Curve, Multiple Comparison Procedures Holm-Sidak method).

3.3. Calcium Concentrations in Isolated Ventricular Myocytes Subjected to Ischemic or Simulated Cardioplegic Arrest.

To determine whether intracellular Ca^{2+} levels were altered during cardioplegic arrest in the presence and absence of SEA0400, Ca^{2+} transients were measured in isolated ventricular myocytes from hearts of young Fischer 344 rats. Figure 3.3 shows representative examples of Ca^{2+} transients recorded from isolated myocytes paced at 1 Hz. At baseline, Ca^{2+} transients were similar in cells from all three groups. During the arrest period, Ca^{2+} transients were abolished in cells treated with cardioplegia or cardioplegia plus SEA0400. However, upon reperfusion the peak Ca^{2+} transient was visibly larger in the myocyte treated with cardiopelgia plus SEA0400.

Mean data normalized to the 20-minute baseline time point (Fig. 3.4, 3.5 and 3.6) were consistent with the differences observed in the representative recordings. Throughout baseline and most of reperfusion, there was no difference in diastolic Ca^{2+} levels between groups (Fig 3.4). During the arrest period, the cells treated with ischemic control showed a rapid and significant increase in diastolic Ca^{2+}. Myocytes treated with cardioplegia showed a more gradual increase in diastolic Ca^{2+}. Near the end of the arrest period and the first 5 minutes of reperfusion, the levels of diastolic Ca^{2+} were significantly higher in cells treated with cardioplegia in comparison to cells treated with cardiopelgia plus SEA0400. In contrast with diastolic Ca^{2+} levels, systolic Ca^{2+} did not differ throughout the arrest period in cells treated with cardioplegia or cardioplegia plus SEA0400. Cells subjected to ischemic control however did show significantly larger systolic Ca^{2+} levels during the first 20 minutes of arrest. Upon reperfusion, systolic Ca^{2+} levels were significantly larger in cells treated with cardioplegia plus SEA0400 in

comparison to cells treated with cardioplegia alone. Figure 3.6 shows calcium transient amplitude normalized to the 20-minute baseline time point. Myocytes treated with ischemic control had larger peak Ca^{2+} transient amplitudes during the first half of arrest compared to cells treated with cardioplegia or cardioplegia plus SEA0400 as Ca^{2+} transients were abolished throughout cardioplegic arrest. Throughout reperfusion, myocytes treated with cardioplegia plus SEA0400 had significantly larger Ca^{2+} transients in comparison to cells treated with cardioplegia alone. Velocities to peak did not differ between groups during the period of arrest and throughout reperfusion (Fig.3.7). Velocities to 50% and 90% decay were significantly larger at several time points during reperfusion in cells subjected to cardioplegia plus SEA0400 in comparison to those subjected to cardioplegia (Fig.3.8 and 3.9).

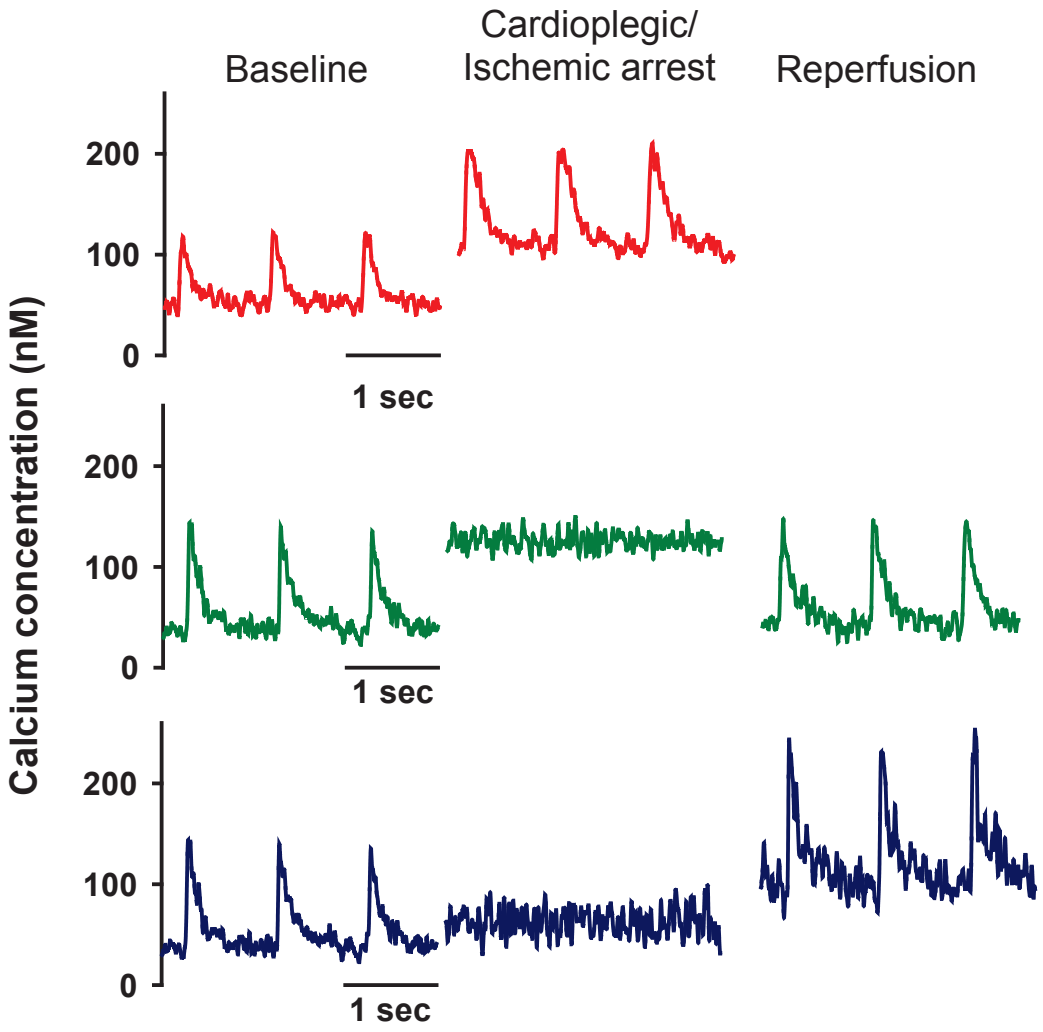

Figure 3.3. Representative examples of calcium transients recorded from cells subjected to ischemic or cardioplegic arrest. Representative examples of Ca^{2+} transients recorded from field stimulated myocytes (1 Hz) isolated from young Fischer 344 rat hearts at baseline, during arrest and upon reperfusion. There is no representative recording during reperfusion in ischemic control as cells did not survive the protocol (red=ischemic control, green=cardiolegia, blue=cardioplegia+SEA0400).

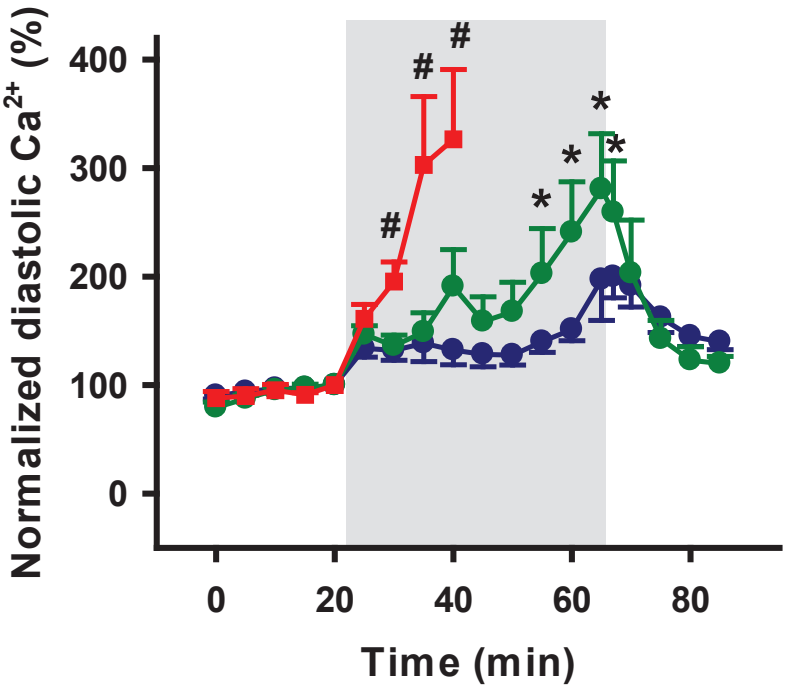

Figure 3.4. Cells treated with cardioplegia containing SEA0400 had significantly less intracellular calcium accumulation during the ischemic period. Mean diastolic calcium levels normalized to 20-minute baseline time point for ischemic control (red), cardioplegia (green) and cardiopelgia+SEA0400 (blue). Cells subjected to ischemia showed a rapid significant rise in diastolic calcium compared to both cardioplegic groups. Cell subjected to cardioplegia+SEA0400 had significantly less calcium accumulation compared to cells in the cardioplegia group. There was no difference in diastolic calcium levels at baseline. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

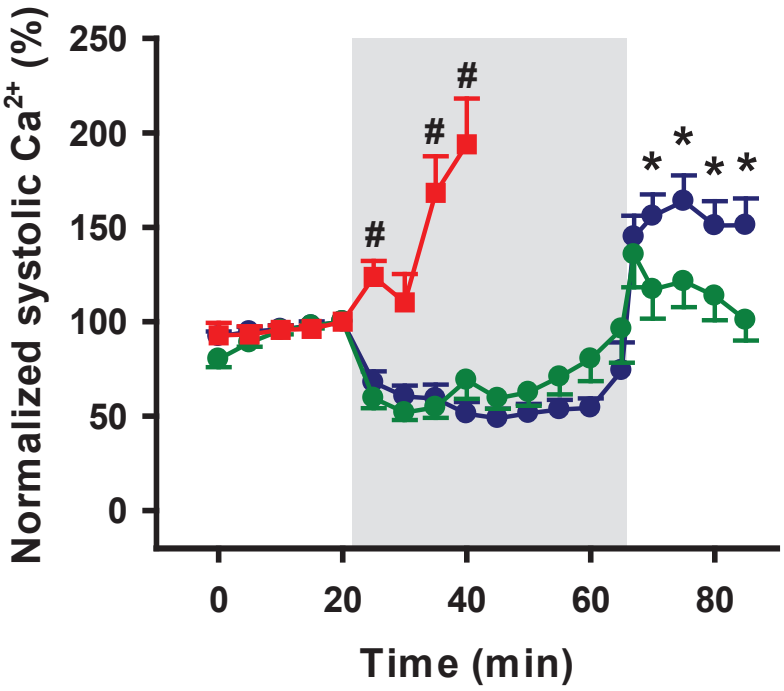

Figure 3.5. Systolic calcium levels were higher during reperfusion in cells treated with cardioplegia+SEA0400. Mean systolic calcium normalized to 20-minute baseline time point for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue). Systolic calcium levels were significantly higher during ischemic arrest in cells treated with ischemic control (red). Cells subjected to cardioplegia and cardioplegia+SEA0400 had no difference in systolic calcium levels throughout the arrest period. Significantly higher systolic calcium levels were present during reperfusion in cells treated with cardioplegia+SEA0400 compared to those treated with cardiopelgia alone. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

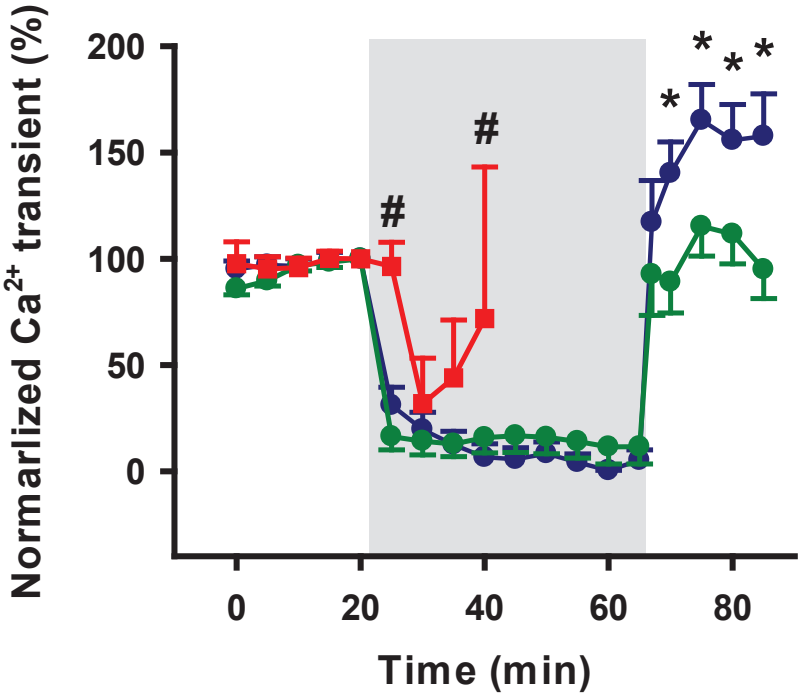

Figure 3.6. Calcium transients were larger during reperfusion in cells treated with cardioplegia+SEA0400. Mean calcium transient amplitude normalized to 20-minute baseline time point for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue). Calcium transients were significantly larger at several time points during ischemic arrest in cells treated with ischemic control solution. Calcium transients were arrested in cells treated with cardiopleia and cardioplegia+SEA0400. During reperfusion mean values for calcium transients were larger at several time points in cells treated with cardioplegia+SEA0400 compared to cells treated with cardioplegia alone. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

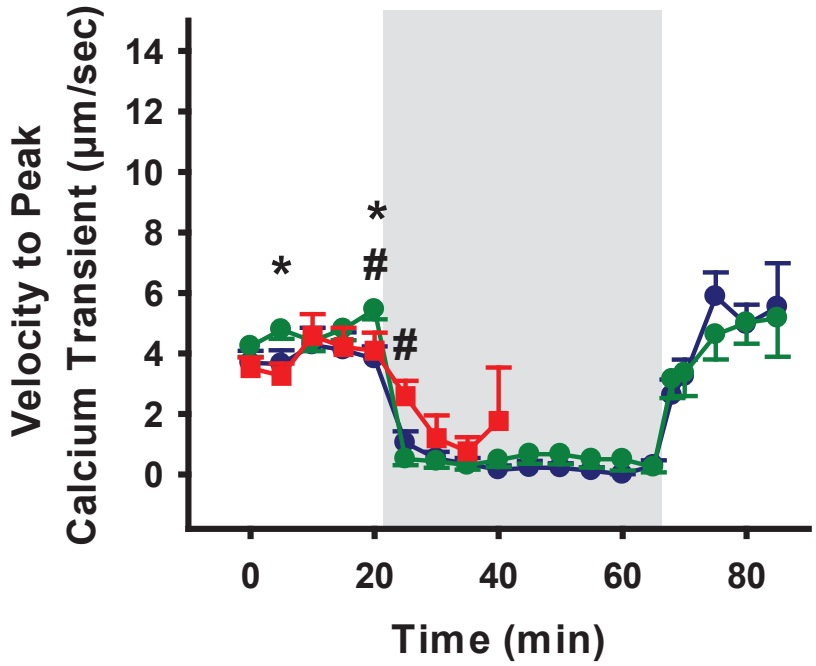

Figure 3.7. Velocity to peak of calcium transients was not different in cells treated with cardiopelgia plus SEA0400. Mean velocity to peak for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue) throughout 85-minute protocol. Velocity to peak did differ during arrest and reperfusion for cells treated with cardioplegia and cardioplegia+SEA0400. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

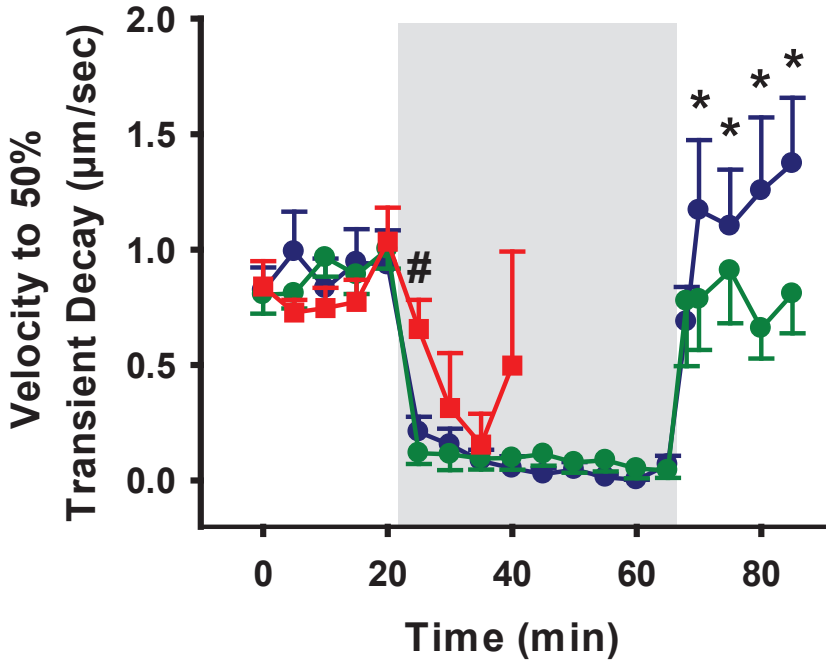

Figure 3.8. Velocity to 50% transient decay was higher during reperfusion in cells treated with cardioplegia plus SEA0400. Mean velocity to 50% transient decay for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue) throughout 85-minute protocol. Velocity to 50% transient decay was significantly larger at several time point during reperfusion in cells treated with cardiopelgia+SEA0400. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

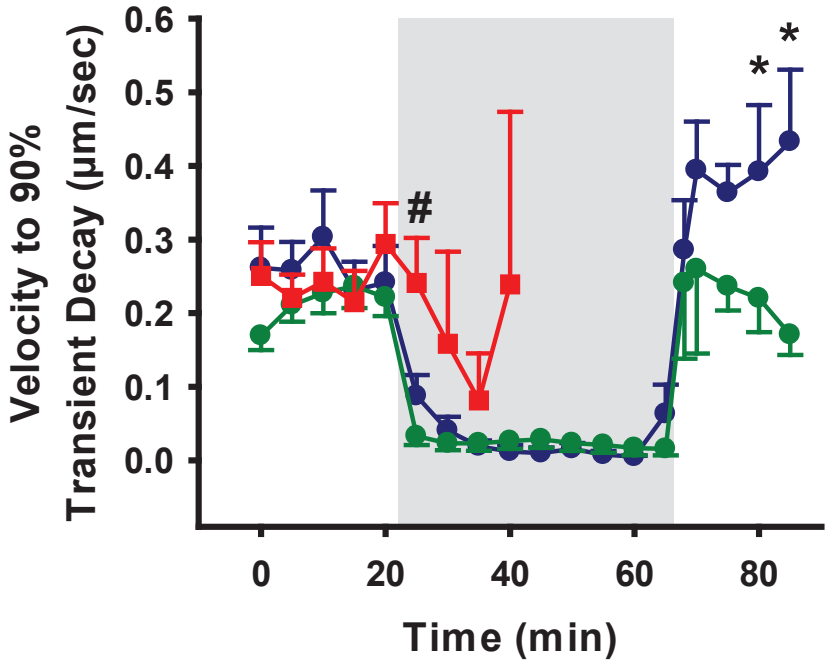

Figure 3.9. Velocity to 90% transient decay was higher during reperfusion in cells treated with cardioplegia plus SEA0400. Mean velocity to 90% transient decay for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue) throughout 85-minute protocol. Velocity to 90% transient decay was significantly larger at several time point during reperfusion in cells treated with cardiopelgia+SEA0400. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

3.4. Contractile Responses in Isolated Ventricular Myocytes Subjected to Ischemic or Simulated Cardioplegic Arrest.

To determine whether myocyte contractile function was altered during ischemic or cardioplegic arrest, contractions were measured in ventricular myocytes isolated from young male Fischer 344 rats. Figure 3.10 shows representative recordings of cell shortening. The downward deflection is the amount the cell shortened with each beat while stimulated at a frequency of 1 Hz. Contraction amplitudes were similar at baseline in all groups. By contrast, during ischemic arrest contractions remained but contractions were abolished during cardioplegic arrest in the absence and presence of SEA0400. Still, the myocyte treated with cardioplegia plus SEA0400 had larger contractions during reperfusion compared to the cell treated with cardioplegia alone. Figure 3.11 shows mean data for cell length when the cell was at rest. These data indicate that cell length did not significantly change throughout the protocol in any group.

Mean data in figure 3.12 shows the contractile function of myocytes throughout the 85-minute protocol. Contractions were normalized to resting cell length to determine fractional shortening and then normalized to the 20-minute baseline time point. Consistent with the representative contractions in figure 3.10, fractional shortening was similar between groups at baseline. However, cells treated with cardioplegia stopped contracting throughout the arrest period, whereas cells subjected to ischemic control continued contracting until cell death occurred. As well, during reperfusion the cells subjected to cardioplegia+SEA0400 had significantly larger fractional shortening compared to cells treated with control cardioplegia.

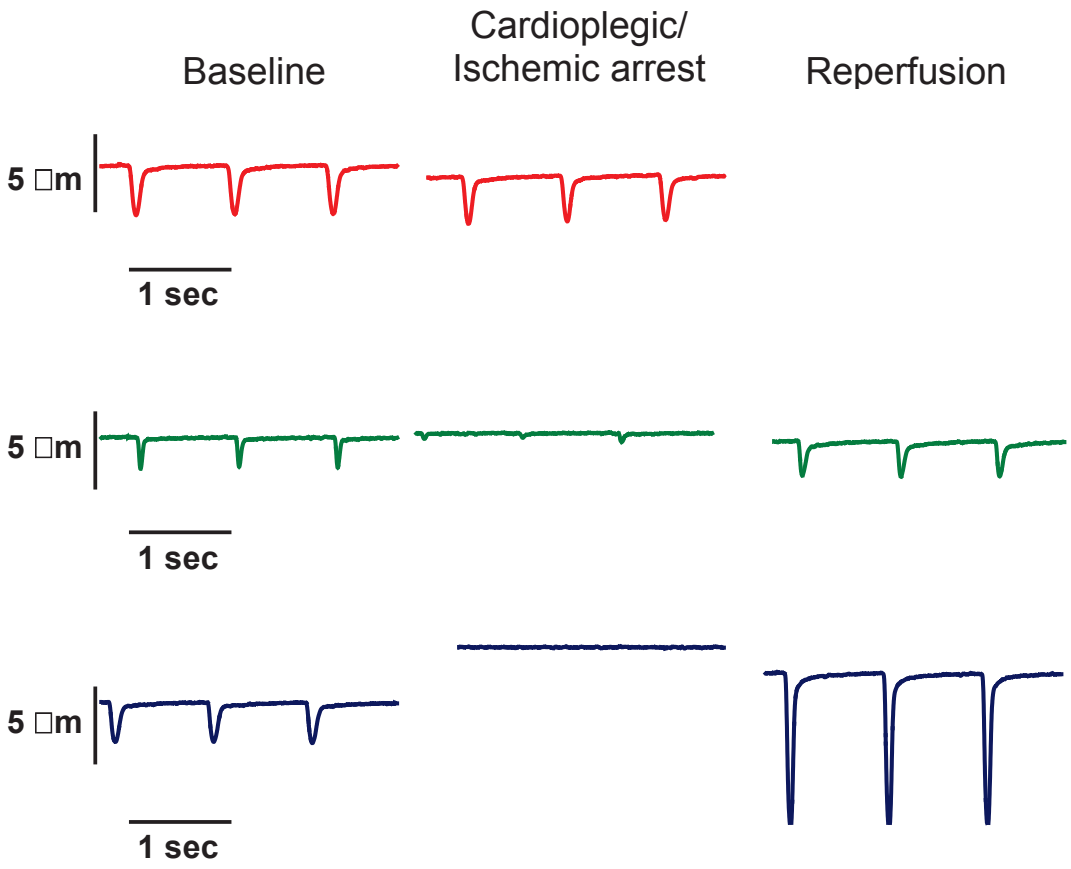

Figure. 3.10. Representative examples of cellular contractions recorded from cells subjected to ischemic or cardioplegic arrest. Representative examples of contractions recorded from field stimulated myocytes (1 Hz) isolated from young Fischer 344 rat hearts at baseline, during arrest and upon reperfusion (red=ischemic control, green=cardiolegia, blue=cardioplegia+SEA0400).

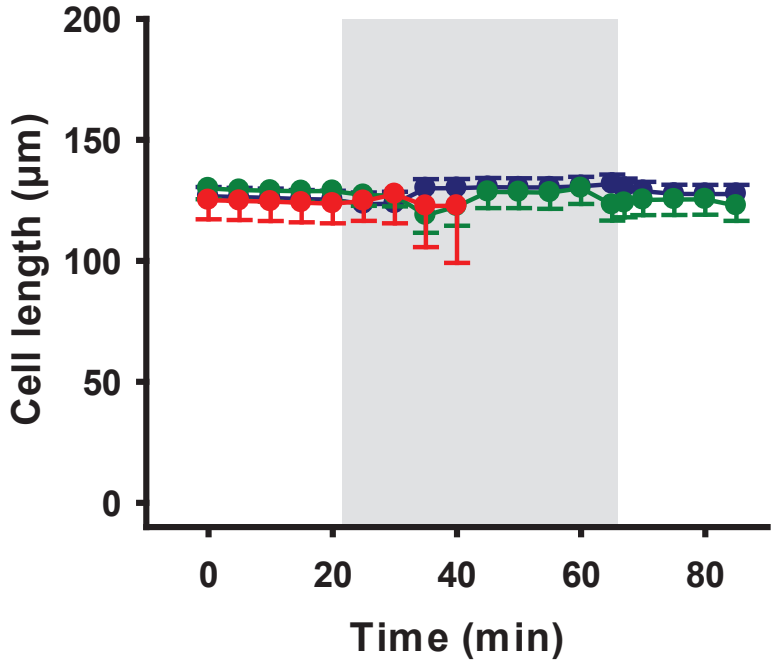

Figure 3.11. Myocyte length did not differ between groups. Mean cell length for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue) did not significantly change throughout 85-minute protocol. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

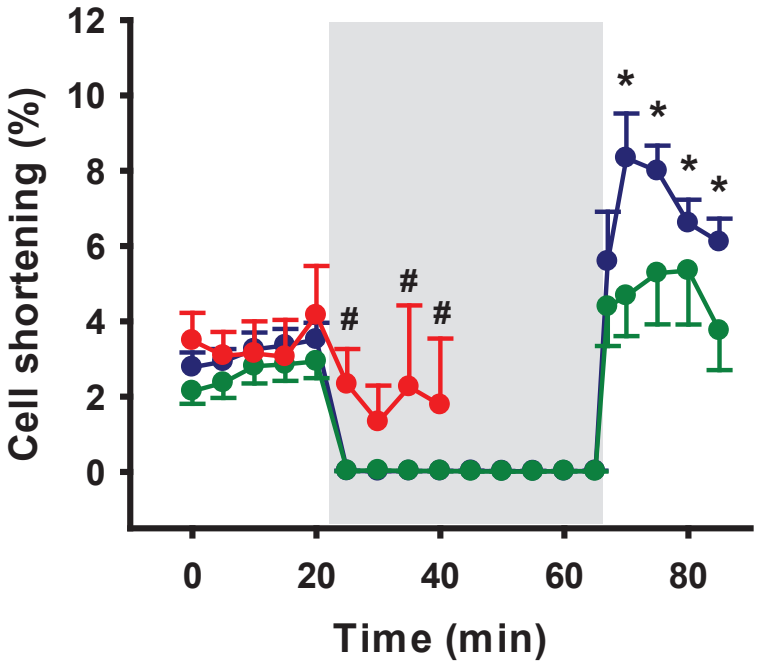

Figure 3.12. Cellular shortening increased during reperfusion in cells subjected to cardioplegia plus SEA0400. Mean cellular shortening for ischemic control (red), cardioplegia (green) and cardioplegia+SEA0400 (blue) throughout the 85-minute protocol. Mean cellular shortening was significantly higher during the first 20 minutes of ischemic arrest in cells subjected to ischemic control in comparison to cells subjected to cardioplegia and cardioplegia+SEA0400. Cellular shortening was abolished in cells subjected to cardioplegic solutions. During reperfusion cells treated with cardioplegia+SEA0400 had significantly larger cellular shortening compared to cells treated with cardioplegia. (n=25 cardiopelgia+SEA0400, n=24 cardiopelgia, n=8 ischemic control; # denotes $p<0.0.5$ vs cardioplegia and cardioplegia+SEA0400; *denotes $p<0.05$ vs cardioplegia+SEA0400, 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

3.5. Effects of SEA0400 on Calcium Homeostasis in Myocytes Subjected to Tyrode's Control Solution.

To determine whether SEA0400 affected the Ca^{2+} handling of myocytes in the absence of cardioplegia, cells were treated with oxygenated Tyrode's solution for 85-minutes. During the regular 45-minute arrest period, cells were exposed to the same oxygenated Tyrode's solution supplemented with 1.0 μM SEA0400 or vehicle control (DMSO). Mean data (Fig. 3.13, 3.14, 3.15) show that diastolic, systolic and Ca^{2+} transient amplitudes (normalized to the 20-minute baseline time point) did not change significantly throughout the 85-minute protocol in both vehicle control and in SEA0400-treated cells. Velocities to peak, 50% and 90% transient decay were also measured in cells treated with Tyrode's solution plus vehicle control or SEA0400. Figure 3.16 shows that velocities to peak, 50% and 90% transient decay did not differ between vehicle control and SEA0400-treated cells.

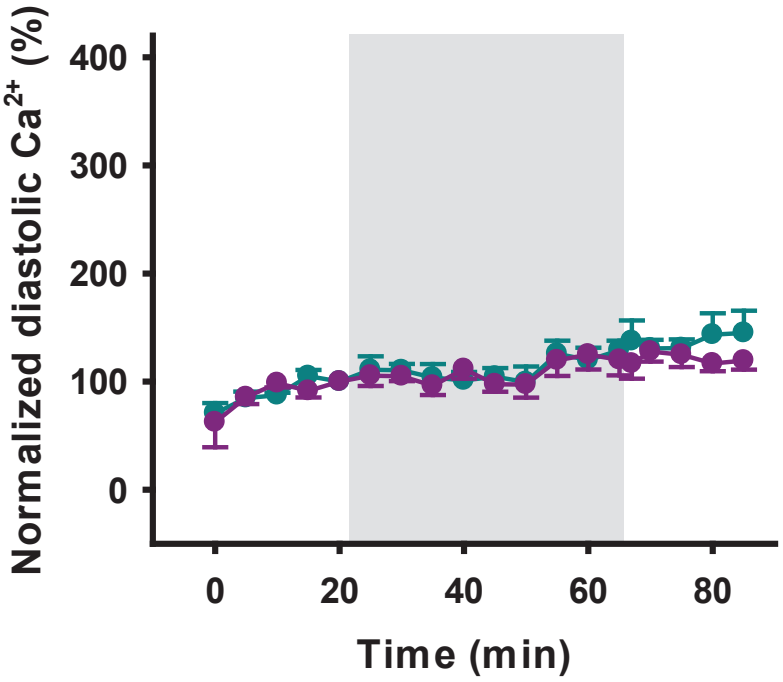

Figure 3.13. Diastolic calcium levels did not differ between control and SEA0400-treated cells. Mean diastolic calcium levels normalized to 20-minute baseline time point for DMSO (teal) and SEA0400 (purple) in Tyrode's solution. Diastolic calcium levels did not differ throughout the 85-minute protocol. (n=5 DMSO, n=4 SEA0400; 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

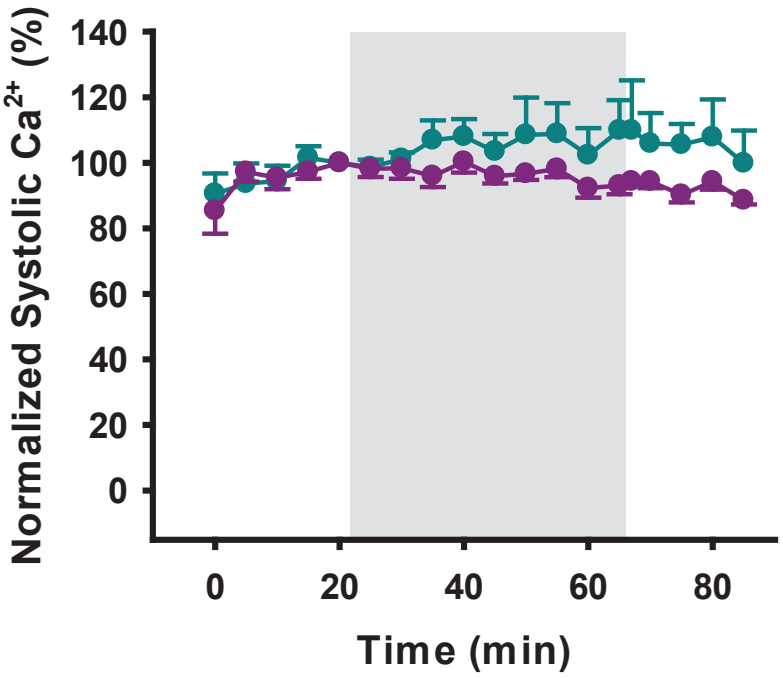

Figure 3.14. Systolic calcium levels did not differ between vehicle control and SEA0400-treated cells. Mean systolic calcium levels normalized to 20-minute baseline time point for DMSO (teal) and SEA0400 (purple) in Tyrode's solution. Systolic calcium levels did not differ between groups throughout the 85-minute protocol. (n=5 DMSO, n=4 SEA0400; 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

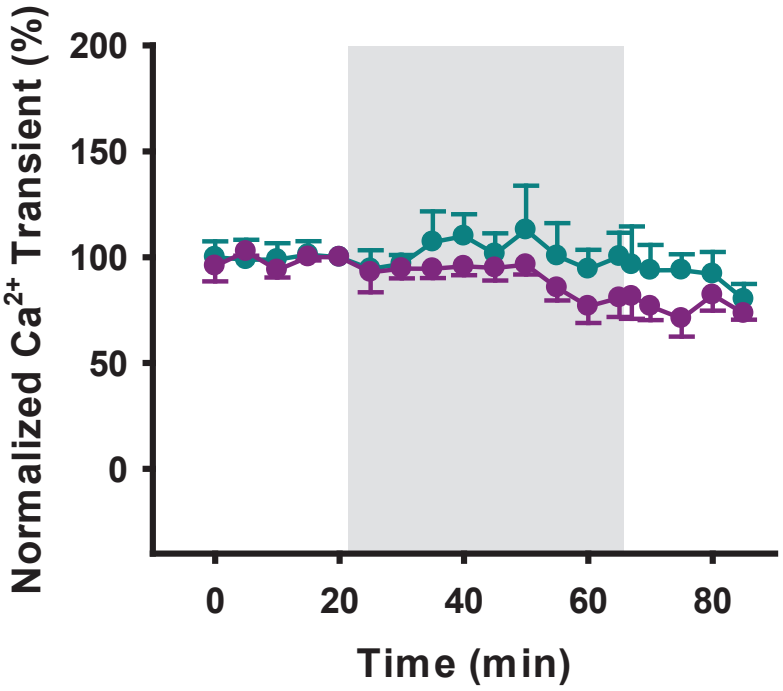

Figure 3.15. Calcium transient amplitudes did not differ between vehicle control and SEA0400-treated cells. Mean calcium transient amplitude normalized to 20-minute baseline time point for DMSO (teal) and SEA0400 (purple) in Tyrode's solution. Calcium transient amplitude did not differ between groups throughout the 85-minute protocol. (n=5 DMSO, n=4 SEA0400; 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

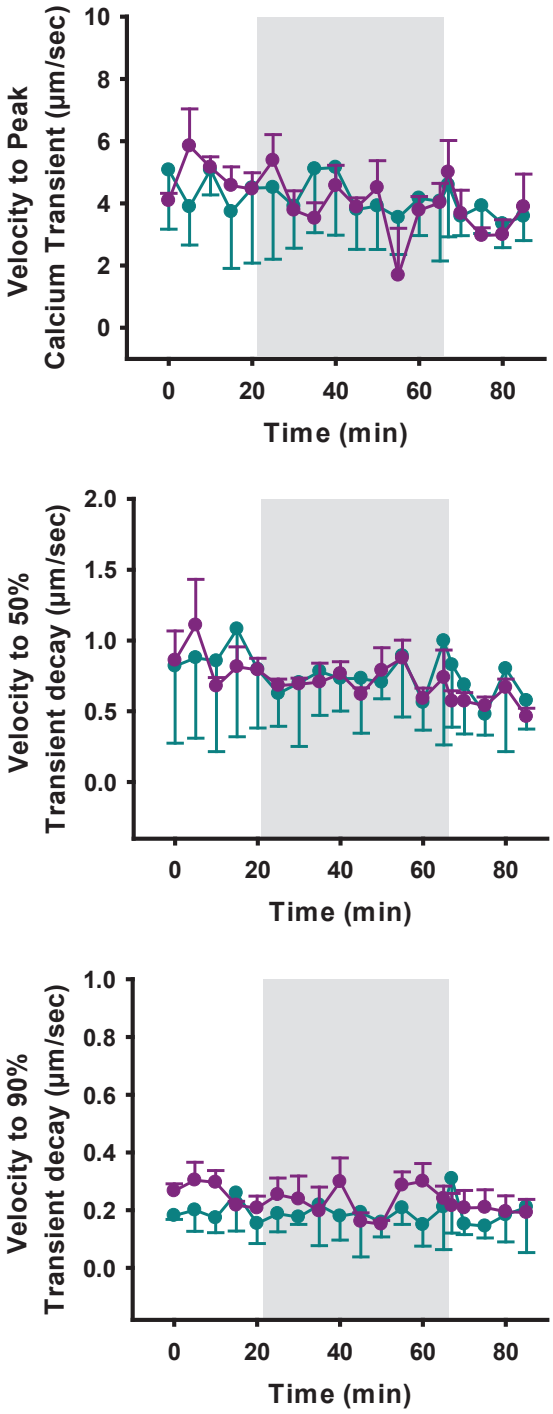

Figure 3.16. Velocity to peak, 50% decay and 90% decay did not differ between vehicle control and SEA0400-treated cells. A. Mean velocity to peak, **B.** 50% and **C.** 90% transient decay for DMSO (teal) and SEA0400 (purple) in Tyrode's solution do not differ throughout the 85-minute protocol. (n=5 DMSO, n=4 SEA0400; 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

3.6. Effects of SEA0400 on Cellular Contraction in Myocytes Subjected to Tyrode's Control Solution.

To determine whether SEA0400 affects contractile function under normal physiological conditions, myocytes were subjected to 85 minutes of oxygenated Tyrode's solution with a 45-minute 'arrest' period in which 1μM SEA0400 or vehicle control (DMSO) was added to the control Tyrode's solution. Figure 3.17 panel A shows mean data for resting cell length throughout the protocol. It is evident that there is no change in cell length during the 85 minutes in either group of cells. Panel B shows that fractional shortening was significantly greater in cells treated with Tyrode's containing SEA0400 in comparison to cells treated with Tyrode's containing vehicle control.

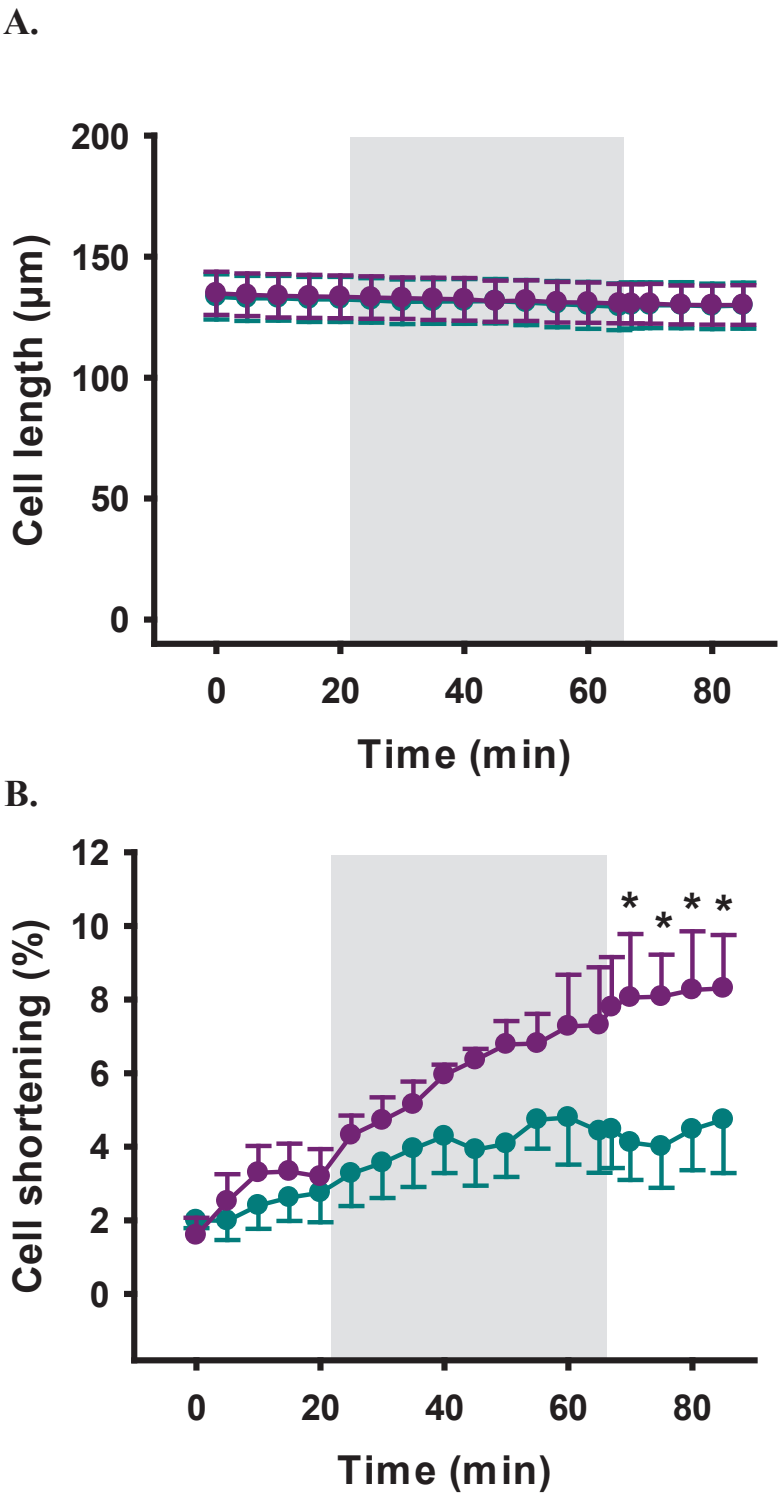

Figure 3.17. Cellular shortening increased in cells treated with SEA0400 in Tyrode's. A. Mean myocyte cell length did not differ in cells treated with vehicle control (teal) in Tyrode's in comparison to cells treated with SEA0400 (purple) in Tyrode's. B. Mean cellular shortening increased throughout the 85-minute protocol in cells treated with SEA0400 in Tyrode's solution. (n=5 DMSO, n=4 SEA0400; *denotes $p<0.05$; 2 way repeated measures ANOVA, Multiple Comparison Procedures Holm-Sidak method).

3.7. Sarcoplasmic Reticulum Calcium Stores in Myocytes Treated with Tyrode's Solution, Cardioplegia, and Cardioplegia+SEA0400.

To determine whether cardioplegic arrest with or without SEA0400 altered the Ca^{2+} content of the SR, SR Ca^{2+} stores were measured in myocytes using a one-second application of 10 mM caffeine. Figure 3.18. shows mean data for caffeine transients measured after caffeine was applied to the cells. Panel A shows no difference in SR Ca^{2+} stores 2 minutes into reperfusion (67 mins of protocol). Panel B shows preliminary results of caffeine transients obtained from cells 10 minutes into reperfusion (75 mins of protocol). Preliminary results also suggest no difference in the SR Ca^{2+} load of the cells subjected to any of the cardioplegic solutions.

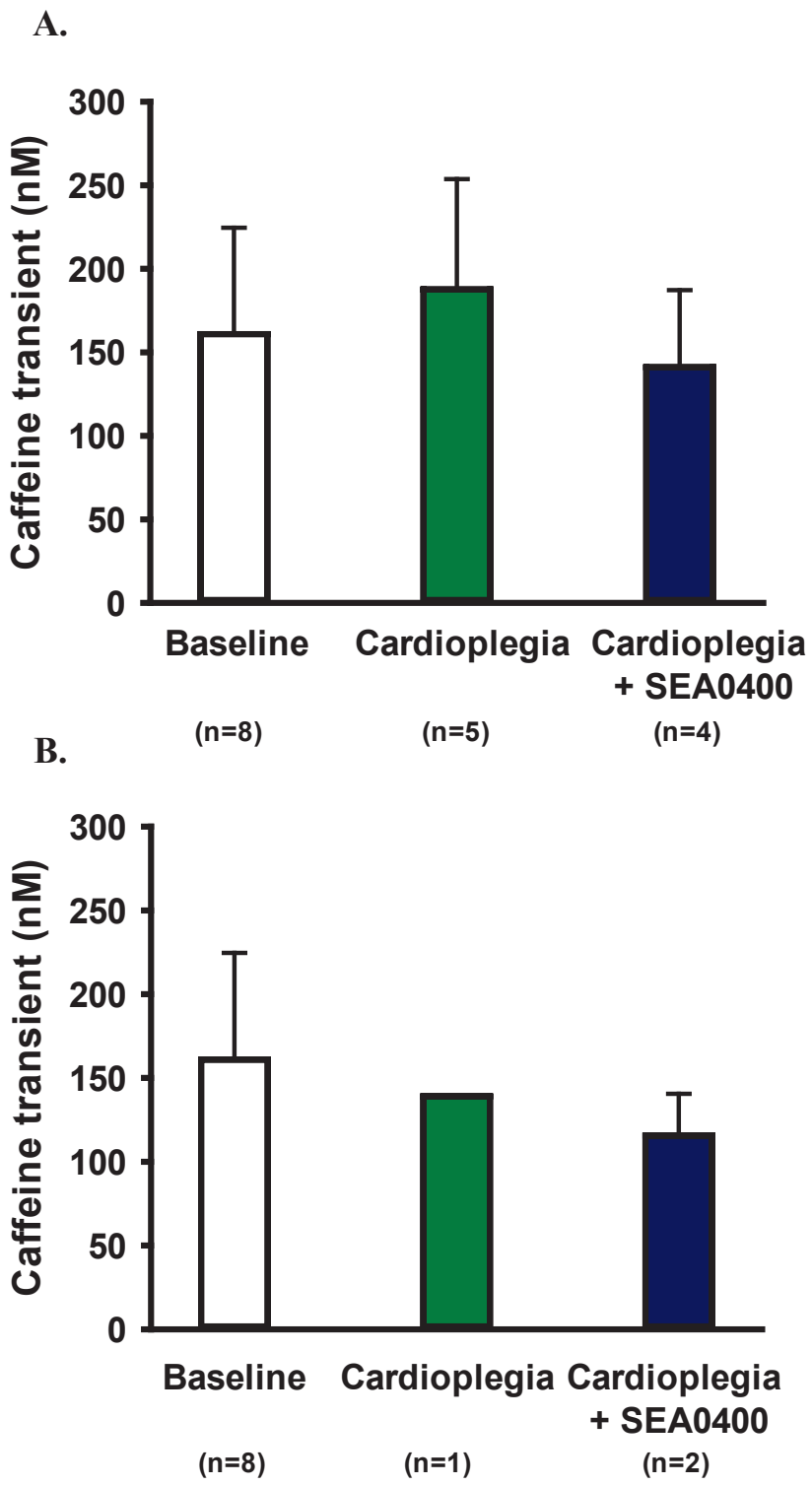

Figure 3.18. SR calcium stores were not affected by SEA0400 in cardioplegia. Mean amplitudes of caffeine transients did not differ between myocytes subjected to Tryode's (black), cardioplegia (green), or cardiopelgia+SEA0400 (blue) at **A.** 2 minutes or **B.** 10 minutes of reperfusion.

CHAPTER 4. DISCUSSION

4.1. Overview

The overall objectives of this study were to evaluate the effects of SEA0400 during ischemia-reperfusion and to determine the mechanism of protection in an isolated myocyte model of cardioplegic arrest. The specific objectives of this study were: 1) to develop an isolated cell model that represents ischemic conditions during cardioplegic arrest, 2) determine if inhibition of NCX with SEA0400 during simulated cardioplegic arrest affects $[Ca^{2+}]_i$, 3) to determine whether SEA0400 affects cardiomyocyte contractile function, 4) to determine whether SEA0400 has an effect on $[Ca^{2+}]_i$ and contractility of myocytes under normoxic conditions, and 5) determine if SEA0400 affects SR Ca^{2+} stores in ischemia and reperfusion.

4.2. Cell Model

In order to develop an isolated cell model that represents typical ischemic conditions encountered during cardioplegic arrest, the pO_2 and pH of the simulated cardioplegia were altered. The pH was altered simply by reducing the concentration of $NaHCO_3$ from 30 mM to 20 mM. After 20 minutes bubbling with 90% N_2/ 10% CO_2 gas, the pH of the solutions was measured and consistently read 6.7-6.8. These values for pH correlate with the pH of cardiac tissue during an ischemic episode (Carrier et al., 1998; Bers, 2001; Karmazyn et al., 2001). Although the solutions were bubbled with 90% N_2/ 10% CO_2 gas throughout the ischemic protocol, and 90% N_2/ 10% CO_2 gas was blown over the plexiglass dish containing the myocytes, the pO_2 of the solutions was still

approximately 38 mmHg. Studies indicate that, during an ischemic episode of 40 minutes, the pO_2 of the myocardium decreases to values as low as approximately 16 mmHg (Carrier et al., 1998).Therefore, $Na_2S_2O_4$ was added in concentrations from 0.5 to 6.0 mM and the pO_2 was measured. Concentrations as low as 0.5 mM and as high as 10 mM $Na_2S_2O_4$ have been used in previous studies to create ischemic solutions for ventricular myocytes (Seki and MacLeod, 1995; Ho et al., 2002; Coutinho et al., 2013). Our results indicated that, at a concentration of 5.0 mM $Na_2S_2O_4$, the pO_2 was approximately 10 mmHg and cells could survive the 85-minute protocol. Therefore, the cell model developed for use in this study included 5 mM $Na_2S_2O_4$ in the ischemic solutions to accurately reflect pO_2 encountered in cardioplegia.

4.3. Effect of SEA0400 as a Cardioplegia Additive on Survival, Ca^{2+} Concentrations and Contractile Responses in Isolated Ventricular Myocytes.

Cell survival was evaluated throughout the 85-minute protocol. The results of this study show that 87.5% (7 of 8 cells) of cells subjected to global ischemia did not survive the 45-minute ischemic period. When cells were subjected to simulated cardioplegia, cell survival was increased so that 10 of 24 cells survived the entire protocol. However, this increase in cell survival was not significant compared to ischemic control. Interestingly, when 1 μM SEA0400 was added to cardioplegia, cell survival was significantly increased compared to ischemic control, with 17 of 25 cells surviving the entire protocol. Still, this increase was not significantly different from treatment with cardioplegia alone. This is the first study to examine the effects of SEA0400 added to cardioplegia in an isolated cell model. However, some previous studies have examined the effect of SEA0400 on rat myocyte survival in a model of Ca^{2+} paradox injury, as well as its effects on infarct size

in the intact heart in ischemia-reperfusion models. Takahashi et al. (2003) reported increased survival in cultured rat myocytes when 1 μM and 3 μM SEA0400 were added to Ca^{2+} paradox injury solutions compared to control solution. As well, Magee et al. (2002) reported that 1 μM SEA0400, whether administered before or after regional ischemia, significantly reduced infarct size and improved postischemic contractility (dp/dt) when compared to control hearts. As infarct size represents cell death in an intact heart, these results concure with the results of this study, in that 1 μM SEA0400 added to cardioplegia protected against cell death compared to global ischemic conditions. As well, a previous Masters student in our lab showed decreased troponin release from whole rat hearts subjected to cardioplegia plus SEA0400 in a Langendorff perfusion model (unpublished Ali, 2011). Troponin release is indicative of cellular damage, further suggesting a decrease in cell death during cardioplegic arrest with SEA0400 (Xiong et al., 2011).

As previously stated, cell death from ischemia-reperfusion is thought to be caused by Ca^{2+} overload (Piper at al., 1998; Murphy and Steenbergen, 2008). As well, Ca^{2+} overload is a cause of potentially fatal arrhythmias (Kloner and Jennings, 2001; Pogwizd et al., 2001). It is understood that reverse mode NCX due to an accumulation of $[Na^{+}]_i$ is the driving force for Ca^{2+} overload at the onset of ischemia and again in early reperfusion (Karmazyn et al., 2001; Allen and Xiao, 2003). Therefore, another objective of this study was to determine the effects of blocking reverse mode NCX on $[Ca^{2+}]_i$ during simulated cardioplegic arrest. This study showed that the addition of 1 μM SEA0400 to cardioplegia significantly reduced Ca^{2+} accumulation throughout the ischemic period compared to both ischemic control and cardioplegia without SEA0400. Although the

effects of SEA0400 on $[Ca^{2+}]_i$ have not been investigated during cardioplegic arrest, previous studies have shown that adding SEA0400 to a chemical ischemic solution significantly decreased $[Ca^{2+}]_i$ accumulation in mouse myocytes compared to their ischemic control (Wang et al., 2006). The results from our study concur with this earlier study in that SEA0400 reduced Ca^{2+} overload in the ischemic period and extend these findings to cardioplegia.

Our study also showed that SEA0400 actually increased Ca^{2+} transient and contraction amplitudes in reperfusion. By contrast, Wang et al. (2006) reported significantly lower $[Ca^{2+}]_i$ throughout reperfusion in myocytes subjected to SEA0400 during the ischemic period. The differing results during reperfusion found in the present study compared to those found by Wang et al. (2006) could be due to a variety of factors. For instance, the concentration of SEA0400 used by Wang et al. (2006) was lower (0.3 μM) than the concentration used in the present study (1 μM). As well, Wang et al. (2006) conducted their studies in quiescent, non-stimulated myocytes, whereas the myocytes in our study were paced throughout the protocol at a frequency of 1 Hz. There was no difference in diastolic Ca^{2+} throughout reperfusion in the cells treated with cardioplegia plus SEA0400 and those treated with cardioplegia alone. Therefore, the difference in $[Ca^{2+}]_i$ during reperfusion between these two studies may be due to the fact that the myocytes in the present study were contracting and therefore had Ca^{2+} transients. By contrast, Wang et al. (2006) only reported $[Ca^{2+}]_i$ in quiescent cells, therefore there were no Ca^{2+} transients to measure.

Velocity to peak and velocities to 50% and 90% decay of Ca^{2+} transients were also assessed in this study. The results show that there was some variability between cells

at baseline with regard to velocity to peak of Ca^{2+} transients. However, there were no significant differences in the velocity to peak during cardioplegic arrest, as Ca^{2+} transients were abolished. In addition, there was no difference in velocity to peak during reperfusion between cells treated with cardioplegia containing SEA0400 or cardioplegia alone. By contrast, cells treated with cardioplegia containing SEA0400 did have a faster velocity to 50% and 90% Ca^{2+} transient decay at several time points during reperfusion. The velocity of Ca^{2+} transient decay is 92% due to reuptake of Ca^{2+} into the SR by SERCA and 7% due to the removal of Ca^{2+} from the cytosol by the NCX in rat cardiomyocytes (Bers, 2001). Phospholamban (PLB) affects the speed of Ca^{2+} reuptake, as it has an inhibitory effect on SERCA. When PLB is phosphorylated by protein kinase A, inhibition of SERCA is removed and the rate of Ca^{2+} reuptake into the SR increases (Bers, 2002). Therefore, SEA0400 may interact with this pathway to speed Ca^{2+} transient decay in cells treated with cardioplegia containing SEA0400. Conversely, Acsai et al. (2007) showed that there was no difference in the decay of Ca^{2+} transients in rat myocytes subjected to SEA0400, suggesting SEA0400 has little effect on SERCA. Additional experiments that examine the effects of SEA0400 on SERCA activity directly could be informative.

To summarize, NCX blockade by SEA0400 in cardioplegia significantly reduced the Ca^{2+} overload normally seen in myocytes subjected to an ischemic episode. Interestingly, Ca^{2+} transient amplitudes and contraction amplitudes were markedly larger in cells treated with cardioplegia containing SEA0400. As well, velocity of the Ca^{2+} transient to 50% and 90% decay were larger at several time points during reperfusion, yet the velocity to peak of the Ca^{2+} transients were unchanged. Therefore SEA0400 may

have an effect on Ca^{2+} transient amplitude and decay, however previous studies suggest that this does not include an effect on SERCA activity. Further experiments to verify SERCA activity upon reperfusion following cardioplegic arrest are necessary to investigate this further.

4.4. Effect of SEA0400 Under Normoxic conditions on survival, Ca^{2+} Concentrations and Contractile Responses in Isolated Ventricular Myocytes.

To determine whether SEA0400 alone was responsible for the increase in $[Ca^{2+}]_i$ and contraction amplitude in reperfusion, SEA0400 was also evaluated under normoxic conditions in isolated rat ventricular myocytes. Our results showed that all the cells subjected to oxygenated Tyrode's solution, whether exposed to SEA0400 or not, survived the 85-minute protocol.

Diastolic Ca^{2+}, systolic Ca^{2+} and Ca^{2+} transient amplitudes did not differ between the two groups throughout the protocol. Furthermore, velocity to peak and velocities to 50% and 90% decay of the Ca^{2+} transients were not different between groups. Previous studies have sought to determine whether SEA0400 has an effect on $[Ca^{2+}]_i$ in isolated ventricular myocytes under normoxic conditions (Nagy et al., 2004; Tanaka et al., 2005; Acsai et al., 2007, Ozdemir et al., 2008; Farkas et al., 2008; Birinyi et al., 2008). Previous studies on isolated myocytes from different species conducted under normoxic conditions have shown that concentrations from 0.3 – 1 μM of SEA0400 have no effect on $[Ca^{2+}]_i$ and contractility (Nagy et al., 2004; Farkas et al., 2008; Birinyi et al., 2008). In contrast, other studies have shown that NCX inhibition with SEA0400 increases contractility and Ca^{2+} transients in isolated myocytes under normoxic conditions (Acsai et al., 2007, Tanaka et al., 2005; Ozdemir et al., 2008). Variability between species is to be expected,

however the study by Acsai et al. (2007) was conducted on rat ventricular myocytes, indicating that SEA0400 under normal conditions can cause an increase in $[Ca^{2+}]_i$. This would be expected as, under normal conditions, the NCX works in the forward mode removing Ca^{2+} from the cell (Matsuda et al., 2001). Since SEA0400 can block both the forward and the reverse mode at concentrations of 1 μM, Ca^{2+} would not be removed through the exchanger at each beat resulting in a net accumulation of $[Ca^{2+}]_i$. Species variations could play a part in these conflicting results, however as previously discussed. Studies using intact murine ventricular myocytes have shown increased $[Ca^{2+}]_i$ and contractility when 1 μM SEA0400 is added to solution (Tanaka et al., 2005; Acsai et al., 2007). Unlike these earlier studies, the present study showed that cells treated with 1 μM SEA0400 for 45-minutes, exhibited significantly larger contraction amplitudes during the 20-minute washout, or "reperfusion", period. These results clearly show that SEA0400 markedly increased peak contractions with little effect on peak Ca^{2+} transients under normoxic conditions. This suggests that exposure to SEA0400 alone may sensitize the myofilaments to Ca^{2+}, as discussed in detail below.

During cardiac EC-coupling, Ca^{2+} is released from the SR causing a rapid rise in $[Ca^{2+}]_i$ which results in contraction. Myofilaments, which are made up of actin and myosin, translate the increase in $[Ca^{2+}]_i$ into contraction. Ca^{2+} directly interacts with troponin C on actin, exposing the myosin binding site and allowing myosin to bind and form a cross bridge (Katz, 2001). Binding of the myosin head and subsequent hydrolysis of ATP by myosin causes shortening of the myofilaments, which results in a contraction. The sensitivity of myofilaments to Ca^{2+} can be affected by various conditions. For example, myofilament Ca^{2+} sensitivity declines markedly at low pH, such as during

ischemia (Bers, 2002; Ferrari, 2002; Pinnell et al., 2007). Thus, the sensitization of myofilaments to Ca^{2+} is a potential target for drug action.

The results of this study showed that myocytes treated with cardioplegia containing SEA0400 had a nearly 100% increase in contraction size during reperfusion compared to baseline (3.5±0.4 vs. 6.9±0.4 % cell shortening), while Ca^{2+} transients rose only by 46% (105.4±6.8 vs. 153.1±9.2 nM) from baseline to reperfusion. Myocytes treated with cardioplegia alone did not have marked increase in contraction size or Ca^{2+} transient amplitude during reperfusion. As well, cells treated with Tyrode's plus vehicle control showed no change in contraction or Ca^{2+} transient size throughout the protocol. However, cells treated with Tyrode's plus SEA0400 did exhibit an increase in contraction size without an increase in Ca^{2+} transient amplitude even in the absence of cardioplegia. Taken together, these results suggest that SEA0400 sensitizes the myofilaments to Ca^{2+}. As a previous study conducted in dog ventricular myocytes showed that SEA0400 had no effects on myofilament Ca^{2+} sensitivity (Birinyi et al., 2008), further experiments to directly measure myofilament Ca^{2+} sensitivity, such as assessing actomyosin MgATPase activity at various levels of free Ca^{2+} (Venema and Kuo, 1993), are now warranted.

4.5. Effect of SEA0400 on SR Ca^{2+} Stores at Basline and Upon Reperfusion in Isolated Ventricular Myocytes.

Another objective of this study was to determine why the systolic Ca^{2+} levels, and therefore the Ca^{2+} transient amplitudes, were larger during reperfusion in cells subjected to cardioplegia plus SEA0400 compared to cells subjected to cardioplegia alone. There are many possible explanations for the increase in peak Ca^{2+} transients in reperfusion. For

instance, persistent blockade of NCX at the beginning of reperfusion could explain elevated $[Ca^{2+}]_i$, as Ca^{2+} would not leave the cell with each beat and could accumulate in the myocyte. However, this explanation is improbable as SEA0400 is reported to have a rapid washout (Matsuda et al., 2001; Lee and Hryshko, 2004). As well, NCX activity accounts for only 7% of Ca^{2+} transient decay in rat cardiomyocytes, and the velocity to 50% and 90% decay of Ca^{2+} transients is significantly faster in cells treated with cardiopelgia containing SEA0400 compared to those treated with cardioplegia alone. As NCX blockade would be expected to slow decay rates, it is unlikely NCX blockade is the cause for the increased peak Ca^{2+} transient.

Another possibility is that SEA0400 increases SR Ca^{2+} stores in reperfusion. In cells subjected to SEA0400 at the onset of ischemia, Ca^{2+} would be unable to enter the cell through NCX. However, Ca^{2+} would also be unable to exit the cell through NCX. Therefore, it is possible that Ca^{2+} is sequestered in the SR at the onset of ischemia, which could account for larger amounts of Ca^{2+} being released with each beat (larger Ca^{2+} transients) upon reperfusion. To test this hypothesis, 10 mM caffeine solution was rapidly applied to myocytes at baseline (20 minute mark of the protocol) or during reperfusion (67 and 75 minute mark of protocol). The results obtained during early reperfusion showed there is no difference in SR Ca^{2+} stores between groups at any time point. As well preliminary results suggest there is also no difference in SR Ca^{2+} stores 10 minutes into reperfusion. These observations agree with the results of a study by Birinyi et al. (2008), which demonstrated SEA0400 has no effect on SR Ca^{2+} stores in isolated canine myocytes. Still, further experiments to increase sample size need to be carried out to

confirm that SEA0400 has no effect on SR Ca^{2+} stores in isolated ventricular myocytes subjected to simulated cardioplegic arrest.

Another possibility is that SEA0400 sensitizes a component or components of the Ca^{2+} release process. Ca^{2+} is released from the SR through RyR which can be regulated by phosphorylation via various signaling pathways such as the Ca^{2+}/Calmodulin-dependent protein kinase II pathway (Wehrens et al., 2004), and the cyclic AMP-protein kinase A pathways (Takasago et al., 1991**).** Increased phosphorylation of RyR by both pathways is believed to increase SR Ca^{2+} release (Bers, 2002). Therefore, experiments could be carried out to determine whether SEA0400 has any effect on phosphorylation of RyR. If SEA0400 in cardioplegia does enhance phosphorylation of RyR, this could augment Ca^{2+} release and increase Ca^{2+} transients upon reperfusion.

4.6. Limitations

This study investigated the effects of SEA0400 on isolated rat ventricular myocytes exposed to either simulated cardioplegic arrest or oxygenated Tyrode's solution. One limitation of this study is the small sample sizes used to assess the SR Ca^{2+} stores. However, further experiments would easily correct for this limitation. As well, further experiments are required to determine why SEA0400 had different effects on $[Ca^{2+}]_i$ in simulated cardioplegia than in normoxic conditions.

The ultimate goal of this research is to determine whether SEA0400 is a safe and beneficial additive to cardioplegic solutions. Thus, although an isolated cell model is an integral part of understanding the effects of SEA0400, further experiments are required to determine the effects of this compound in an *in vivo* model. Understanding the mechanism of the protective effect is essential in understanding possible side effects of SEA0400. Still, the logical next step would be to test the drug in an *in vivo* animal model.

There are differences with regards to Ca^{2+} handling in rodent and human ventricular myocytes. The NCX accounts for approximately 28% of the Ca^{2+} removal in human cardiomyocytes whereas it only accounts for approximately 7% of the Ca^{2+} removal in ventricular myocytes from rats and mice. As such, it is important to repeat experiments in an animal model that more closely resembles the Ca^{2+} handling seen in humans. Possible research subjects include rabbits and pigs. Pigs would be ideal in conducting *in vivo* experiments, as their organ size closely resembles that of humans.

4.7. Summary

The present study demonstrated that the NCX blocker SEA0400 added to cardioplegia significantly reduced the accumulation of intracellular Ca^{2+} throughout the ischemic period while also increasing cell survival compared to ischemic control. Ca^{2+} transient amplitude and fractional cell shortening were significantly larger in reperfusion in cells treated with SEA0400 in cardioplegia. Interestingly, there was no difference in $[Ca^{2+}]_i$ in cells exposed to SEA0400 in oxygenated Tyrode's solution, yet contraction amplitudes were significantly larger after 45-minutes exposure to the drug. Finally, SR Ca^{2+} stores were unchanged by cardioplegia and cardiolpegia containing SEA0400. This is the first study to examine the effects of SEA0400 as a cardioplegia additive in an isolated cellular model and show improved cell survival. The results of this study have potentially important clinical implications. As Ca^{2+} overload during ischemic arrest is thought to be a main factor in cardiac injury following surgery, the reduction in $[Ca^{2+}]_i$ brought on by the addition of SEA0400 to cardioplegia, as well as the increased cell survival, could be greatly beneficial to patients undergoing open heart surgery.

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