Poloxamer 188 Protects Isolated Mouse Cardiomyocytes from Hypoxia/Reoxygenation Injury
-Implications for Cardioprotection after Cardiac Arrest

By

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Thesis

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Dedication

To everyone who has believed in me all these years!

“Shoot for the moon.
Even if you miss, you’ll land among the stars.”

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1. Introduction

1.1. Cardiac Arrest and CPR

Cardiac arrest is a leading cause of both debilitating illness and death worldwide (Benjamin et al, 2017; Coute et al, 2017; Mozaffarian, 2017). In the United States (U.S.) alone, over 450,000 people died due to cardiac arrest in 2016 (Benjamin et al, 2017; Coute et al, 2017). More than 380,000 people experienced an out-of-hospital cardiac arrest. Of those given cardiopulmonary resuscitation (CPR) by emergency medical services, only 10.6% survived. While in the hospital, ~209,000 people suffered a cardiac arrest and received CPR, with better survival rates of ~24%. If a bystander provided CPR, 31.4% survived due to the earlier delivery of the resuscitation. Therefore, even with delivery of the best, most up-to-date CPR, survival rates are still too low, and ~90% of survivors still suffer cardiac, as well as neurological, deficits (Benjamin et al, 2017; Coute et al, 2017; Mozaffarian, 2017).

Experiencing a cardiac arrest can lead to severely limited quality of life, loss of productivity, and premature death. Moreover, the economic costs associated with cardiac arrest can exceed $300 billion dollars per year (Benjamin et al, 2017). As a consequence of the dismal survival rates of both in-hospital and out-of-hospital cardiac arrests, and the high personal and economic costs associated, the 2015 Institute of Medicine report “Strategies to Improve Cardiac Arrest Survival: A Time to Act” has made it a “*national responsibility… to improve the likelihood of survival and favorable outcomes following a cardiac arrest*” (Institute of Medicine, 2015).

Risk factors leading to cardiac arrest include high levels of low-density lipoprotein (LDL) and low levels of high-density lipoprotein (HDL), diabetes, hypertension, obesity, and smoking (Benjamin et al, 2017; Rafieian-Kopaei et al, 2014). While prevention of cardiac arrest through decreasing risk factors remains the highest priority, timely reperfusion to shorten the duration of the primary ischemic event affecting vital organs, such as the heart, is of utmost importance (Rafieian-Kopaei et al, 2014).

1.2. Ischemia/Reperfusion Injury

Ischemia occurs when there is a disruption of the blood flow, along with the supply of oxygen and nutrients it carries to organs, tissues, and cells. Cellular consequences can include hypoxia, increased intracellular pH, altered ion exchange/transport, cell swelling, membrane disruptions, and cell death. Reperfusion is the return of the blood flow, and the oxygen and nutrients it carries, after the ischemic period. However, reperfusion itself, after ischemia, results in additional detrimental cellular dysfunction, including continued calcium (Ca^{2+}) ion transport dysfunction, altered redox state, production of reactive oxygen species, further membrane damage, initiation of inflammation, and complement cell activation. Both phases contribute to the subsequent ischemia/reperfusion (I/R) injury, and potential increases in cell death (Gottlieb et al, 2011; Kalogeris et al, 2012; Martindale & Metzger, 2014). Therefore, I/R injury, occurring during cardiac arrest and CPR, is a complex pathological event with multiple processes (Kalogeris et al, 2012; Maneechote et al, 2017)(Figure 1).

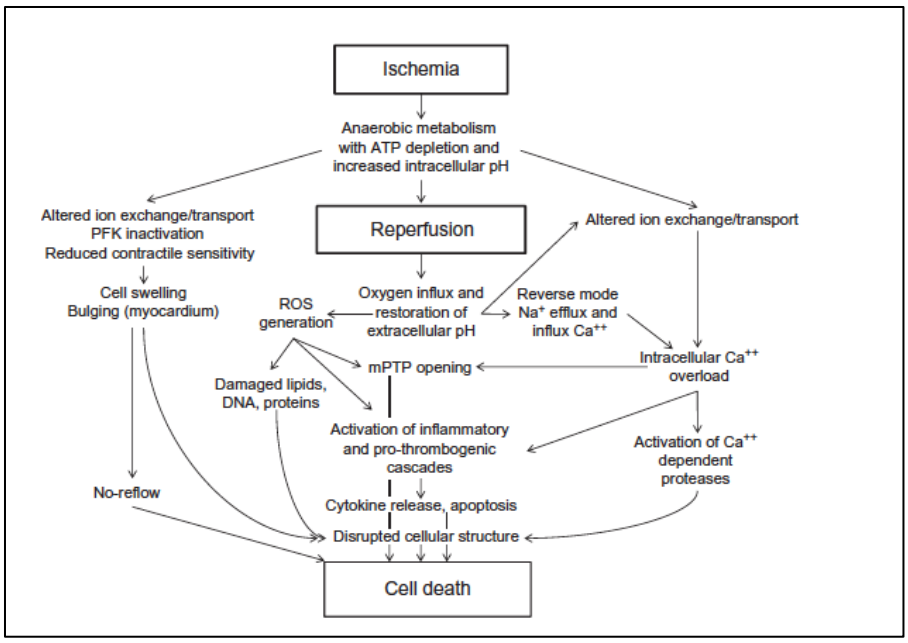

Figure 1. Schematic diagram of major pathological cellular events that occur during I/R injury (Kalogeris et al, 2012).

In cardiomyocytes, many of the same pathological events shown in Figure 1 also occur, and lead to disruption of the cell membrane. Loss of the membrane integrity can allow an excessive influx of Ca^{2+} ions into the cardiomyocytes, leading to altered redox, hypercontracture, mitochondrial dysfunction, and cell death (Figure 2).

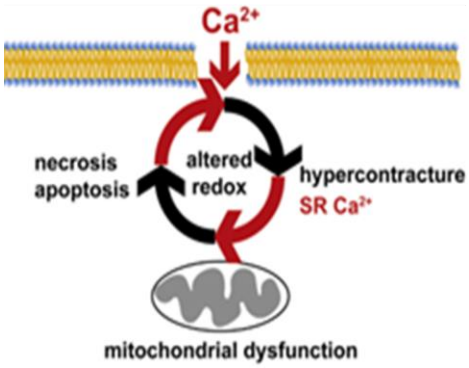

Figure 2. Model of cardiomyocyte membrane disruption and subsequent pathological events (Martindale & Metzger, 2014).

1.3. Calcium (Ca^{2+}) in normal healthy cardiomyocytes

Calcium (Ca^{2+}) is a major regulator of cardiomyocyte function. It plays a role in the heart's electrical signaling and cardiac myocyte contraction, and acts as an intracellular messenger in numerous cellular processes (Fearnley et al, 2011; Li et al, 2017). Normal intracellular Ca^{2+} concentration in cardiomyocytes is ~1 µM, with the extracellular concentration typically averaging 2 mM (Klabunde, 2016; Li et al, 2017). To regulate proper Ca^{2+} concentrations, cardiomyocytes rely upon a critical working balance between Ca^{2+} channels in the cell membrane that bring Ca^{2+} into the cell (predominantly voltage-gated L-type Ca^{2+} channels), Ca^{2+} release into the cell from ryanodine receptors on the sarcoplasmic/endoplasmic reticulum (S/ER), and

Ca^{2+} efflux by the Na^+/Ca^{2+} exchanger on the cell membrane (Fearnley et al, 2011; Kalogeris et al, 2012; Li et al, 2017).

1.4. Calcium (Ca^{2+}) overload in cardiomyocytes during I/R injury

Extreme increases in intracellular [Ca^{2+}] ([Ca^{2+}]$_i$), such that occur during I/R injury, can have major effects on cardiomyocytes (Garcia-Dorado et al, 2012). The Ca^{2+} may not only enter the cell through membrane disruptions, but also through altered functioning of Ca^{2+} channels, exchangers, and/or modulating receptors. In dystrophic mouse cardiac myocytes, altered functioning voltage-gated L-type Ca^{2+} channels contribute to the Ca^{2+} influx (Viola et al, 2013). This Ca^{2+} influx was pharmacologically replicated by activating the mouse cardiac myocytes voltage-gated L-type Ca^{2+} channels with the agonist (activator) S-(-)-BayK8644, which significantly increased the [Ca^{2+}]$_i$ (Viola et al, 2013). During I/R injury, voltage-gated L-type Ca^{2+} channels may also play a role in the Ca^{2+} overload that starts during ischemia, and more importantly, continues during the early part of reperfusion.

During I/R injury, depleted ATP concentrations inactivate various Ca^{2+}-ATPases, including the ones located on the cell membrane and the S/ER Ca^{2+}-ATPase (SERCA), decreasing active Ca^{2+} efflux and reuptake of Ca^{2+} into the calcium stores of the S/ER, respectively, and thus causing Ca^{2+} overload (Kalogeris et al, 2012) (Figure 3). Pharmacologically, thapsigargin, a potent inhibitor of the SERCA, has been used to block Ca^{2+} from entering the calcium stores (Kwan et al, 2003). Depletion of these intracellular Ca^{2+} stores occurs because the ryanodine receptors continue to pump Ca^{2+} out into the cytoplasm, causing an increase in [Ca^{2+}]$_i$ (Feng et al, 2011) (Figure 3).

In addition, during I/R injury, an increase in cellular pH due to a switch to cellular anaerobic metabolism causes the Na^+/H^+ exchanger to increase its efflux of H^+ ions. This increased H^+ efflux allows a greater influx of Na^+ ions. As reperfusion begins, this efflux of H^+ ions and influx of Na^+ ions becomes even greater. This significant increase in intracellular Na^+ causes the Na^+/Ca^{2+} exchanger to switch to reverse mode, pumping Na^+ ions out and bringing Ca^{2+} ions in to the cell (Kalogeris et al, 2012) (Figure 3). The β_1 receptor agonist, dobutamine, in concentrations ranging from 0.1-1 µM, has been pharmacologically used to activate the voltage-gated Na^+ channel in the cell membrane. The increased intracellular Na^+ concentration, similar to that which can occur through the upregulation of the Na^+/H^+ exchanger activity during I/R injury, causes the Na^+/Ca^{2+} exchanger to switch to its reverse mode, increasing the influx of Ca^{2+} ions in to the cell. This increase in Ca^{2+} influx can be abolished by selectively blocking the reverse mode of the Na^+/Ca^{2+} exchanger with 3 µM KB-R7943 (Satoh et al, 2000; Yan et al, 2015).

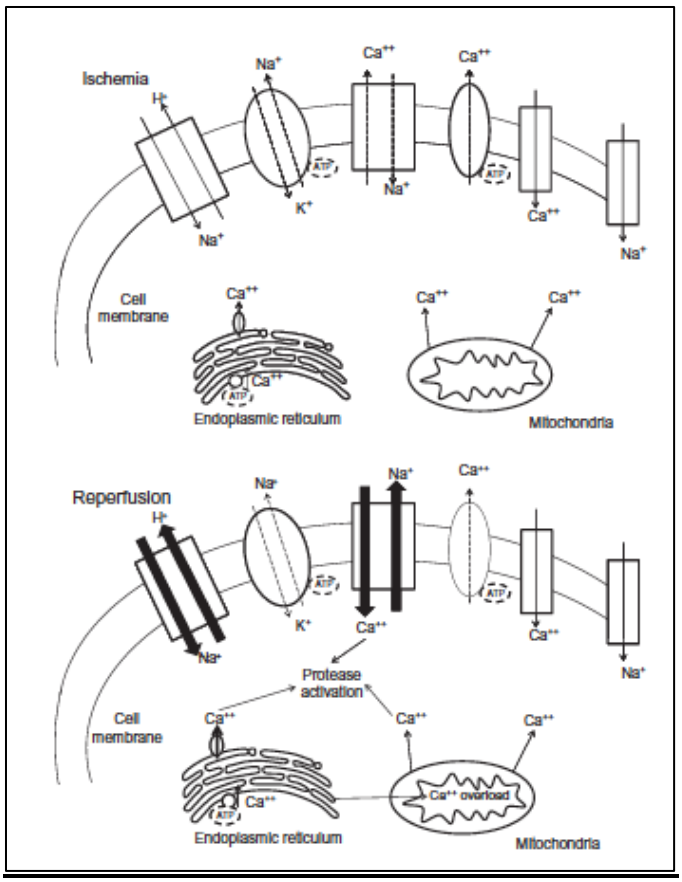

Figure 3. Schematic diagrams of changes in Ca^{2+} handling during ischemia and reperfusion (Kalogeris et al, 2012).

The Ca^{2+}-sensing receptor (CaSR) is a member of the G-protein coupled receptor superfamily, and is expressed on the membranes of cardiomyocytes. Its main function is to aid in regulating intracellular Ca^{2+} homeostasis. However, during I/R, this receptor can dysfunction, and cause excessive Ca^{2+} influx, most likely through activation of several Ca^{2+} channels (Feng et al, 2011; Zhang et al, 2016). Pharmacologically, spermine can stimulate the CaSR into activating an abnormal influx of Ca^{2+} (Feng et al, 2011).

1.5. Potential clinical interventions for I/R injury

A critical focus of clinical interventions is to improve survival rates and quality of life for thousands of patients each year by attenuating I/R injury when reperfusing the organs and tissues after a cardiac arrest. However, there unfortunately is no current effective therapy available for combating reperfusion injury following cardiac arrest. The challenge lies in the fact that a cardiac arrest is an unplanned event, and often not witnessed, so medical assistance is often delayed, thus prolonging the ischemic period. When medical assistance is initiated, including the delivery of CPR, this necessary reperfusion can cause additional detrimental organ and cellular damage. This is unlike planned surgical procedures, such as coronary artery bypass graft surgeries and heart transplants, which experience some degree of I/R injury, but can be pre- and peri-operatively treated with interventions like hypothermia and cardioplegia. These interventions, however,

would be difficult to translate to most cardiac arrest and CPR scenarios. A successful therapy for reperfusion injury following a cardiac arrest would need to be easily transported, since a large number of cardiac arrests occur outside of a medical facility, and easily administered at the start of CPR, since the majority of damage occurs during the initial minutes of reperfusion (Garcia-Dorado et al, 2012). Therefore, this is an important unmet medical need, and novel therapeutic interventions capable of limiting the reperfusion-induced cellular dysfunctions are needed to reduce the organ and tissue damage, preserve function, and improve clinical outcomes.

A variety of therapeutic interventions have been investigated over the years to limit I/R injury, with a few focusing on the additive injury that occurs during the early reperfusion phase to target any potentially salvageable cells. Ischemic post-conditioning, which is the intentional introduction of several short ischemic pauses during the early part of reperfusion, has been successfully employed in I/R models of myocardial infarction (MI), stroke, and cardiac arrest (Vinten-Johansen et al, 2005; Segal et al, 2012; Dezfulian et al, 2013; Yannopoulos et al, 2013; Dongworth et al, 2014). The additional reperfusion injury following ischemia can also be attenuated by pharmacological post-conditioning. For example, volatile anesthetics, such as sevoflurane, when given immediately upon reperfusion for several minutes, have been shown to improve functional recovery in hearts (Weber et al, 2005; Riess et al, 2014). Unfortunately, results of studies with both the above mentioned interventions in clinical settings have been mixed (Hausenloy et al, 2010; Hausenloy et al, 2013).

Another therapeutic option may involve methods to improve the endogenous membrane resealing capacity of cells that are overwhelmed due to pathological disruptions, such as I/R. Improving the membrane integrity may prevent cardiomyocyte loss, because the ability of the myocardium to regenerate is limited (Moloughney & Weisleder, 2012).

1.6. Copolymer-based cell membrane stabilizers

Copolymer-based cell membrane stabilizers (CCMS) have the potential to play a role as a therapeutic option for I/R injury. CCMS, also called Poloxamers or Pluronics, are synthetic non-ionic block copolymer molecules. They are available in varying molecular weights (MW), as well as varying ratios of hydrophobic polypropylene oxide (PPO) to hydrophilic polyethylene oxide (PEO) chains (Bates et al, 2012). CCMS are thought to directly stabilize cell membranes by adhering to the membrane surface, thus sealing gaps and/or tears caused by severe cellular stress, such that occurs during I/R injury. This may prevent unregulated Ca^{2+} ion exchange between intra- and extra-cellular compartments, altered redox state, hypercontracture, mitochondrial dysfunction, and cell death, and thus, preserve cellular function (Yasuda et al, 2005; Townsend et al, 2010) (Figure 4).

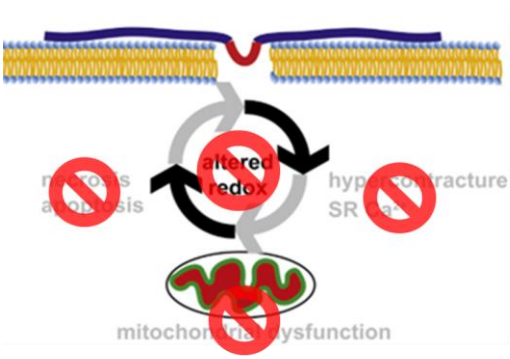

Figure 4. Model of CCMS membrane stabilization preventing I/R-induced cellular damage (Martindale & Metzger, 2014).

Recent work using x-ray scattering, atomic force microscopy, and computer simulations has shown the interaction of poloxamers and lipid bilayers. These studies show that the hydrophilic portions interact with the lipid headgroups of the bilayer and remain at the surface of the membrane, while the hydrophobic portion inserts into the damaged region of the bilayer, ultimately closing the tear (Maskarinec et al, 2002; Adhikari et al, 2016; Goliaei et al, 2016) (Figure 5).

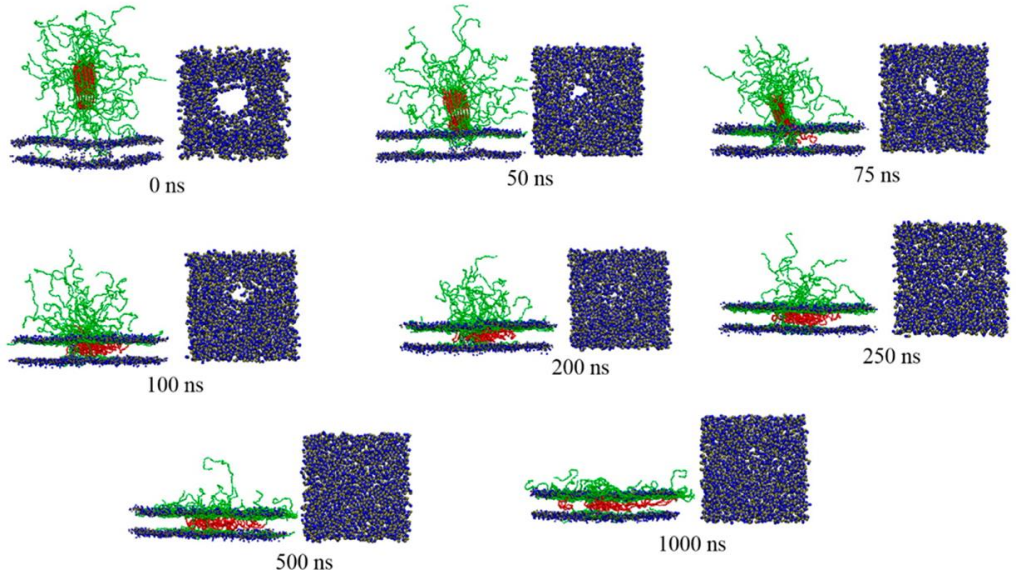

Figure 5. Snapshots from a computer simulation of a tri-block CCMS micelle interacting with an artificial tear in a lipid bilayer. The picture on the right shows the top view of the tear in the membrane. PEO and PPO chains are colored green and red, respectively (Adhikari et al, 2016).

Additional studies have shown that following the poloxamer insertion, which stimulates tighter lipid packing, and thus restored membrane integrity, the once inserted poloxamer is squeezed out (Wu et al, 2004; Wu et al, 2005; Wu & Lee, 2009). This may allow the molecule to potentially interact with another damaged area of the membrane, or with another cell.

Even though these studies better illustrate the proposed mechanism by which CCMS interact with tears in the membrane, the mechanisms on how exactly CCMS may provide cellular protection, when administered at the start of reperfusion, remain largely undefined. Thus, further research into this mechanism is warranted.

1.7. Poloxamer 188

Poloxamer 188 (P188) is a tri-block CCMS composed of a central hydrophobic PPO chain that covalently connects two hydrophilic PEO chains. The structure is designated as $PEO_{75}PPO_{30}PEO_{75}$, with 75 PEO units on each side of the central 30 PPO units (Figure 6). This makes P188 ~20% hydrophobic and ~80% hydrophilic, with an average MW of 8,400 g/mol.

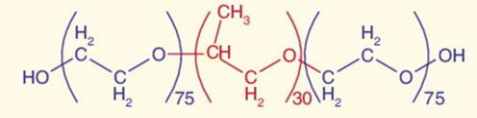

Figure 6. Structure of a P188 molecule (Bates et al, 2012).

P188 has been FDA approved for ~50 years as a therapeutic agent to reduce the viscosity of blood before transfusions and as a drug-delivery carrier molecule. It has a half-life of 18 hours in humans, and has been demonstrated to be safe when given for up to 72 hours (Maloughney & Weisleder, 2012).

In research settings examining the injury protection potential of P188, it has been shown to protect skeletal muscle (Murphy et al, 2010; Walters et al, 2011; Houang et al, 2015) and neurons (Gu et al, 2013) from I/R injury, and preserve the blood-brain barrier during cerebral hemorrhage and after traumatic brain injury (TBI), leading to reduced brain edema and neuronal apoptosis (Wang et al, 2015; Serbest et al, 2006; Bao et al, 2012). In dystrophic mouse hearts, both *ex-vivo* and *in-vivo*, subjected to I/R injury, P188 significantly reduced infarct size and fully blocked cellular markers of cell death (both apoptosis and necrosis)(Martindale & Metzger, 2014).

Studies from our research group show that in Langendorff isolated perfused rat hearts from different rat strains (Brown Norway [BN] and Dahl salt-sensitive [SS]), 1 mM P188 infused during 120 minutes of reperfusion following 30 minutes of no-flow global ischemia led to sustained improvement of coronary and myocardial function, and decreased infarct size (Figure 7).

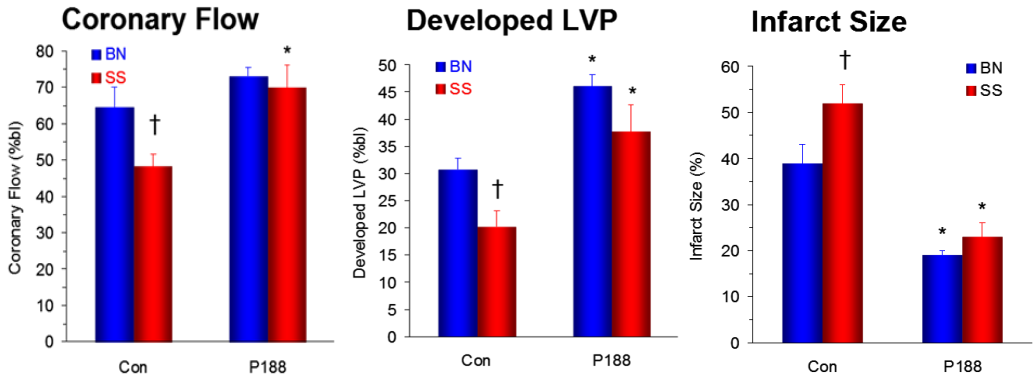

Figure 7. Graphs showing improved coronary and myocardial function (coronary flow, developed left ventricular pressure [LVP]), and decreased infarct size, in isolated rat hearts from BN and SS rats infused with 1 mM P188 during reperfusion following global ischemia (Salzman et al, 2015).

Further studies in our rat stroke model of 60 minute occlusion of the left-side middle carotid artery show that a bolus intravenous (IV) injection of 250 mg/kg P188 in saline given at the start of reperfusion dramatically decreased cerebral infarct size and improved neurological function compared to untreated stroke animals (Riess et al, 2015)(Figure 8). Neurological improvements were evidenced by improved velocity and distances run as assessed during open field assessments 24 hours post-stroke and treatment (Desland et al, 2014).

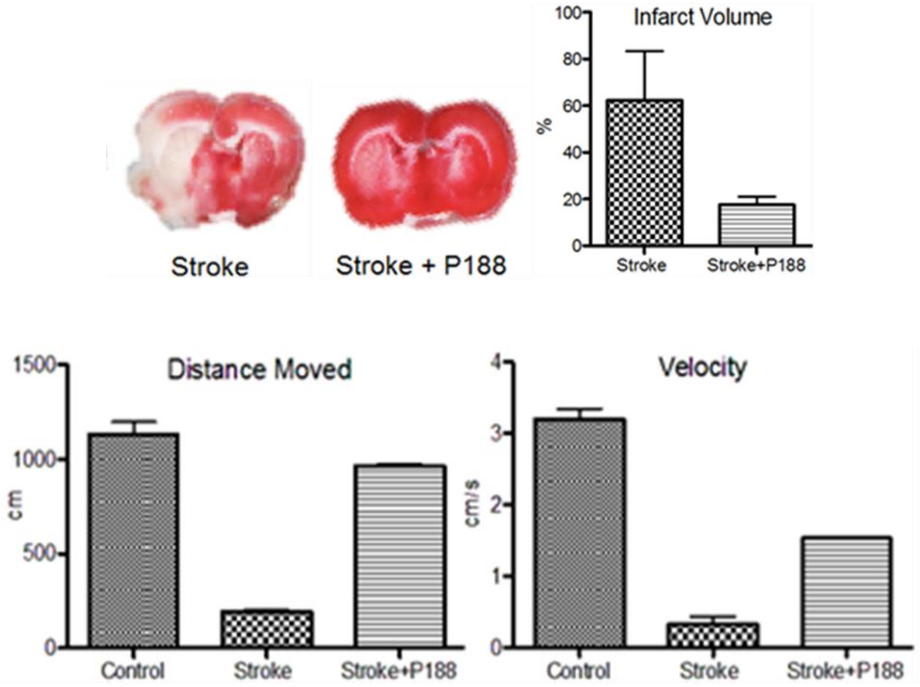

Figure 8. Top left: Photographic evidence of decreased brain infarct size 24 hours after stroke and P188 treatment administered at reperfusion. Top right: Graph depicting percentage of brain infarct volume 24 hours after stroke and P188 treatment administered at reperfusion. Bottom: Graphs depicting improved neurological function (distance moved, velocity of movement) 24 hours after stroke and P188 treatment administered at reperfusion (Riess et al, 2015).

In our collaborator's pig model of ST-Segment Elevation Myocardial Infarction (STEMI) induced by 45 minutes of endovascular coronary occlusion, 250 mg/kg P188 infusion during the 4 hour reperfusion led to significant decrease in infarct size and protection of mitochondrial integrity and function (Bartos et al, 2016)(Figure 9).

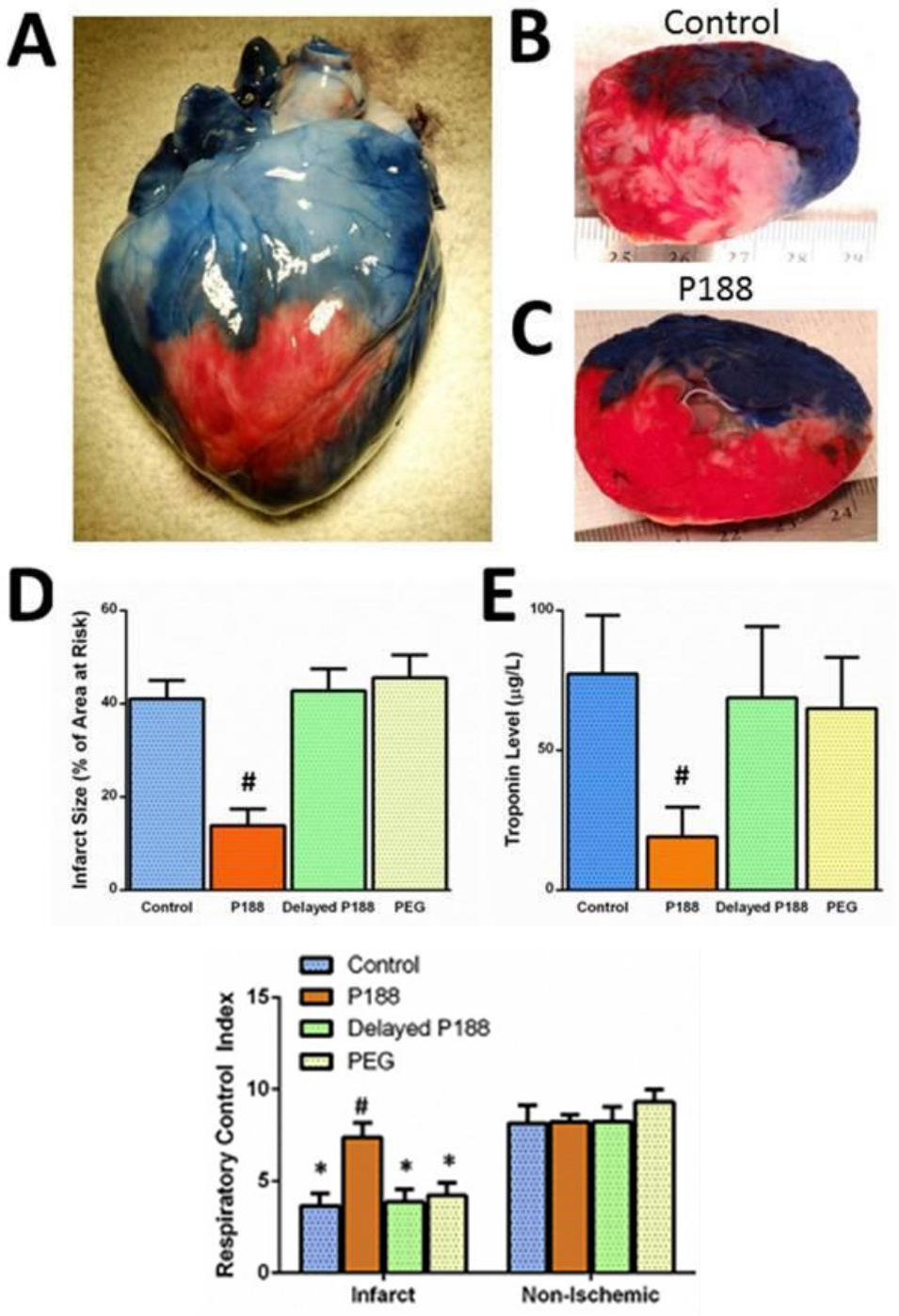

Figure 9. A: Photographic evidence of occluded region of heart (occluded area is red, viable tissue is stained blue). B: Photographic evidence of infarct area (white region) compared to area at risk (red area) and viable tissue (blue staining). C: Photographic evidence of decreased infarct size when P188 is infused during reperfusion following a myocardial infarction (MI). D: Graph depicting infarct sizes of hearts that underwent I/R (Control), hearts that received P188 infused during reperfusion following the MI, hearts that received P188 infusion 30 minutes after the start of reperfusion, and hearts that received the control molecule PEG (polyethylene glycol) infused during reperfusion. E: Graph depicting troponin levels of hearts that underwent I/R (Control), hearts that received P188 infused during reperfusion following the MI, hearts that received P188 infusion 30 minutes after the start of reperfusion, and hearts that received the control molecule PEG (polyethylene glycol) infused during reperfusion. Bottom: Graph depicting the respiratory control index of mitochondria isolated from the infarct area and non-ischemic area of hearts that underwent I/R (Control), hearts that received P188 infused during reperfusion following the MI, hearts that received P188 infusion 30

minutes after the start of reperfusion, and hearts that received the control molecule PEG (polyethylene glycol) infused during reperfusion. (Bartos et al, 2016).

On the other hand, treatment of MI with P188 in humans has shown mixed results. Although infarct size, left ventricular function, and in-hospital re-infarction rates were improved when P188 was infused immediately after thrombolytic therapy (Schaer et al, 1996), the larger follow-up RheothRx study failed to corroborate these findings (CORE trial, 1997). It is likely that the different findings are due to differences in the timing of P188 administration, such as immediately upon reperfusion versus a delay in its delivery, as demonstrated in Bartos et al (2016).

Nonetheless, studies conducted in a number of different *in-vitro*, *ex-vivo* and *in-vivo* models of I/R-type injuries to skeletal muscle and heart have shown improved function and decreased injury markers with P188 treatment. Therefore, determining the mechanism of action of this protective molecule is needed, with a critical focus being the determination of its cellular protection mechanism when P188 is administered at the start of reperfusion. Knowing this mechanism could improve clinical practices in the future.

1.8. Objectives of this study

The objectives of this study were to 1) develop an *in-vitro* cardiomyocytes model that closely mimics the status of cardiomyocytes in a normal physiologic environment, 2) develop a simulated I/R injury using hypoxia/reoxygenation (H/R) to cause 40-60% damage as assessed by several well-established indices of cellular function, 3) determine a protective concentration of P188 when administered at the start of reoxygenation following a hypoxic period, and 4) determine potential protective mechanisms of P188 when provided only during reoxygenation. The overall objective is based on the hypothesis that the tri-block copolymer P188, with its unique hydrophobic / hydrophilic chemical properties, protects cardiomyocytes against H/R injury by stabilizing the damaged cellular membrane, thus allowing the cells to maintain normal, or nearly normal, function.

2. Materials and Methods

2.1. Cell culture

Cardiomyocytes isolated from adult male C57BL/6J mouse heart ventricles were obtained from Celprogen (Torrance, CA, USA)(Figure 10). Vials of cells from three different mice were purchased, shipped frozen, and used across all experiments of this project. These cardiomyocytes harvested from adult mice were chosen for these studies since cardiomyocytes of adult humans experience I/R injury during cardiac arrest and CPR more often than those of children.

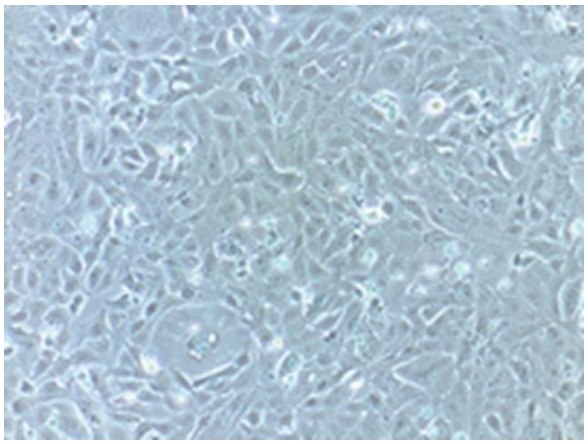

Figure 10. Photo of cardiomyocytes isolated from mouse heart ventricles (40x magnification) (Celprogen product insert, 2016).

The biochemical and molecular properties of these primary cardiomyocytes resemble those of cardiomyocytes *in-vivo* when they are maintained under the culture conditions specified by Celprogen, such as cardiomyocyte growth media containing 5% FBS, 5 mM glucose, and antibiotics obtained from Celprogen; plated on vessels coated with extracellular matrix; and exposed to an environment of humidified 21% O_2:74%N_2:5% CO_2 at 37^{o}C. These cells express a number of cardiomyocyte markers, including troponin-T and I, myogen, and α-actin. They also contain abundant mitochondria and contractile proteins. The sarcomeres, however, appear to have become disrupted, and the cells are unable to contract.

An additional benefit of these cardiomyocytes is that they have regained the ability to divide in culture conditions. This is a similar benefit used by researchers in other studies who have used cell lines (i.e., human microvascular endothelial cells, mouse atrial cardiomyocytes [HL-1]) to determine the protective effect of an endogenous compound on cellular function after H/R injury (Vellota et al, 2011).

Cardiomyocytes between passages 4-10 in cardiomyocyte growth media were plated at a seeding density of 50,000 cells/cm^2 in 96-well black-walled, clear-bottom plates coated with extracellular matrix and placed in a normal culture environment (humidified 21% O_2:74% N_2:5% CO_2, 37^{o}C). Six replicate wells for each control and treatment group were plated in each experiment. At this plating density, the cells reached confluency in 48-72 hours. The cardiomyocytes were grown to confluency to mimic the normal *in-vivo* physiological state of the cells. Plates of cells for each experiment were then randomized to either control/normoxia (C/N) or hypoxia/reoxygenation (H/R) conditions.

2.2. Optimization of H/R conditions for confluent cultures of cardiomyocytes

Previous studies with human cardiomyocytes at 80% confluency showed 40-60% damage in cell number and viability, as well as lactate dehydrogenase (LDH) release after 3 hours of hypoxia (0.1% O_2) in serum- and glucose-free media followed by 2 hours of reoxygenation (Salzman et al, 2017). We performed a series of experiments to optimize the H/R protocol in our confluent cultures of mouse cardiomyocytes (MCMs) to replicate the 40-60% reduction in cell number and viability, as well as increase in LDH release, observed in

our non-confluent cultures of human cardiomyocytes. This would allow us to examine the protective actions of a given agent (e.g., P188) over the linear segment of the cytotoxicity response curve. We determined that the optimal parameters to replicate the cytotoxicity response observed in the non-confluent human cardiomyocytes was 5 hours hypoxia (0.01% O_2) in serum- and glucose-free media followed by 2 hours reoxygenation. As shown in Figure 11, these conditions produced 40-60% cytotoxicity in our confluent mouse cardiomyocytes as assessed by cell number, as well as LDH release. We speculate that the requirement of a significantly greater hypoxia challenge is due to two factors: 1) confluent cardiomyocytes rely more on glycolysis than aerobic respiration (Piao et al, 2013), and 2) these cardiomyocytes are not consuming ATP by beating.

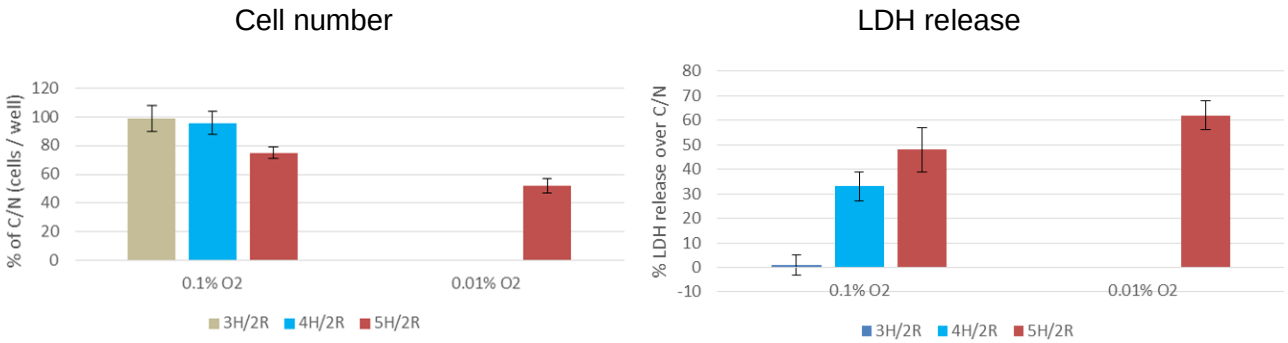

Figure 11. Graphs depicting the evolution of decreased oxygen percentage (0.1% to 0.01%) and increased hypoxia time (3 hours, 4 hours, 5 hours) followed by 2 hours of reoxygenation needed for the confluent mouse cardiomyocytes model to sustain 40-60% decrease in cell number and increase in LDH release (N = 4 experiments; cells isolated from two different mice; 4 different passages).

2.3. In-vitro I/R injury (H/R)

To simulate I/R, the cells underwent 5 hours of hypoxia (0.01% O_2:5% CO_2:94.99% N_2; serum- & glucose-free cardiomyocyte media obtained from Celprogen; 37°C) in a humidified Billups-Rosenthal plexiglass hypoxia chamber (Stemcell Technologies; Vancouver, BC, Canada) followed by 2 hours of reoxygenation (normal environment of humidified 21% O_2:74% N_2:5% CO_2, 37°C, cardiomyocyte growth media) ± P188 or polyethylene glycol (PEG). PEG is considered the optimal control molecule for P188 since it has a similar molecular weight (8,000 g/mol) and produces similar osmolarity, but is purely hydrophilic (Figure 12).

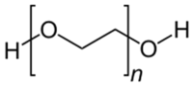

Figure 12. Structural unit of polyethylene glycol (PEG) (Sigma-Aldrich Chemical Co. product insert, 2016).

To control for any effects of media replacement, the media of the C/N cells was changed at the same time the hypoxic media was changed to the normoxic media for the reoxygenation phase.

2.4. P188 dose-response curves

The therapeutic potential of P188 was tested at concentrations ranging from 10 µM – 1 mM. This range was chosen based on previous studies investigating P188. In mouse muscle myoblast cultures (~80% confluent) exposed to hypo-osmotic stress and isotonic recovery, P188 concentrations as low as 14 µM were protective when assessed by LDH release (Kim et al, 2017). In addition, P188 (150 µM) fully restored dystrophic myocyte stretch compliance (Yasuda et al, 2005), and in a pig model of STEMI, a 250 mg/kg dose of P188 infused at the start of reperfusion significantly decreased infarct size, as well as improved mitochondrial integrity and function (Bartos et al, 2016).

P188 and PEG were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). P188 and PEG were dissolved in cardiomyocyte growth media obtained from Celprogen to achieve the desired concentrations. At these concentrations, both P188 and PEG are ≥ 96% soluble, do not affect the pH of the media, and exist as a dynamic solution of single molecules, as well as grouped micelles (personal communication with our collaborator, Benjamin J. Hackel, PhD, at the University of Minnesota).

2.5. Cell number and viability assay

Cell number and viability were assessed using the CyQUANT Direct Cell Proliferation Assay Kit (Molecular Probes, Inc., Eugene, OR, USA). Based on a cell-permeant DNA-binding dye combined with a background suppression reagent, the kit fluorescently measures the DNA content only in healthy cells, while blocking the staining of dead cells and cells with compromised membranes. Since DNA content is highly regulated, cell number estimates, as well as cytotoxicity assessments, are very accurate.

At the end of each experiment (i.e., the reperfusion period), the cells were washed once with 1x phosphate buffered saline (PBS) before 100 µl of fresh cardiomyocyte growth media was added to the wells. An equal volume of 100 µl of prepared 2x detection buffer was then added, and the cells incubated for 60 minutes at 37^{o}C. The fluorescence of each well was read at an excitation (Ex) of 480 nm and emission (Em) of 535 nm using a Bio-Tek bottom-reading plate reader. Number of experiments = 4-8, with 6 replicate wells plated per control or treatment group. Data are expressed as average number of cells per well.

2.6. Cellular injury assay (LDH release)

Cellular injury was assessed by the colorimetric measurement of the intracellular enzyme lactate dehydrogenase (LDH) released from damaged cells into the cell culture media using the LDH Cytotoxicity Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The leakage of LDH is widely used as an indicator of reduced cell membrane integrity and cytotoxicity. Analysis of extracellular LDH involves a two-step enzymatic reaction. In the first step, LDH catalyzes the conversion of lactate to pyruvate by reduction of NAD^{+} to NADH. Diaphorase then uses the NADH to reduce a tetrazolium salt to a red formazan product that is measured at 490 nm. The amount of formazan produced is directly proportional to the amount of LDH measured in the media.

At the end of each experiment, 50 µl of media from each well of the plate used to assess cell number/viability was transferred to a corresponding well of a new 96-well plate and mixed with 50 µl of prepared reaction mixture. After a 30 minute incubation at room temperature, the reactions were terminated by the addition of 50 µl of stop solution, which halts the reduction of the tetrazolium salt to the formazan product. Absorbance was measured at 490 nm using a Bio-Tek top-reading plate reader. Number of experiments = 4-8, with 6 replicate wells plated per control or treatment group. Data are expressed as average absorbance units (AU) per well, with well equaling the average number of cells determined in the original corresponding plate well.

2.7. Cell membrane injury and repair assay (FM1-43 dye incorporation)

To directly test whether our model of H/R causes cell membrane damage, and whether P188 provides membrane repair, the membrane impermeant styryl dye FM1-43 (Molecular Probes, Inc., Eugene, OR, USA) was used. FM1-43 remains extracellular unless damage to the membrane allows it to become incorporated into the lipid bilayer of the cell membrane, where it specifically fluoresces (Amaral et al, 2011). It has been used to assess membrane damage and repair in a range of cell systems (Yasuda et al, 2005; Townsend et al, 2010).

Following the method of Yasuda et al (2005), 5 µM of FM1-43 was added to the media at the initiation of the reoxygenation period. At the end of each experiment, the cells are washed twice with 1x PBS to remove any remaining extracellular, non-incorporated dye. Fresh normal media was added to the wells and the fluorescence at Ex = 488 nm and Em = 568 nm was read using a Bio-Tek bottom-reading plate reader. Number of experiments = 4-8, with 6 replicate wells plated per control or treatment group. Data are expressed as average relative fluorescent units (RFU) per well.

2.8. Intracellular [Ca^{2+}] assay (Fluo-4 fluorescence)

Intracellular [Ca^{2+}] was assessed using the Fluo-4 Direct Calcium Assay Kit (Molecular Probes, Inc., Eugene, OR, USA). Fluo-4 is a fluorescent in-cell Ca^{2+} indicator. The kit also utilizes a suppression dye to reduce background fluorescence, and probenecid to inhibit transport of the internalized Fluo-4 outside the cells.

Prior to the start of each experiment, cells were loaded with Fluo-4 by adding 100 µl of the prepared 2x Fluo-4 Direct loading solution to the wells containing cells and 100 µl of culture media. Following a 60 minute incubation at 37^{o}C, the cells were washed twice with 1x PBS to remove any remaining non-internalized Fluo-4. The appropriate experimental media (100 µl) was then added and the experiment conducted. At the end of each experiment, the fluorescence of each well was read at Ex = 494 nm and Em = 576 nm using a Bio-Tek bottom-reading plate reader. Number of experiments = 3-8, with 6 replicate wells plated per control or treatment group. Data are expressed as average relative RFU per well.

2.9. Calcium (Ca^{2+}) channel, exchanger, and modulating receptor agonists / antagonists

To address the role Ca^{2+} channels, exchangers, and/or modulating receptors may play in our *in-vitro* model of cardiomyocytes exposed to H/R, and whether P188 protection targets any these when their function is altered, we used several pharmacological agonists (activators) and antagonists (blockers). Each agonist or antagonist was used under both C/N and H/R conditions (Figures 13 and 14), and added at the start of the reoxygenation period (or final 2 hour period in the C/N condition). EC_{50} and IC_{50} values were estimated based on the dose response curves obtained for each agonist and antagonist, respectively.

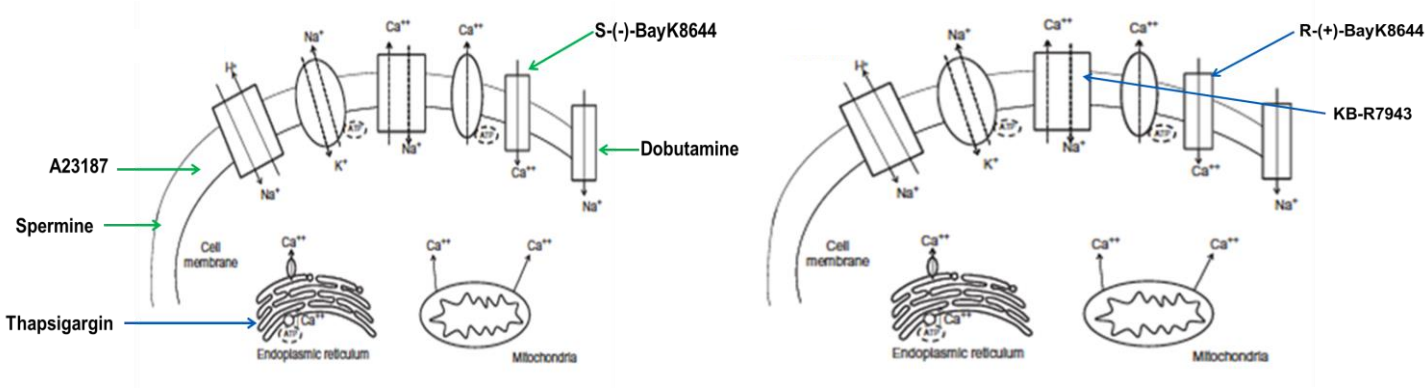

Figure 13. Pharmacologic compounds used under C/N conditions and their cellular targets. Left panel: Compounds used to increase $[Ca^{2+}]_i$. Right panel: Compounds used to block Ca^{2+} entry.

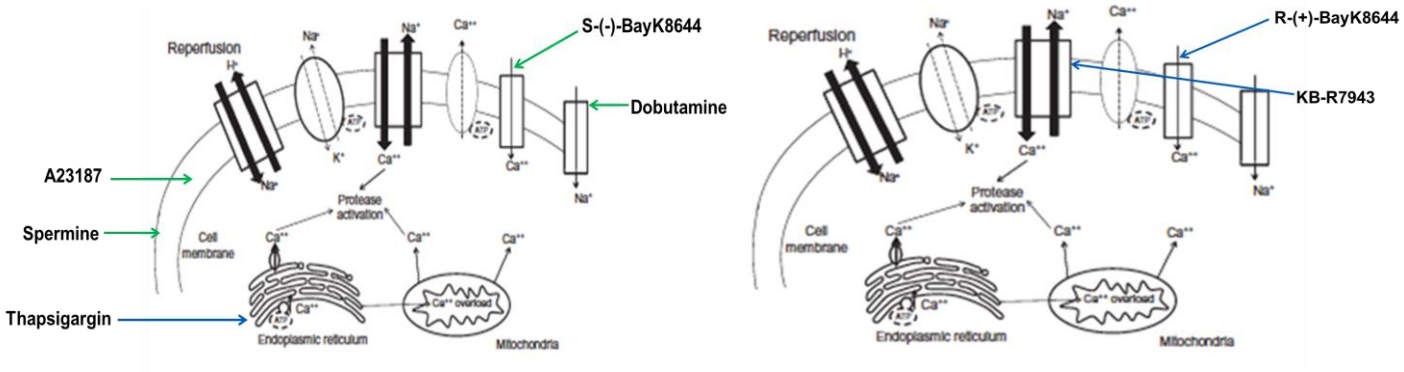

Figure 14. Pharmacologic compounds used under H/R conditions and their cellular targets. Left panel: Compounds used to increase $[Ca^{2+}]_i$. Right panel: Compounds used to block Ca^{2+} entry.

The Ca^{2+} ionophore A23187 was purchased from Alomone Labs (Jerusalem, Israel). This compound forms pores in the membrane that allow Ca^{2+} ions to cross and increase $[Ca^{2+}]_i$. Within an *in-vitro* system, A23187 can provide insight into the maximum $[Ca^{2+}]_i$ a cell may contain by allowing equilibration of extra- and intracellular $[Ca^{2+}]$ levels. The voltage-gated L-type Ca^{2+} channel agonist S-(-)-BayK8644 and antagonist R-(+)-BayK8644 were also purchased from Alomone Labs. The β_1 receptor agonist and voltage-gated Na^+ channel activator dobutamine, as well as the Na^+/Ca^{2+} exchanger (reverse mode) antagonist KB-R7943 and the S/ER Ca^{2+}-ATPase (SERCA) antagonist thapsigargin were purchased from Abcam (Boston, MA, USA). The Ca^{2+}-sensing receptor agonist spermine was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Stocks of each compound, except spermine, were made in DMSO; spermine stocks were made in

deionized water. All stocks were stored at -20^{o}C, and then diluted to the appropriate experimental concentration in cardiomyocyte growth media prior to each experiment. Experimental concentrations were chosen based on information in the product literature received with each compound and other studies using isolated cardiomyocytes (Skeberdis et al, 1997; Satoh et al, 2000; Kwan et al, 2003; Feng et al, 2011; Viola et al, 2013; Li et al, 2015; Yan et al, 2015; Zhang et al, 2016). For A23187, the concentrations of 0.2 µM, 2 µM, and 20 µM were used. Concentrations of 15 nM, 150 nM, and 1.5 µM were used for S-(-)-BayK8644 and R-(+)-BayK8644. The concentrations of 0.1 µM, 1 µM, and 10 µM were chosen for dobutamine and thapsigargin. For spermine, the concentrations of 50 µM, 100 µM, and 500 µM were used. And, concentrations of 0.5 µM, 5 µM, and 50 µM were used for KB-R7943.

2.10. Statistics

All data are expressed as the mean ± the standard error of the mean (SEM). For one comparison between groups, Student's t-test (two-tailed) was used. For multiple comparisons between groups, one-way analysis for variance (ANOVA) was used. Student-Newman-Keuls (SNK) post-hoc comparisons were applied to all significant results. Differences were considered statistically significant when $p<0.05$. All statistical analyses were performed using PRISM 5 (GraphPad) software. Graphically, significance is expressed as: *vs control/normoxia (C/N), **vs hypoxia only or H/R, † vs vehicle.

3. Results

3.1. Reoxygenation potentiates cardiomyocyte injury caused during hypoxia

Two hours of reoxygenation following 5 hours of hypoxia resulted in a significant increase in cytotoxicity as assessed by cell number/viability and LDH release compared to hypoxia alone. As shown in Figure 15, hypoxia alone reduced cell number/viability by ~20%; reoxygenation following this hypoxia reduced cell number/viability by nearly 50% compared to control/normoxia (C/N). Corresponding results were obtained in studies measuring LDH release. Hypoxia alone increased LDH release by ~20%, whereas reoxygenation following the hypoxia doubled the increase in LDH release compared to C/N (Figure 15). Studies using the dye FM1-43 to assess membrane damage revealed an increase in FM1-43 incorporation. Hypoxia caused a statistically significant ~33% increase in FM1-43 incorporation, and an ~45% increase was observed following reoxygenation compared to C/N (Figure 15). Finally, measurement of $[Ca^{2+}]_i$ revealed an ~30% increase with hypoxia and an ~60% increase with reoxygenation compared to C/N (Figure 15). These data confirm that reoxygenation following hypoxia in our H/R model produces an increase in cytotoxicity relative to hypoxia alone. The focus of our studies with P188 is this reoxygenation injury.

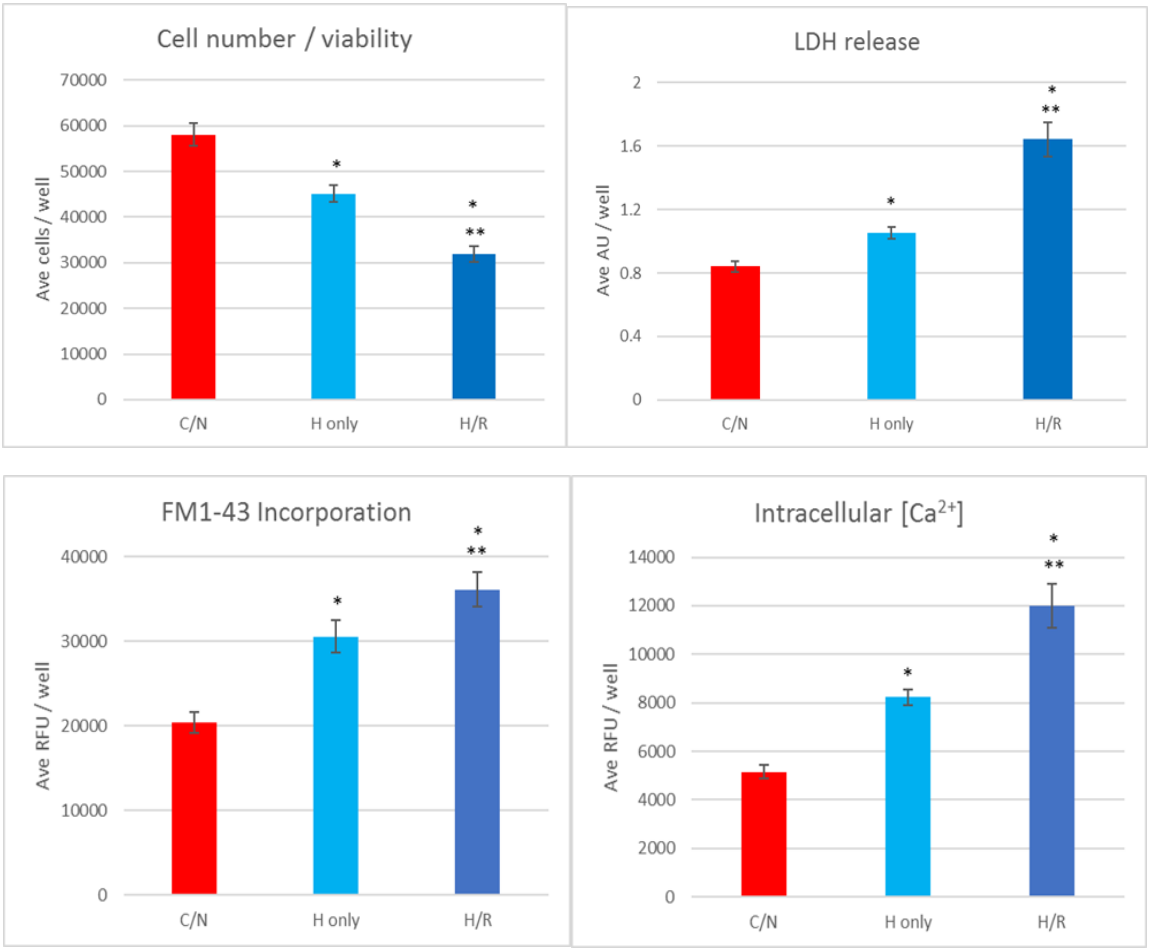

Figure 15. Potentiation of injury by reoxygenation following hypoxia in the four indices of cellular function. Reoxygenation following hypoxia further decreases cell number/viability, and further increases LDH release, membrane damage assessed by FM1-43 incorporation, and intracellular [Ca^{2+}].
*(N = 4 experiments; *vs C/N*, **vs hypoxia only*).*

3.2. Cell number and viability

There was a significant decrease of nearly 50% in cell number/viability during H/R compared to cells under C/N conditions. A range of P188 concentrations from 30 µM - 1 mM present during reoxygenation significantly increased cell number/viability in H/R conditions, with 100 µM P188 offering the most effective protection and nearly returning the cell number/viability to C/N levels. No significant effect of any P188 concentration was observed under C/N conditions (Figure 16).

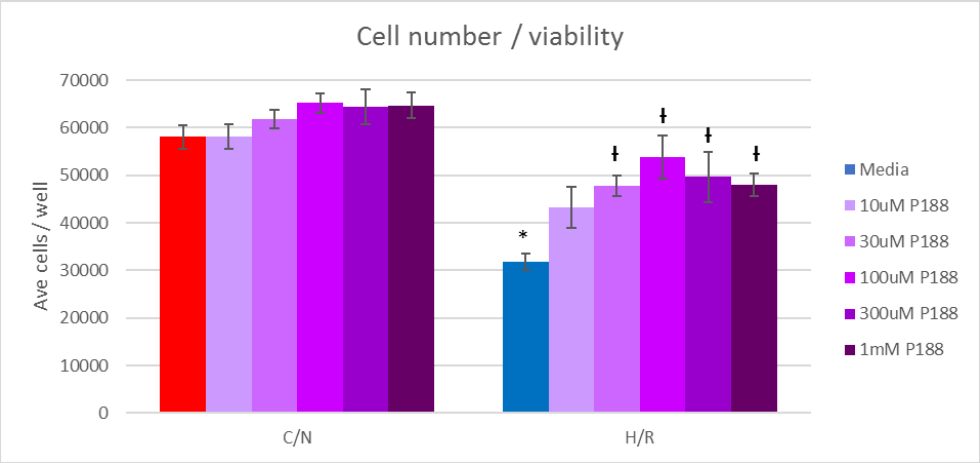

*Figure 16. The significant decrease in cell number/viability during H/R compared to cells under C/N conditions was attenuated with P188. No significant effect of P188 was observed under C/N conditions (N = 8 experiments; *vs C/N, † vs vehicle).*

H/R caused a significant decrease of nearly 50% in cell number/viability compared to cells under C/N conditions. No protective or negative effects by PEG were observed on cell number/viability (Figure 17).

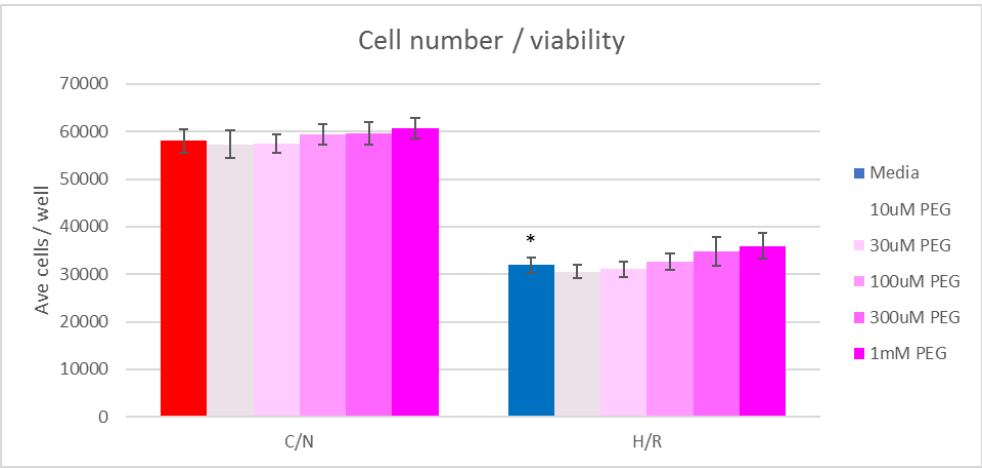

*Figure 17. No significant effect of PEG on cell number/viability was observed under C/N or H/R conditions (N = 4 experiments; *vs C/N).*

3.3. LDH release

There was a significant, approximately 2-fold increase of LDH release during H/R compared to cells under C/N conditions. A range of P188 concentrations from 10 µM - 1 mM present during reoxygenation significantly decreased LDH release in H/R conditions, with 100 µM providing the most effective protection against LDH release by decreasing LDH release ~33% compared to H/R. No significant effect of any P188 concentration was observed under C/N conditions (Figure 18).

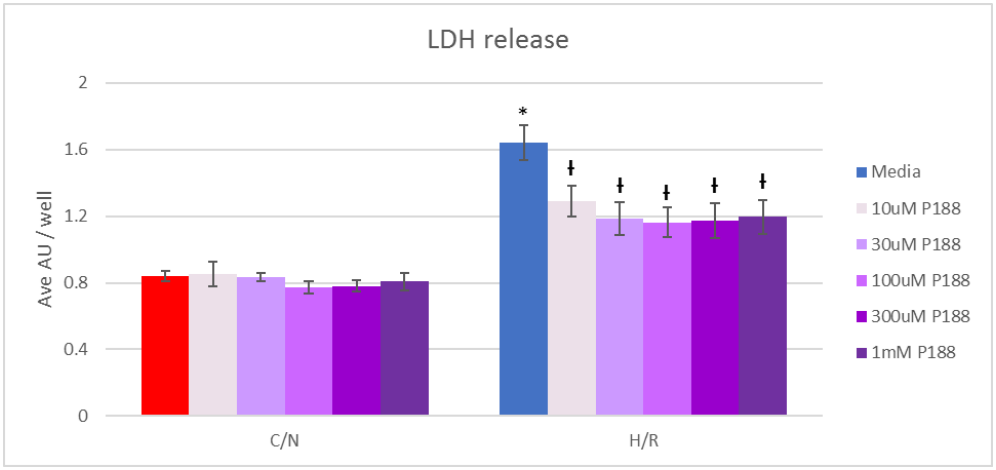

*Figure 18. The significant increase in LDH release during H/R compared to cells under C/N conditions was decreased with P188. No significant effect of P188 was observed under C/N conditions (N = 8 experiments; *vs C/N, † vs vehicle).*

During H/R, there was a significant, approximately 2-fold increase of LDH release compared to cells under C/N conditions. No protective or negative effects by PEG on LDH release were observed under either C/N or H/R conditions (Figure 19).

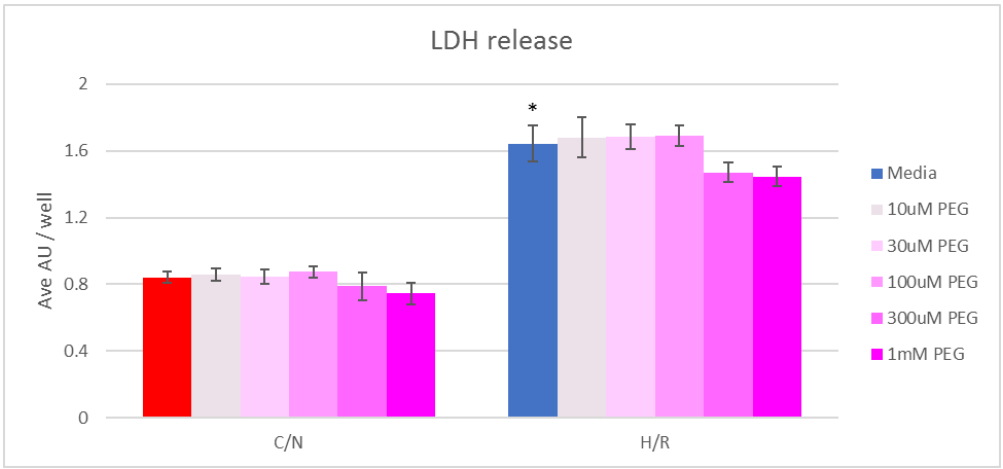

*Figure 19. No significant effect of PEG on LDH release was observed under C/N or H/R conditions (N = 4 experiments; *vs C/N).*

3.4. Cell membrane injury

There was a significant ~45% increase in FM1-43 incorporation during H/R compared to cells under C/N conditions. A range of P188 concentrations from 100 µM – 1 mM present during reoxygenation significantly decreased FM1-43 incorporation during H/R conditions, with 300 µM P188 decreasing FM1-43 incorporation by ~35%. No significant effect of any P188 concentration was observed under C/N conditions (Figure 20).

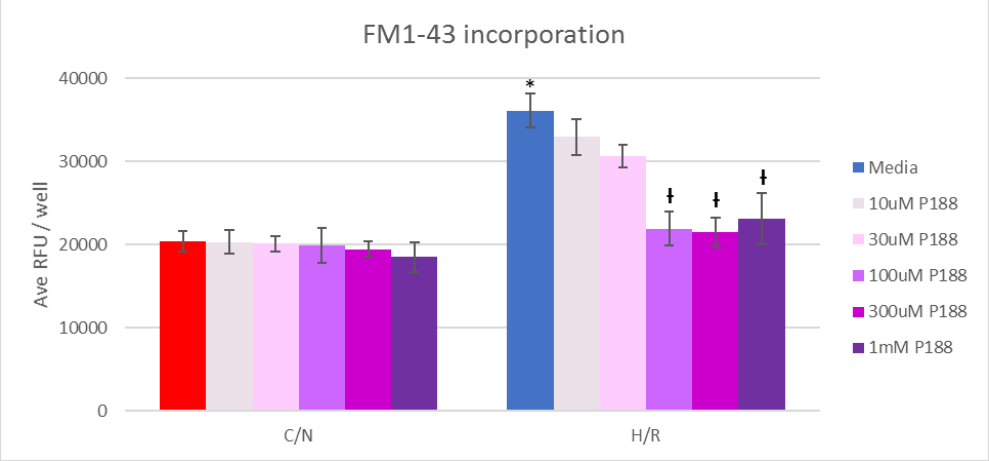

*Figure 20. The significant increase in FM1-43 incorporation during H/R compared to cells under C/N conditions was decreased with P188. No significant effect of P188 was observed under C/N conditions (N = 8 experiments; *vs C/N, † vs vehicle).*

H/R caused a significant ~45% increase in FM1-43 incorporation compared to cells under C/N conditions. Under both C/N and H/R conditions, no protective or negative effects by PEG on FM1-43 were observed (Figure 21).

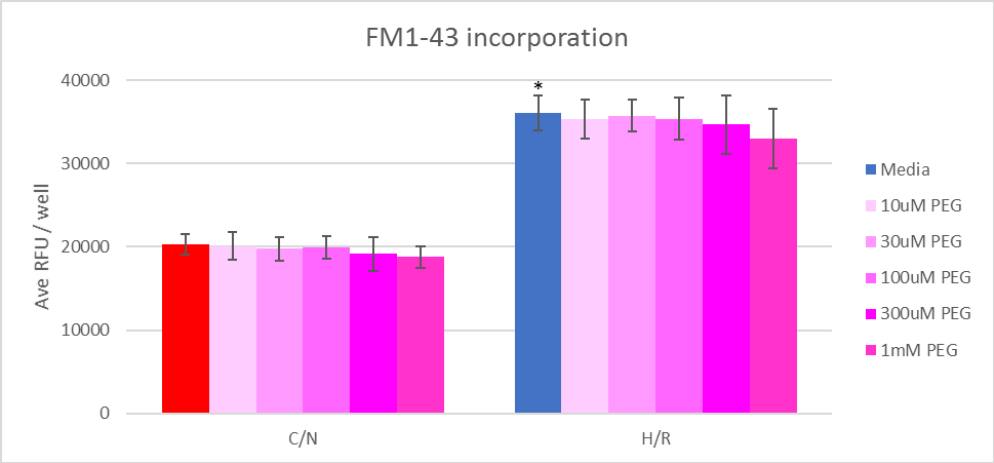

*Figure 21. No significant effect of PEG on FM1-43 incorporation was observed under C/N or H/R conditions (N = 4 experiments; *vs C/N).*

3.5. Intracellular [Ca^{2+}]

There was a significant ~60% increase in $[Ca^{2+}]_i$ during H/R compared to cells under C/N conditions. A range of P188 concentrations from 100 µM – 1 mM present during reoxygenation significantly decreased $[Ca^{2+}]_i$ under H/R conditions, with 300 µM P188 decreasing $[Ca^{2+}]_i$ by ~30%. No significant effect of any P188 concentration was observed under C/N conditions (Figure 22).

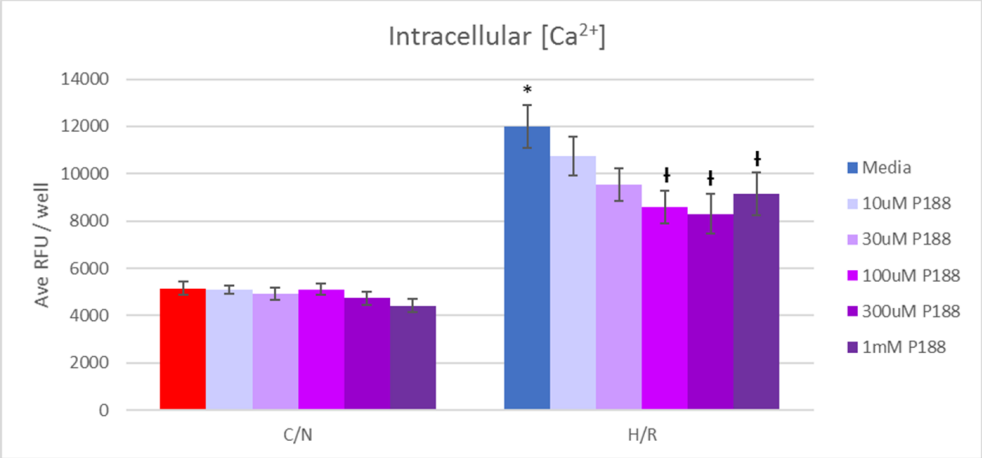

*Figure 22. The significant increase in $[Ca^{2+}]_i$ during H/R compared to cells under C/N conditions was decreased with P188. No significant effect of P188 was observed under C/N conditions (N = 8 experiments; *vs C/N, † vs vehicle).*

During H/R, there was a significant ~60% increase in $[Ca^{2+}]_i$ compared to cells under C/N conditions. Under both C/N and H/R conditions, no protective or negative effects by PEG on $[Ca^{2+}]_i$ were observed (Figure 23).

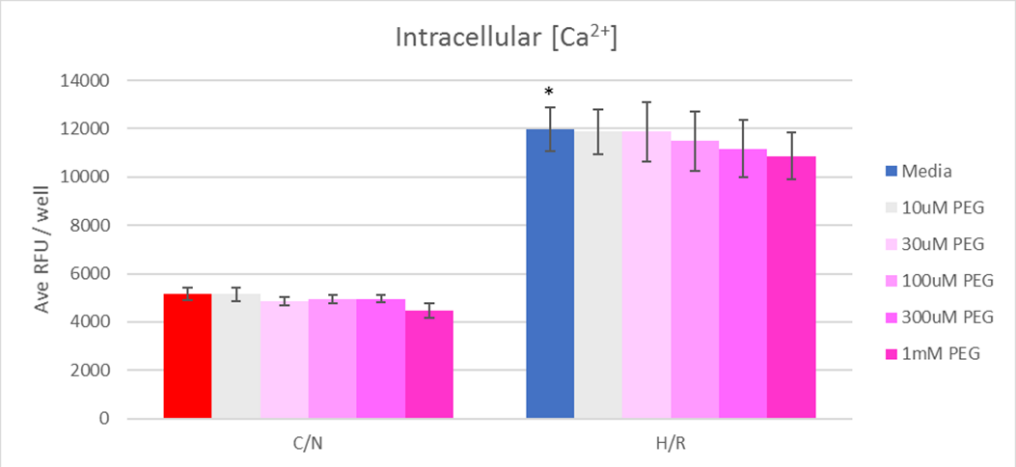

*Figure 23. No significant effect of PEG on $[Ca^{2+}]_i$ was observed under C/N or H/R conditions (N = 4 experiments; *vs C/N).*

Alterations in cardiomyocyte Ca^{2+} homeostasis are known to play key roles in ischemia, as well as the injury caused by reperfusion. The following studies investigated the role of several channels, exchangers, and a receptor that play a role in cardiomyocyte Ca^{2+} homeostasis and possibly also I/R injury. Pharmacologic compounds were used to modify Ca^{2+} homeostasis, and assess the effect of P188 (100 µM) present during reoxygenation, on these Ca^{2+} modifications. The concentration of 100 µM P188 was chosen because it was the most consistent protective concentration in the four previously discussed endpoint measurement dose-response studies.

3.6. Effects of different compounds to increase Ca^{2+} entry under C/N conditions in the presence or absence of P188

Compound	Action
A23187	Ca^{2+} ionophore
S-(-)-BayK8644	L-type Ca^{2+} channel agonist
Dobutamine	Na^{+} channel activator
Spermine	Ca^{2+}-sensing receptor agonist
Thapsigargin	SERCA antagonist

Table 1. Pharmacologic compounds used under C/N conditions, and their actions.

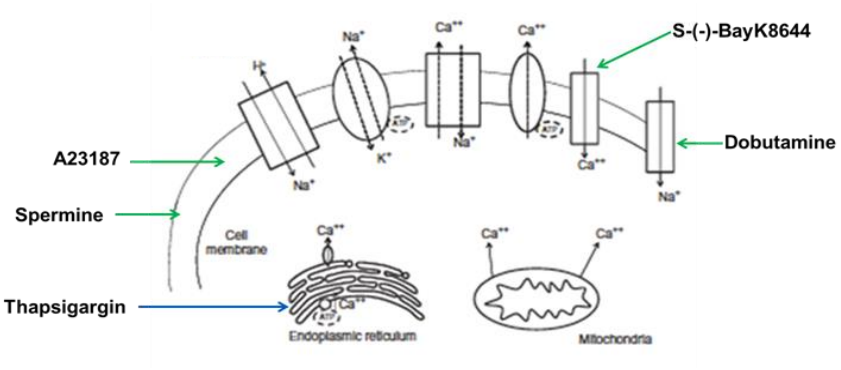

Figure 24. Pharmacologic compounds used under C/N conditions, and their cellular targets.

3.6.1. The Ca^{2+} ionophore A23187

A23187 dose-dependently increased $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period equaling the reoxygenation time in H/R conditions. Concentrations of 2 µM and 20 µM caused significant increases. The estimated EC_{50} is ~10 µM. P188 did not significantly decrease Ca^{2+} influx at any A23187 concentration examined (Figure 25).

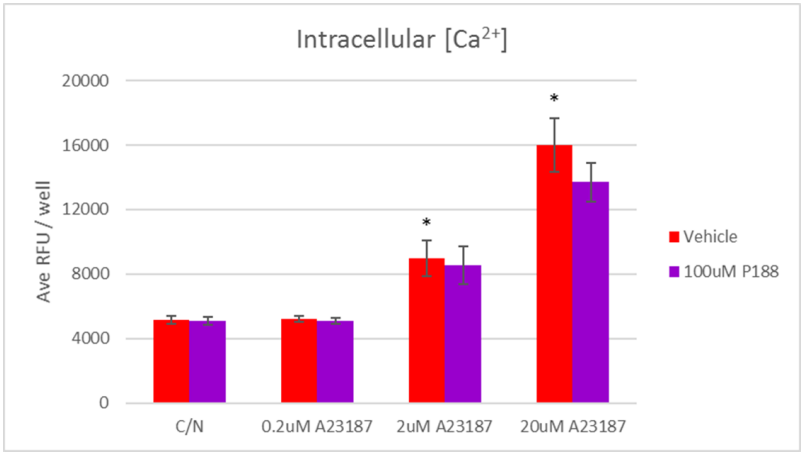

*Figure 25. A23187 dose-dependently increased $[Ca^{2+}]_i$ under C/N conditions. P188 had no effect on decreasing this Ca^{2+} influx (N = 4 experiments; *vs C/N).*

3.6.2. The voltage-gated L-type Ca^{2+} channel agonist S-(-)-BayK8644

S-(-)-BayK8644 significantly increased $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period at a concentration of 150 nM. Therefore, the estimated EC_{50} is ~75 nM. P188 did not significantly decrease Ca^{2+} influx at any concentration of S-(-)-BayK8644 examined (Figure 26).

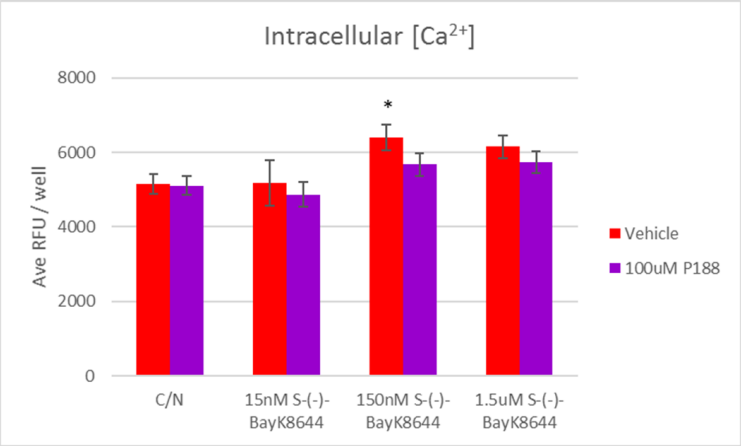

*Figure 26. S-(-)-Bayk8644 increased $[Ca^{2+}]_i$ under C/N conditions. P188 had no effect on decreasing this Ca^{2+} influx (N = 4 experiments; *vs C/N).*

3.6.3. The β_1 receptor agonist and voltage-gated Na^+ channel activator Dobutamine

No concentration of dobutamine significantly increased $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period. P188 did not significantly decrease Ca^{2+} influx at any of the dobutamine concentrations used (Figure 27).

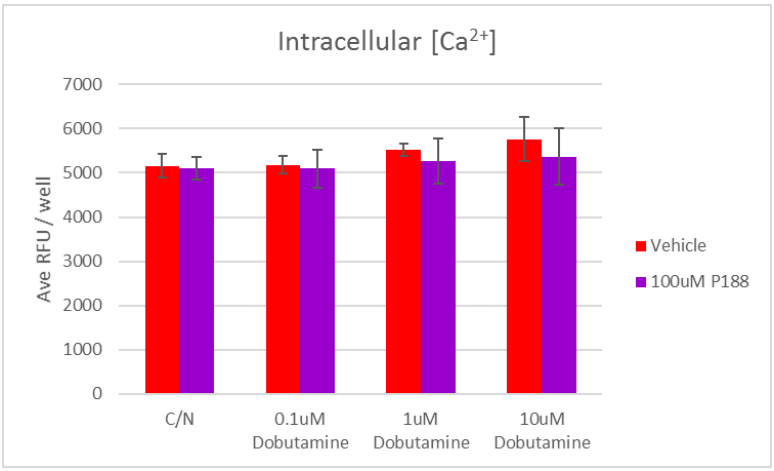

Figure 27. Dobutamine did not increase $[Ca^{2+}]_i$ under C/N conditions. P188 had no effect on decreasing Ca^{2+} influx (N = 4 experiments).

3.6.4. The calcium-sensing receptor agonist Spermine

Spermine (100 µM) significantly increased $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period. The EC_{50} was estimated to be ~50 µM. P188 did not significantly decrease Ca^{2+} influx at any of the concentrations of spermine used (Figure 28).

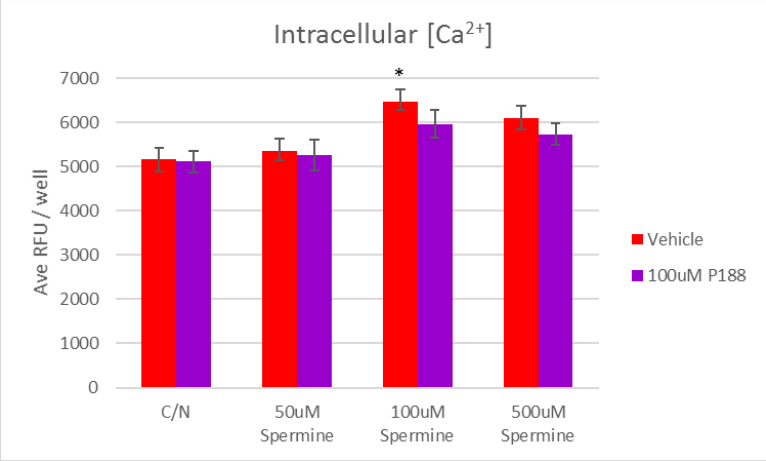

*Figure 28. Spermine increased $[Ca^{2+}]_i$ under C/N conditions. P188 had no effect on decreasing this Ca^{2+} influx (N = 4 experiments; *vs C/N).*

3.6.5. The SERCA antagonist Thapsigargin

No concentration of thapsigargin used significantly increased $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period. P188 did not significantly decrease Ca^{2+} influx at any of the concentrations of thapsigargin used (Figure 29).

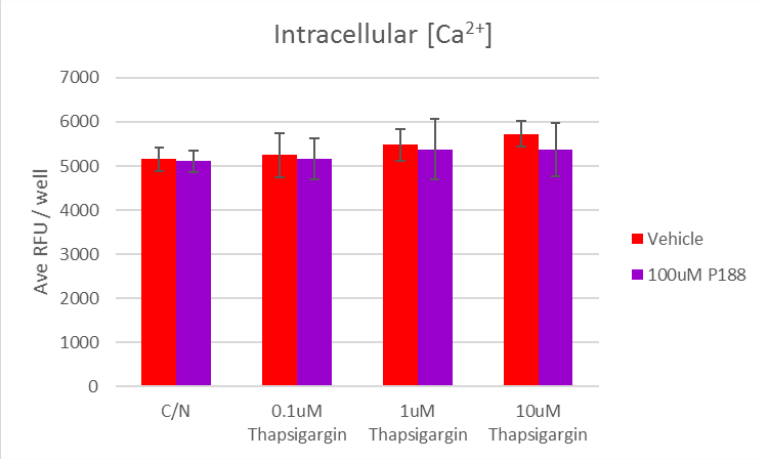

Figure 29. Thapsigargin did not increase $[Ca^{2+}]_i$ under C/N conditions. P188 had no effect on decreasing Ca^{2+} influx (N = 4 experiments).

3.7. Effects of different compounds to decrease Ca^{2+} entry under C/N conditions in the presence or absence of P188

Compound	Action
R-(+)-BayK8644	L-type Ca^{2+} channel blocker
KB-R7943	Na^{+}/Ca^{2+} exhanger (reverse mode) blocker

Table 2. Pharmacologic blockers used under C/N conditions, and their actions.

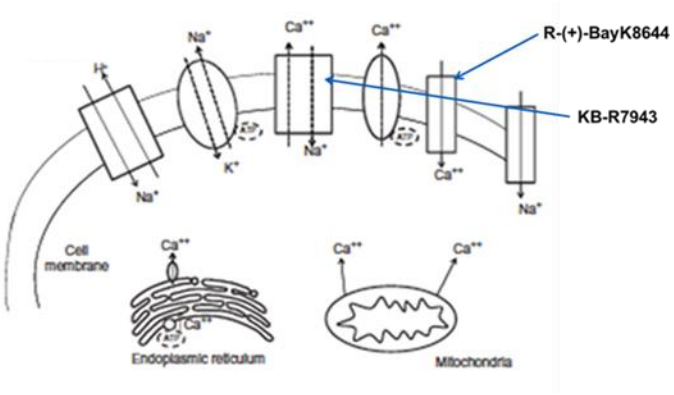

Figure 30. Pharmacologic blockers used under C/N conditions, and their cellular targets.

3.7.1. The voltage-gated L-type Ca^{2+} channel antagonist R-(+)-BayK8644

This channel blocker decreased $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period, with 1.5 µM causing a significant Ca^{2+} decrease. The IC_{50} was estimated to be ~750 nM. P188 did not further decrease Ca^{2+} influx to a significant degree at any of the blocker concentrations used (Figure 31).

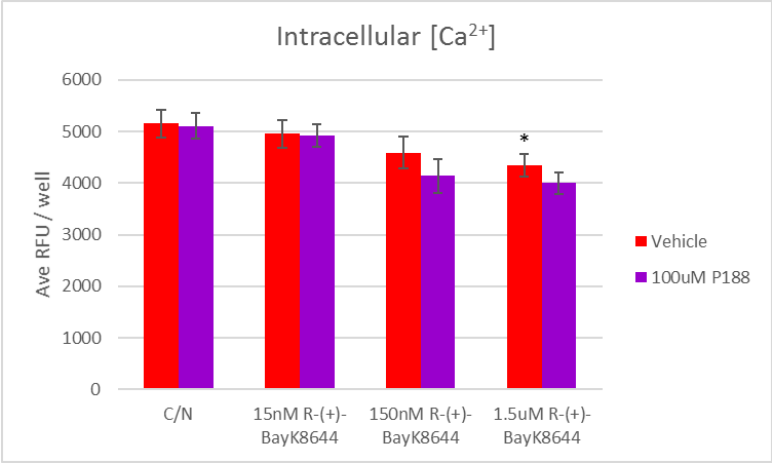

*Figure 31. R-(+)-BayK8644 decreased $[Ca^{2}]_i$ under C/N conditions. P188 did not further decrease Ca^{2+} influx (N = 3 experiments; *vs C/N).*

3.7.2. The Na^{+}/Ca^{2+} exchanger (reverse mode) antagonist KB-R7943

This blocker of the reverse mode of the Na^{+}/Ca^{2+} exchanger did not decrease $[Ca^{2+}]_i$ under C/N conditions over a 2 hour period at any concentration examined. In addition, P188 had not effect on decreasing Ca^{2+} influx (Figure 32).

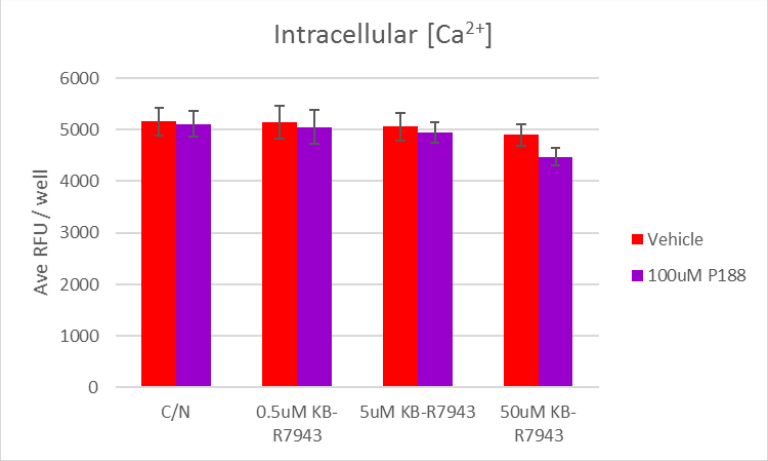

Figure 32. KB-R7943 did not decrease $[Ca^{2+}]_i$ under C/N conditions. P188 also did not have an effect on decreasing Ca^{2+} influx (N = 3 experiments).

3.8. Effects of different compounds to increase Ca^{2+} entry under H/R conditions in the presence or absence of P188

Compound	Action
A23187	Ca^{2+} ionophore
S-(-)-BayK8644	L-type Ca^{2+} channel agonist
Dobutamine	Na^+ channel activator
Spermine	Ca^{2+}-sensing receptor agonist
Thapsigargin	SERCA antagonist

Table 3. Pharmacologic compounds used under H/R conditions, and their actions.

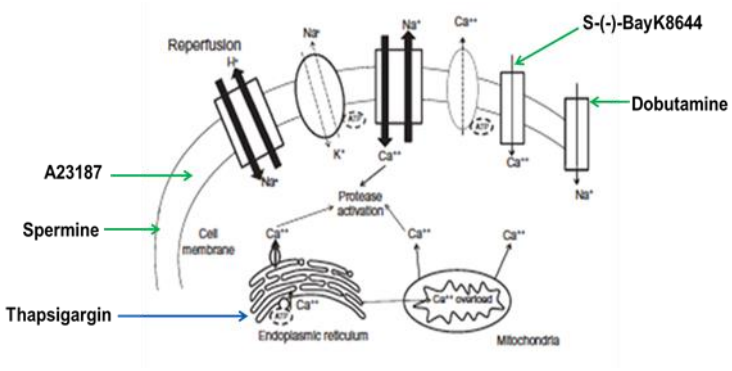

Figure 33. Pharmacologic compounds used under H/R conditions, and their cellular targets.

3.8.1. The Ca^{2+} ionophore A23187

A23187 increased $[Ca^{2+}]_i$ during the 2 hour reoxygenation period following 5 hours of hypoxia, with 20 µM causing a significant increase. The estimated EC_{50} is ~10 µM. P188 significantly decreased Ca^{2+} influx due to H/R, but did not significantly affect the additional Ca^{2+} influx caused by this ionophore (Figure 34).

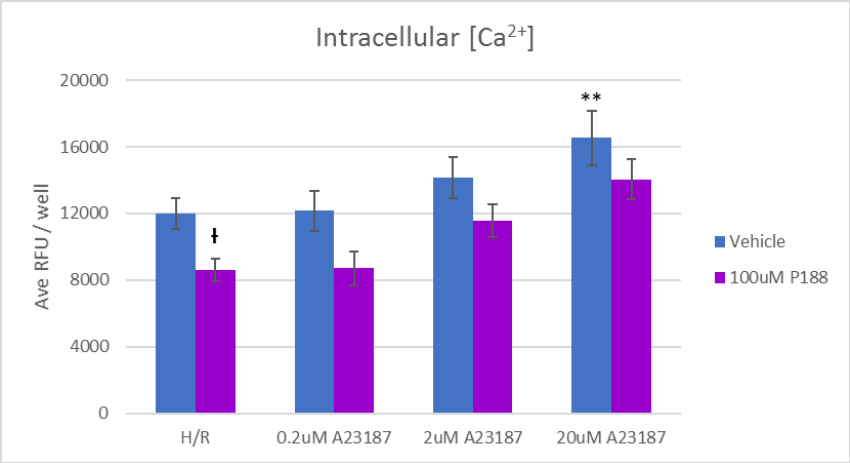

*Figure 34. A23187 increased $[Ca^{2+}]_i$ under H/R conditions. P188 blocked only H/R-induced Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

3.8.2. The voltage-gated L-type Ca^{2+} channel agonist S-(-)-BayK8644

This channel activator significantly increased $[Ca^{2+}]_i$ during the 2 hour reoxygenation at a concentration of 150 nM. The estimated EC_{50} is ~75 nM. P188 significantly decreased Ca^{2+} influx only due to H/R (Figure 35).

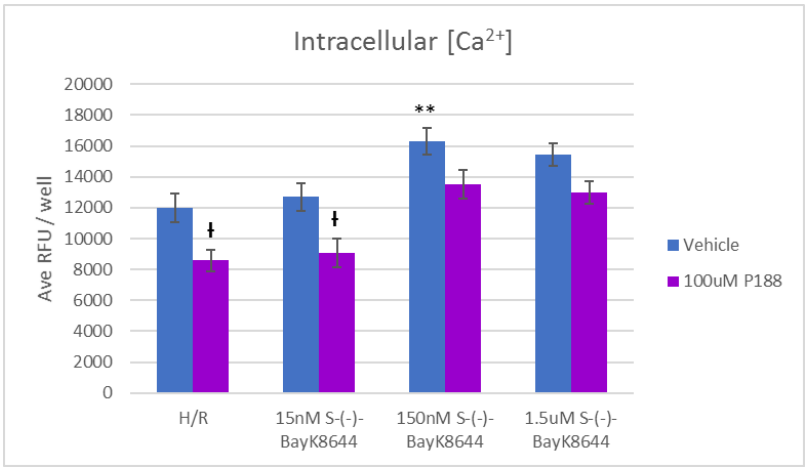

*Figure 35. S-(-)-BayK8644 increased $[Ca^{2+}]_i$ under H/R conditions. P188 blocked only H/R-induced Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

3.8.3. The β_1 receptor agonist and voltage-gated Na^+ channel activator Dobutamine

Intracellular $[Ca^{2+}]$ was increased by this channel activator during the 2 hour reoxygenation, with a concentration of 10 µM causing a significant increase. The estimated EC_{50} is ~5 µM. P188 significantly decreased Ca^{2+} influx due to H/R; however, P188 did not significantly affect the added Ca^{2+} influx caused by 0.1 µM and 1 µM dobutamine (Figure 36).

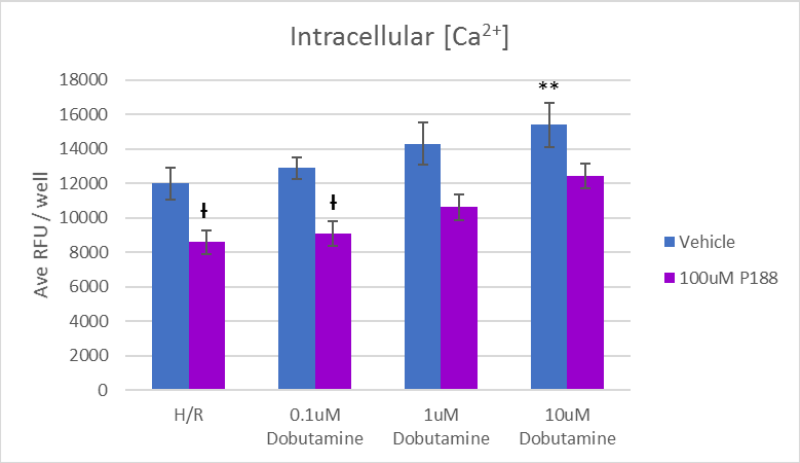

*Figure 36. Dobutamine increased $[Ca^{2+}]_i$ under H/R conditions. P188 blocked only H/R-induced Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

To confirm the Na^+/Ca^{2+} exchanger (reverse mode) could be blocked under these H/R conditions, KB-R7943 (5 µM) was used. KB-R7943 significantly decreased Ca^{2+} influx due to H/R, as well as the additional Ca^{2+} influx caused by all concentrations of dobutamine used. The estimated IC_{50} is ~2.5 µM (Figure 37).

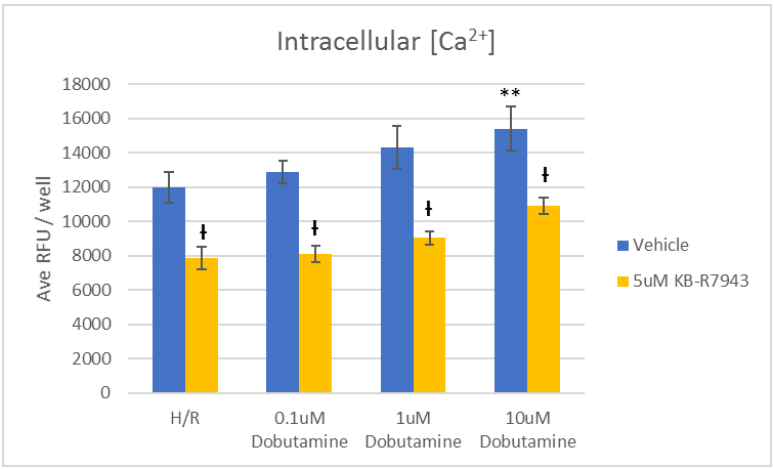

*Figure 37. Dobutamine increased $[Ca^{2+}]_i$ under H/R conditions. KB-R7943 blocked both H/R- and dobutamine-induced Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

3.8.4. The calcium-sensing receptor agonist Spermine

Spermine dose-dependently increased $[Ca^{2+}]_i$ during the 2 hour reoxygenation, with concentrations of 100 µM and 500 µM causing a significant increase. The estimated EC_{50} is ~60 µM. P188 significantly decreased Ca^{2+} influx due to H/R, but did not significantly affect the added Ca^{2+} influx caused by any concentration of spermine used (Figure 38).

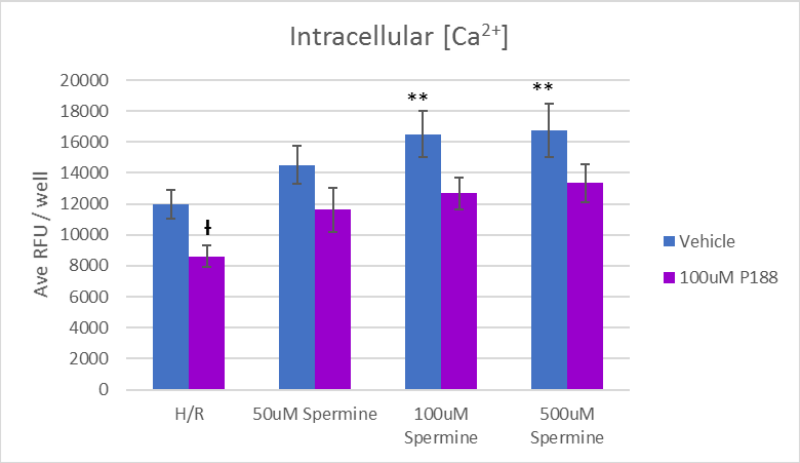

*Figure 38. Spermine increased $[Ca^{2+}]_i$ under H/R conditions. P188 blocked only the H/R-induced Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

3.8.5. The SERCA antagonist Thapsigargin

Thapsigargin increased $[Ca^{2+}]_i$ during the 2 hour reoxygenation, with a concentration of 10 µM causing a significant increase. The estimated IC_{50} is ~5 µM. P188 significantly decreased Ca^{2+} influx due to H/R, but did not significantly affect the added Ca^{2+} influx caused by any concentration of thapsigargin used (Figure 39).

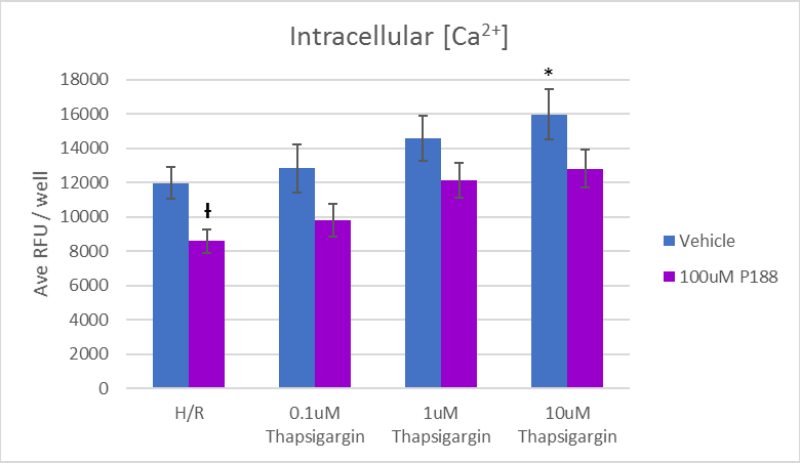

*Figure 39. Thapsigargin increased $[Ca^{2+}]_i$ under H/R conditions. P188 blocked only the H/R-induced Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

3.9. Effects of different compounds to decrease Ca^{2+} entry under H/R conditions in the presence or absence of P188

Compound	Action
R-(+)-BayK8644	L-type Ca^{2+} channel blocker
KB-R7943	Na^{+}/Ca^{2+} exhanger (reverse mode) blocker

Table 4. Pharmacologic blockers used under H/R conditions, and their actions.

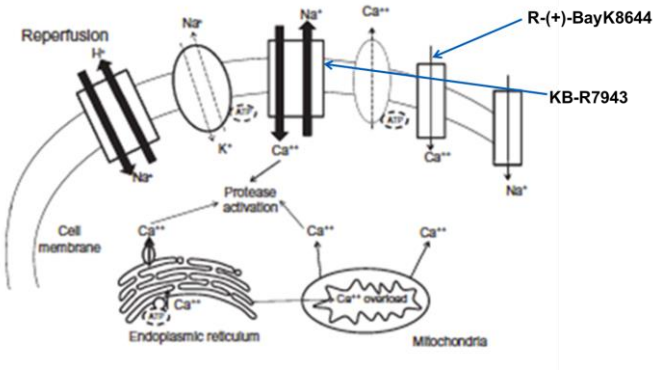

Figure 40. Pharmacologic blockers used under H/R conditions, and their cellular targets.

3.9.1. The voltage-gated L-type Ca^{2+} channel antagonist R-(+)-BayK8644

This channel blocker decreased $[Ca^{2+}]_i$ during the 2 hour reoxygenation, with a concentration of 1.5 µM causing a significant decrease. The estimated IC_{50} is ~750 nM. P188 significantly decreased Ca^{2+} influx due to H/R. It also continued to significantly decrease Ca^{2+} influx in the presence of 150 nM and 1.5 µM of R-(+)-BayK8644 (Figure 41).

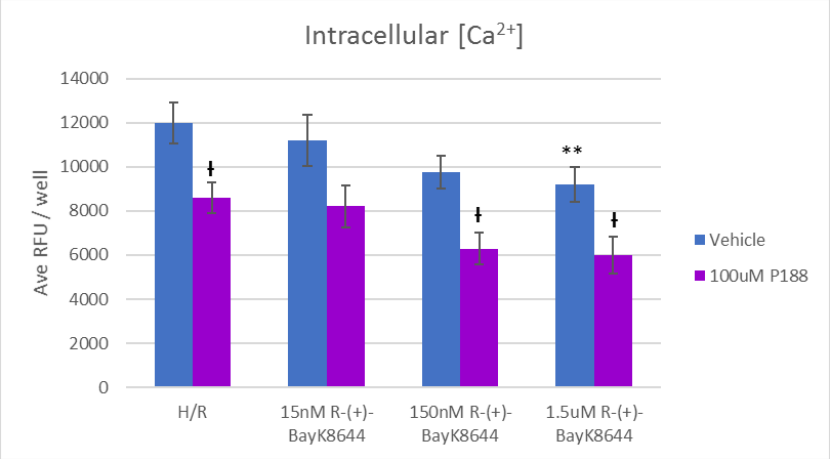

*Figure 41. R-(+)-BayK8644 decreased $[Ca^{2+}]_i$ under H/R conditions. P188 added to the decrease in Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

3.9.2. The Na^{+}/Ca^{2+} exchanger (reverse mode) antagonist KB-R7943

KB-R7943 dose-dependently decreased $[Ca^{2+}]_i$ during the 2 hour reoxygenation, with concentrations of 5 µM and 50 µM causing significant decreases. The estimated IC_{50} is ~25 µM. P188 significantly decreased Ca^{2+} influx due to H/R, and also continued to significantly decrease Ca^{2+} influx in the presence of all the concentrations of KB-R7943 used (Figure 42).

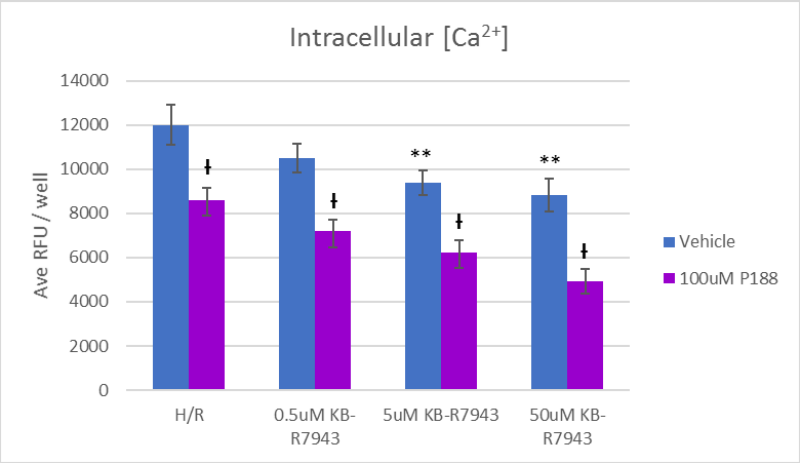

*Figure 42. KB-R7943 decreased $[Ca^{2+}]_i$ under H/R conditions. P188 added to the decrease in Ca^{2+} influx (N = 3 experiments; **vs H/R, † vs vehicle).*

4. Discussion

4.1. I/R injury during cardiac arrest and CPR

With cardiac arrest being a leading cause of debilitating illness and death worldwide, survival rates only reaching ~31% even when the best, most up-to-date CPR is provided, and ~90% of survivors suffering cardiac, as well as neurologic, deficits, further improvements to CPR practices are needed. The increased awareness of the benefit of bystander provided CPR and the teaching of optimal CPR techniques, in addition to the use of mechanical CPR delivery devices to maintain adequate CPR for longer durations, has led to improvements in survival rates (Nassar & Kerber, 2017; Peberdy et al, 2017). However, more adjunct therapies are needed to further increase survival rates and reduce cardiac and neurologic deficits in survivors.

The increased survival rates are most likely due to the limiting of the ischemic period of the cardiac arrest provided by the timely CPR delivered; however, this needed return of blood flow results in additional organ and cellular damage beyond that occurring during ischemia. This I/R injury is the likely cause of the cardiac and neurologic deficits survivors suffer from.

I/R injury is a complex pathological event, with multiple processes underlying the injury (Kalogeris et al, 2012; Maneechote et al, 2017). The ischemic injury in cardiac tissues includes hypoxia, ATP depletion, increased intracellular pH, cell swelling and membrane destabilization, ionic imbalances, and cell death. The subsequent reperfusion itself results in additional detrimental cellular dysfunction, including further membrane damage by lipid peroxidation, continued Ca^{2+} ion influx, altered redox state, hypercontracture, mitochondrial dysfunction, and cell death.

Since the best strategy for reversing cardiac arrest is the return of blood flow to the ischemic organs, such as the heart, but this reperfusion itself causes further organ damage and cellular dysfunction, pharmacological interventions and/or therapeutic strategies which modulate cellular dynamics during the reperfusion phase of I/R injury could provide beneficial effects to organs, tissues, and cells.

4.2. P188 protects cardiomyocytes against H/R injury

P188 has been shown to be a protective compound in various *in-vitro*, *ex-vivo*, and *in-vivo* models of I/R injury (Gu et al, 2013; Martindale & Metzger, 2014; Houang et al, 2015). However, the exact mechanism of P188's protective effect when given during ischemia and reperfusion is still not completely understood. And, when P188 is given only at the more clinically relevant time point of reperfusion, in several similar models of I/R injury, it still provides powerful protective effects. More specifically, when P188 was combined with the use of ischemic and sevoflurane post-conditioning during CPR after prolonged cardiac arrest in pigs, there was significantly improved cardiac and neurological function at 48 hours post-resuscitation compared to pigs that only received standard CPR (Bartos et al, 2015). Using only P188, and immediately infused upon reperfusion following a STEMI, infarct size was significantly reduced, and mitochondrial integrity and function improved compared to pigs that did not receive P188 upon reperfusion (Bartos et al, 2016). Unfortunately, there is even less known about P188's mechanism of action when it is given only at the start of reperfusion and/or whether the mechanisms have any similarities.

To improve our understanding of the protective actions of P188 when administered at the start of reperfusion, studies were conducted in an *in-vitro* model of cardiomyocytes designed to mimic the status of these cells *in-vivo*. Confluent cultures of cardiomyocytes were subjected to a hypoxia/reoxygenation protocol to simulate I/R. This caused significant damage, especially upon reoxygenation following a period of hypoxia. In four different cellular indices analyzed, 5 hours of hypoxia (0.01% O_2, serum- and glucose-free media) caused a significant decrease in cell number/viability, as well as significant increases in LDH release, FM1-43 incorporation, and $[Ca^{2+}]_i$ compared to cells in C/N conditions. When reoxygenation followed, additional cellular injury was observed, with continued significant decrease in cell number/viability, and continued significant increases in LDH release, FM1-43 incorporation, and $[Ca^{2+}]_i$ compared to cells in C/N conditions. These data indicate that our *in-vitro* cardiomyocytes model experiences additional injury upon reoxygenation (i.e., reperfusion), which is the phase of I/R injury targeted by our P188 administration.

I/R injury causes cardiomyocytes to become less viable and potentially die. Cell membranes become compromised due to cell swelling, damage to bilayer lipids, and/or activation of Ca^{2+}-dependent proteases (Kalogeris et al, 2012). Intracellular $[Ca^{2+}]$ overload due to damaged cell membranes can trigger a cascade of pathological events. The H/R protocol used in this study mimicked the effects of I/R *in-vivo* by significantly decreasing cell number/viability, and significantly increasing LDH release, membrane damage, and $[Ca^{2+}]_i$.

P188 treatment during reoxygenation produced a concentration-dependent attenuation of cytotoxic parameters of H/R with optimal effects observed at a concentration of 100 µM P188. The significant decrease in cell number and viability observed during H/R was attenuated by the presence of P188 during reoxygenation. LDH release from damaged cells, often used as an indicator of reduced cell membrane integrity and cytotoxicity, was significantly increased during H/R. All concentrations of P188 added during reoxygenation caused a significant decrease in the release of LDH. Membrane disruption and repair was assessed using the styryl dye FM1-43. This dye remains extracellular unless damage to the cell membrane

allows it to incorporate into the lipid environment of the membrane, where it specifically fluoresces (Amaral et al, 2011). It also has been used to characterize membrane damage and repair in dystrophic cardiac myocytes (Yasuda et al, 2005). During H/R, incorporation of FM1-43 significantly increased. However, with several concentrations of P188 present during reoxygenation, the amount of FM1-43 incorporated into membranes was significantly attenuated. Using the fluorescent in-cell Ca^{2+} indicator Fluo-4, $[Ca^{2+}]_i$ was measured. H/R caused a significant increase in $[Ca^{2+}]_i$, which was significantly reduced with the presence of several concentrations of P188 added during reoxygenation.

It has been proposed that the single molecules of P188 in solution are most likely to confer protection compared to grouped P188 micelles. This is because the hydrophobic portion of the molecule is more readily available for insertion into, and interaction with, the damaged section of the membrane. It is likely that there were insufficient individual P188 molecules available to provide adequate protection in those assessed cellular parameters (i.e., cell number/viability, cell membrane injury, $[Ca^{2+}]_i$) when lower concentrations of P188 (e.g., 10 µM, 30 µM) were used.

Under C/N conditions, where no measurable cellular injury occurred in any of the cellular parameters examined, no beneficial effect of any P188 concentration was observed, as well as no negative effect. This may be due to the fact that there were no detectable damaged sections of membrane for the hydrophobic portion of P188 to interact with.

Also, under both C/N and H/R conditions, no concentration of PEG administered had a protective, or negative, effect. Several studies using PEG compared to P188 have shown this same non-protective effect. The Bartos et al (2016) study showed that P188 infused immediately upon reperfusion following a STEMI reduced infarct size and preserved mitochondrial integrity and function. However, when PEG was used, no protective benefit was provided. *In-vitro* studies using C2C12 myoblasts exposed to a hypo-osmotic stress/isotonic recovery insult showed similar results. Various concentrations of P188 ranging from 14 µM to 150 µM were protective when added during the isotonic recovery based on assessments of LDH release. Comparable concentrations of PEG added during isotonic recovery had no effect on decreasing the release of LDH from these myoblasts (Houang et al, 2017; Kim et al, 2017). This may be due to the fact that PEG does not contain a hydrophobic portion, which is proposed to be the component of copolymers needed to insert into, and interact with, the damaged sections of a cell membrane.

4.3. P188 does not target Ca^{2+} channels, exchangers, and/or modulating receptors

Isolated dystrophic cardiac myocytes have been shown to have significantly greater Ca^{2+} influx from passive stretch compared to non-dystrophic myocytes, and P188 at a concentration as low as 60 µM has corrected this increased Ca^{2+} influx (Yasuda et al, 2005). To determine the mechanism of Ca^{2+} entry during passive stretch, Yasuda et al (2005) treated these myocytes with the L-type Ca^{2+} channel blocker nifedipine. The nifedipine did not block the stretch-induced Ca^{2+} entry that was reduced by P188. They concluded that the

Ca^{2+} influx may come from channels not blocked by nifedipine, other Ca^{2+} channels or exchangers, or most likely, through small tears in the myocyte's membrane that are prevented by P188 (Yasuda et al, 2005).

During I/R injury, the altered functioning of several Ca^{2+} channels, exchangers, and/or modulating receptors has been implicated in the excess Ca^{2+} influx that occurs (Satoh et al, 2000; Feng et al, 2011; Kalogeris et al, 2012; Viola et al, 2013; Yan et al, 2015; Zhang et al, 2016). To further examine the possibility of altered Ca^{2+} channels, exchangers, and/or modulating receptors contributing to excess Ca^{2+} influx, and being targets of P188, during reoxygenation, we used pharmacological compounds known to modify $[Ca^{2+}]_i$ under both C/N and H/R conditions. These studies were conducted in the presence and absence of 100 µM P188 during reoxygenation. The normal $[Ca^{2+}]_i$ of the cardiomyocytes in our model system was determined to be ~1 µM, while the cardiomyocyte growth media used contained 7 µM $CaCl_2$.

Over a 2 hour period of time under C/N conditions, the Ca^{2+} ionophore A23187, at concentrations of 2 µM and 20 µM, allowed significant increases in $[Ca^{2+}]_i$ to occur. P188 did not significantly reduce this Ca^{2+} influx into the cardiomyocytes. These data suggest that the interaction of P188 with the cellular membrane does not alter the ability of A23187 to form pores that allow Ca^{2+} entry into the cells.

In these same conditions, the agonists, S-(-)-BayK8644 and spermine, which activate the voltage-gated L-type Ca^{2+} channel and the Ca^{2+}-sensing receptor, respectively, also increased $[Ca^{2+}]_i$. Again, P188 did not significantly decrease these influxes of Ca^{2+}. Therefore, P188 probably does not target this Ca^{2+} channel, or this receptor and the channels and/or exchangers it regulates.

Intracellular $[Ca^{2+}]$ was not significantly increased under these same C/N conditions by the voltage-gated Na^+ channel activator dobutamine, or thapsigargin, the antagonist of the SERCA. And, P188 had no effect in reducing $[Ca^{2+}]_i$ in any of these conditions. From this data, it is difficult to determine whether P188 would target the Na^+/Ca^{2+} exchanger in its reverse mode, or the SERCA directly, or even remotely.

Compound	Action	C/N	C/N + P188
		Effect on $[Ca^{2+}]_i$	Effect on $[Ca^{2+}]_i$
A23187	Ca^{2+} ionophore	increased	no effect
S-(-)-BayK8644	L-type Ca^{2+} channel agonist	increased	no effect
Dobutamine	Na^+ channel activator	no effect	no effect
Spermine	Ca^{2+}-sensing receptor agonist	increased	no effect
Thapsigargin	SERCA antagonist	no effect	no effect

Table 5. The pharmacologic compounds used under C/N conditions, their actions, and effects on $[Ca^{2+}]_i$ in the absence or presence of 100 µM P188.

To confirm the voltage-gated L-type Ca^{2+} channel is present and active in our cardiomyocytes, the antagonist R-(+)-BayK8644 was used. At a concentration of 1.5 µM, it significantly decreased Ca^{2+} influx in C/N conditions. Addition of P188 did not produce a further decrease in Ca^{2+} influx, reconfirming the notion that P188 probably does not target this channel. Additionally, the blocker of the reverse mode of the Na^+/Ca^{2+}

exchanger, KB-R7943, was used. No concentration of KB-R7943 used caused a significant decrease in $[Ca^{2+}]_i$, and P188 had no effect on decreasing $[Ca^{2+}]_i$ either. This data indicates that the reverse mode of the exchanger is not activated under C/N conditions; therefore, it is difficult to determine whether P188 targets the reverse mode of the Na^+/Ca^{2+} exchanger.

Compound	Action	C/N	C/N + P188
		Effect on $[Ca^{2+}]_i$	Effect on $[Ca^{2+}]_i$
R-(+)-BayK8644	L-type Ca^{2+} channel blocker	decreased	no effect
KB-R7943	Na^+/Ca^{2+} exhanger (reverse mode) blocker	no effect	no effect

Table 6. The pharmacologic blockers used under C/N conditions, their actions, and effects on $[Ca^{2+}]_i$ in the absence or presence of 100 µM P188.

We next examined these same agonists and antagonists during experiments under H/R conditions to determine whether more information could be obtained about the protective actions of P188. During the 2 hour reoxygenation period, following 5 hours of hypoxia, 20 µM of A23187 significantly increased $[Ca^{2+}]_i$. P188 did not significantly decrease the additional Ca^{2+} influx caused by A23187, but did significantly attenuate the increased Ca^{2+} influx caused only by the H/R conditions. This data further suggests that P188 does not interfere with the pore-forming ability of A23187 that allows Ca^{2+} to enter the cell, but probably targets tears in the membrane created by the H/R injury, into which its hydrophobic central portion can insert and interact.

Under the same H/R conditions, S-(-)-BayK8644, dobutamine, spermine, and thapsigargin, all caused significant increases in $[Ca^{2+}]_i$ with at least one of their concentrations examined. The influx of Ca^{2+} that occurred during reoxygenation itself was significantly decreased by P188; however, the additional Ca^{2+} influx caused by the agonists, and antagonist thapsigargin, were not affected by P188. This information agrees with our previous findings that P188 probably does not target the voltage-gated L-type Ca^{2+} channel, the CaSR, or the channels and/or exchangers it regulates, the reverse mode of the Na^+/Ca^{2+} exchanger, or the SERCA.

Compound	Action	H/R	H/R + P188
		Effect on $[Ca^{2+}]_i$	Effect on $[Ca^{2+}]_i$
A23187	Ca^{2+} ionophore	increased	no effect
S-(-)-BayK8644	L-type Ca^{2+} channel agonist	increased	no effect
Dobutamine	Na^+ channel activator	increased	no effect
Spermine	Ca^{2+}-sensing receptor agonist	increased	no effect
Thapsigargin	SERCA antagonist	increased	no effect

Table 7. The pharmacologic compounds used under H/R conditions, their actions, and effects on $[Ca^{2+}]_i$ in the absence or presence of 100 µM P188.

To confirm the reverse mode of the Na^+/Ca^{2+} exchanger is activated by the H/R injury, as well as by dobutamine activation, in these cardiomyocytes, 5 µM KB-R7943 was used to block this reverse mode. This concentration of KB-R7943 significantly blocked the influx of Ca^{2+} caused by the reoxygenation, as well as that contributed by several concentrations of dobutamine during the 2 hour reoxygenation period.

Finally, under H/R conditions, several concentrations of the antagonists R-(+)-BayK8644 and KB-R7942, which block the voltage-gated L-type Ca^{2+} channel and the reverse mode of the Na^+/Ca^{2+} exchanger, respectively, significantly decreased the Ca^{2+} influx that occurred during the 2 hour reoxygenation. P188 was able to significantly reduce the influx of Ca^{2+} that occurred due to the reoxygenation itself, as well as the additional Ca^{2+} influx caused by the blockers.

Compound	Action	H/R	H/R + P188
		Effect on $[Ca^{2+}]_i$	Effect on $[Ca^{2+}]_i$
R-(+)-BayK8644	L-type Ca^{2+} channel blocker	decreased	enhanced decrease
KB-R7943	Na^+/Ca^{2+} exhanger (reverse mode) blocker	decreased	enhanced decrease

Table 8. The pharmacologic blockers used under H/R conditions, their actions, and effects on $[Ca^{2+}]_i$ in the absence or presence of 100 µM P188.

The additive decrease in Ca^{2+} influx when both P188 and the blockers are present during reoxygenation may be due to the channel and/or exchanger being an "innocent bystander". When P188's hydrophobic center inserts into the tear of the cell membrane that occurs during the H/R injury, the two hydrophilic end portions interact with the surface of the cell membrane to stabilize the molecule in place. If a Ca^{2+} channel and/or exchanger happens to be in the area of the membrane covered by the hydrophilic portions of the P188 molecule, their function could possibly be compromised, since a Ca^{2+} channel or exchanger is ~80 – 100 Å wide, while a hydrophilic end portion of P188 is ~825 Å long (Figure 43). This theory can only be further addressed with carefully designed experimental scenarios in follow-up to these findings.

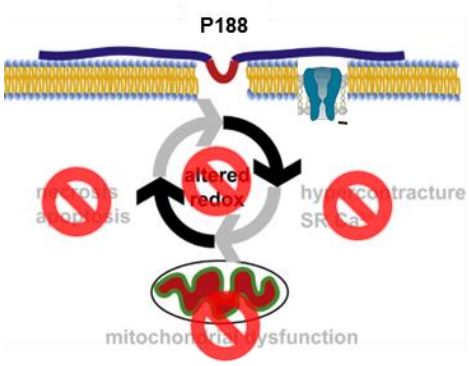

Figure 43. Hypothetical model depicting the hydrophobic center of a P188 molecule inserted in a cell membrane tear, while the two hydrophilic end portions interact with the surface of the cell membrane. One hydrophilic end is shown covering a Ca^{2+} channel, potentially causing the channel's function to be compromised.

4.4. Study conclusions

The major findings from this body of work is that: 1) P188, at the concentrations used in these experiments and administered during reoxygenation, can protect cardiomyocytes from H/R-induced injury, 2) PEG does not protect cardiomyocytes from H/R-induced injury, most likely due to the absence of a hydrophobic component, 3) the predominant source of excess Ca^{2+} influx during H/R most likely occurs through tears in the cardiomyocytes cell membrane caused by the insult, and these tears were repaired by P188, and finally, 4) P188 does not appear to target Ca^{2+} channels, exchangers, and/or modulating receptors. The protective effect of P188 most likely comes from its ability to seal membrane disruptions, thus restoring the cell's membrane integrity and normal, or near normal, function. The P188 must be drawn to damaged areas of the cell membrane, where the unique chemical properties of P188, both its hydrophobic central portion and hydrophilic ends, are required to insert into the damaged section of the membrane, and interact with the external membrane surface, respectively, to stabilize both the molecule and membrane.

As shown in this study using an *in-vitro* cardiomyocytes model, P188 may also be able to protect other cell types in similar ways when they are affected by an injury, such as H/R, in an *in-vitro* single cell type model. However, in more complex models containing multiple cell types, other additional protective mechanisms may come into play. In our proposed model of cardiomyocyte protection shown below (Figure 44), when P188 is delivered in a way where it interacts with endothelial cells first, such as when administered intravascularly, it would most likely protect the endothelial cells similar to how the cardiomyocytes were protected in this study. The stabilized endothelial cells would be allowed to function normally, and in one scenario, continue to release the cardioprotective signaling molecule nitric oxide (NO)(Lundberg et al, 2015).

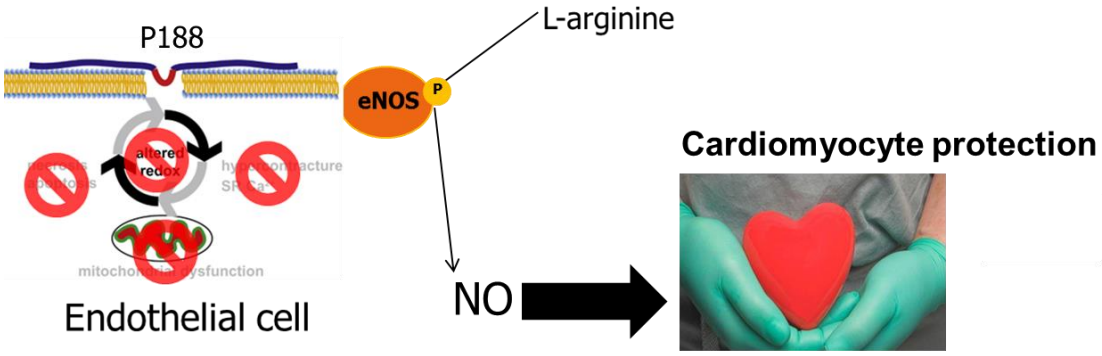

Figure 44. Proposed model of P188 protection of endothelial cells providing cardioprotective nitric oxide (NO) signaling to cardiomyocytes.

4.5. Study limitations

The present study does have limitations, such as: 1) the use of only an *in-vitro* model of I/R injury, 2) only four indices of cellular function were measured, 3) not all potential sources of increased $[Ca^{2+}]_i$ were examined, and 4) limited statistical analyses. However, the results still support the protective role that P188

has against I/R injury when it is administered at the start of reperfusion. The findings also improve the understanding of the mechanism of cellular protection by P188 when it is given at the start of reperfusion to attenuate I/R injury.

4.4. Future directions

Future research directions should include the determination of the exact location(s) P188 works to convey it protective effects. Currently, it is not known if P188 can pass through the tears in the cell membrane caused by an injury, such as I/R (H/R), and migrate to intracellular organelles, or even to other cells. However, several studies have indicated that P188 does not pass through the membrane; that it only interacts with the damaged portion and adheres to the cell membrane (Martindale & Metzger, 2014). More recent evidence from our University of Minnesota collaborator, Benjamin J. Hackel, PhD, shows that P188 interacts only on the cell membrane. Confocal microscopy shows that AlexaFluor647-tagged P188 (orange fluorescence) remains on the cellular membrane, while the interior of the cell (mouse muscle myoblast) fluoresces green with Calcein (Figure 45). Thus, P188's protective effects likely come from its ability to preserve the cell membrane's integrity, and thus, the cell's proper functioning.

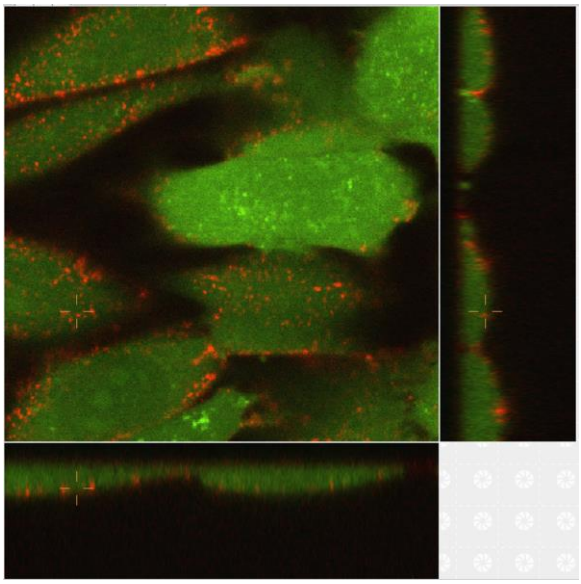

Figure 45. Confocal microscopy image showing AlexaFluor647-tagged P188 (orange fluorescence) remaining on the cellular membrane, while the interior of the cell (muscle myoblast) fluoresces green with Calcein (100x magnification)(image courtesy of our collaborator, Benjamin J. Hackel, PhD, at the University of Minnesota).

Additionally, the proposed role of P188 preservation of endothelial cell function against I/R injury, when it is administered intravascularly, should be pursued. These answers will only help to develop a more defined mechanism of action of P188 that can be tested in more translational models, because developing effective and safe treatments to prevent further membrane damage and Ca^{2+}-induced cellular consequences during reperfusion is an unmet challenge with important therapeutic implications and large potential clinical impact.

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