MODULATION OF CARDIAC MYOCYTE FUNCTION BY REACTIVE OXYGEN SPECIES

by

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A thesis submitted to the Department of Physiology

In conformity with the requirements for

the degree of Master of Science

Queen's University

Kingston, Ontario, Canada

(March, 2009)

ABSTRACT

Previous investigations have demonstrated that reactive oxygen species such as hydrogen peroxide (H_2O_2) have the ability to alter electrophysiological and mechanical properties of rat ventricular cardiac myocytes. However, despite the breadth of the literature, there is little definitive consensus on the cellular mechanisms. The purpose of this study, therefore, was to study the cellular mechanism of action of H_2O_2 and test whether H_2O_2-mediated affects were partially a result of reverse-mode Na^+/Ca^{2+} exchanger (NCX) activity. Unloaded cell shortening, intracellular Ca^{2+} transients, caffeine induced Ca^{2+} transients, L-type Ca^{2+} channel recordings, and action potential waveforms were recorded in the presence of combinations of different compounds including Cd^{2+}, H_2O_2, and KB-R7943. H_2O_2 was found to cause significant positive inotropy by an increase in contractility of 80 ± 20 % (n=6) and an increased amplitude of Ca^{2+} transients by 24 ± 14 % (n=8), relative to pre-treatment values. Interestingly, H_2O_2 caused an increase in contractility even in the presence of Cd^{2+} block from 4 ± 1 % (n=9) to 15 ± 3 % (n=5) of resting cell length. Using caffeine pulse experiments to induce unloading of the sarcoplasmic reticulum (SR), we found that 100µM H_2O_2 did not significantly alter SR Ca^{2+} load. Under control conditions, H_2O_2 significantly increased L-type Ca^{2+} currents while this H_2O_2-induced increase was not observed in myocytes pretreated with Cd^{2+}. Positive inotropy in the presence of H_2O_2 was blocked using 10µM KB-R7943, a selective reverse-mode inhibitor of the NCX. However, it was found that 10µM KB-R7943 alone altered action potential profile and suppressed normal contraction. Altogether, the major finding of this study is that H_2O_2 has the ability to

enhance myocardial contractility, even under conditions of L-type Ca^{2+} channel inhibition, through a mechanism that likely involves reverse-mode of the NCX.

ACKNOWLEDGEMENTS

I would like to thank the members of the lab who made research infinitely easier and more enjoyable. Thank you Gina, for always being there to listen, and for your humble advice, which helped me through many difficult times. Thank you Lian, for being the intelligent and genuine person you are, and for all the philosophical conversations you humored me with. Thank you Sarah, for helping keep me on my toes and being someone who I could both laugh at and laugh with. And thank you Jesse for being my friend, the childish yet wise researcher, whose competitive nature gave us both many fond memories.

I would like to thank my mentors, Chris and Luis, for their help and encouragement throughout the years. I am grateful for your guidance. With your help, I learned more in these years of research than I could have imagined.

I would like to thank all of those who have been close to me throughout my research. Thank you Jennifer, your free spirit and contagious laughter helped me through many of my most stressful times.

And lastly, I would like to thank my family. To my father, thank you for your continued support. To my mom, thank you for your endless love, patience, and for all the times you drove to the lab just to drop off hot food for me. And to my sister, thank you for your smiles, your laughs, and all the times you made living at home a pleasure.

I would also like to acknowledge the mice and rats that allowed me to complete my research. I will strive to use this knowledge and experience I gained to benefit others.

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LIST OF ABBREVIATIONS

APD50…….Action Potential Duration for 50% repolarization

APD90…….Action Potential Duration for 90% repolarization

ATP……..Adenosine Triphosphate

cAMP……...Cyclic Adenosine Monophosphate

CICR……Ca^{2+}-induced Ca^{2+} Release

DADs……...Delayed Afterdepolarizations

DTDP……...4,4’-Dithiodipyridine

DTNB……..5.5'-Dithio-bis(2-nitrobenzoic acid)

DTT…….Dithiothreitol

ECC…….Excitation Contraction Coupling

ETC…….Electron Transport Chain

FADH…….……Flavin Adenine Dinucleotide

HEK293…….…….Human Embryonic Kidney 293 cells

HEPES…….……...4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IC50……Half Maximal Inhibitory Concentration

I_{CaL}……..L-Type Ca^{2+} Current

I_{Na}……...Na^{+} Current

MPT……Mitochondrial Permeability Transition

NADPH…….……Nicotinamide Adenine Dinucleotide Phosphate

NCX……Na^{+}/Ca^{2+} Exchanger

RIRR……...ROS-Induced ROS Release

ROS…….Reactive Oxygen Species

RyRs……Ryanodine Receptors

SERCA…….……Sarcoplasmic Reticulum Ca^{2+} ATPase

SH…….Sulfhydryl

SR…….Sarcoplasmic Reticulum

TTX……...Tetrodotoxin

VSRM……...Voltage-sensitive Release Mechanism

CHAPTER 1

Reperfusion injury

Cardiovascular disease is a leading cause of morbidity and mortality in Canada, being responsible for more than 36% of all deaths (Statistics Canada, 1999). Acute myocardial infarction, and the complications arising from it, such as cardiac arrhythmia, represents a significant area of heart disease research. A primary cause of tissue injury and necrosis/apoptosis is due to myocardial ischemia, defined as a lack of oxygen supply to cardiac myocytes, which results in an imbalance of metabolic supply and demand. To minimize the effects of myocardial ischemia, procedures have been developed to restore blood supply and oxygen to affected cells through methods such as the use of thrombolytic agents, percutaneous coronary intervention, and coronary angioplasty. These strategies have proven effective for reducing the size of infarct and thus improving clinical outcome after acute myocardial infarct (Becker & Ambrosia, 1987).

However, immediate reperfusion of tissue is most effective if it occurs within approximately 15 minutes after ischemia develops (Kloner & Jennings, 2001). Following this brief period, ischemic tissue undergoes additional damage after reintroduction of oxygenated blood supply to previously ischemic tissue (Verma *et al*. 2002). This damage is termed myocardial ischemia-reperfusion injury and has been found to be associated with Ca^{2+} overload and arrhythmias, free-radical production, aberrations in myocardial energetics and metabolism, damage to mitochondria, endothelial dysfunction, sarcoplasmic reticulum dysfunction, hypercontracture, and necrosis/apoptosis (Zhao & Vinten-Johansen, 2006; Zima & Blatter, 2006; Murphy & Steenbergen, 2008). Although,

immediate reperfusion is still the most effective way to conserve cardiac function known to date, understanding reperfusion injury is an important part of the search for better treatments and therapies for heart disease. The early phase of reperfusion remains a window of therapeutic opportunity, still largely unused clinically, and that if taken advantage of, could further improve patient prognosis after treatment of myocardial infarction.

Cardiac arrhythmia

Cardiac arrhythmia is a broad term that encompasses a heterogeneous group of conditions involving abnormalities in the rhythm, conduction, or coordination of electrical activity in heart cells. Given the breath of the term arrhythmia, the effects of such conditions are also varied such that some cardiac arrhythmias may have no symptoms, while others may be debilitating to daily life. Likewise, both the severity and prognosis of an arrhythmia can vary greatly, and depending on severity, may predispose patients to potentially life threatening conditions such as ventricular tachyarrhythmia and ventricular fibrillation, which have the potential to induce sudden cardiac death (Wit & Janse, 2001).

Theories that exist to explain arrhythmias most often attribute them to problems with either impulse conduction involving re-entrant impulses, or ectopic impulse formation involving abnormal triggered activity in cells of the heart. In the case of ischemia-reperfusion induced arrhythmias, both types of theories may be relevant although more evidence exists for the latter. Ischemia-reperfused cells, for example, have

been found to have elevations in intracellular Ca^{2+} (Ribeiro *et al*. 1981; Sharma *et al*. 1983). This elevation in Ca^{2+} can is linked to spontaneous release of Ca^{2+} from the SR independent of sarcolemmal depolarization if the intracellular Ca^{2+} is sufficiently high. Importantly, increased intracellular Ca^{2+} has been suggested to be associated with potentially life-threatening ventricular tachyarrhythmias (Kihara & Morgan, 1991; Lakatta & Guarnieri, 1993; Zaugg *et al*. 1996).

In the last few decades, studies to elucidate the cellular mechanisms that underlie arrhythmogenesis have demonstrated that many chemical and bodily substances, such as reactive oxygen species (ROS), are produced in higher concentrations during reperfusion injury and may be the cause of abnormal automaticity (Hool, 2007). The focus of our study will primarily be to better understand the mechanism by which ROS, produced in excess during ischemia-reperfusion, modify electrophysiological function in the heart.

Reperfusion injury and arrhythmia

The first observation that ventricular fibrillation sometimes followed restoration of blood flow to ischemic myocardium was made in the 19th century and later confirmed (Tennant & Wiggers, 1935). Since then the phenomenon has become much more documented. More recent studies have shown reperfusion-induced injury in patients with ischemia after subsequent reintroduction of oxygenated blood supply (Ferrari *et al*. 1990; Hansen, 1995; Weman *et al*. 2000). For example, the use of thrombolytic therapy and coronary artery bypass in humans to initiate reperfusion has been shown to result in severe arrhythmias (Lockerman *et al*. 1987; Solimene *et al*. 1988).

Mechanisms through which arrhythmias arise have been extensively studied in terms of electrophysiology and the underlying ion channels involved. However, many potentially arrhythmogenic substrates such as ROS are present in high concentrations in an infarct after reperfusion injury, and of these arrhythmogenic substrates, many of their mechanisms of action are not well characterized or understood. ROS can be created through a variety of mechanisms during reperfusion injury and one that has previously been found to cause electrophysiological disturbances is hydrogen peroxide (H_2O_2). H_2O_2 is a particularly interesting target for study since it is a relatively stable ROS in comparison to the oxygen free radicals such as superoxide anion, or hydroxyl radical, and thus has the ability to diffuse further and cause injury to a greater tissue mass.

Reperfusion-induced reactive oxygen species

Reactive oxygen species is a generalized term that includes oxygen ions, free radicals, and peroxides. Oxygen ions and free radicals are highly reactive chemical species due to the presence of an oxygen atom with unpaired valence shell electrons such as superoxide anions or the hydroxyl radical. The unpaired valence electrons of the oxygen in ROS lead to electrical strain and result in a highly reactive molecule seeking to complete its electron pair (McCord, 1985). Although they are not free radicals, peroxides such as H_2O_2 are also classified as ROS because of their highly oxidative and reactive nature. ROS are formed in normal cellular metabolism and respiration and have roles in cell signaling (Salmeen *et al*. 2003). However, during certain stressful events such as reperfusion after sustained myocardial ischemia, ROS can buildup to levels far exceeding

those found in normal cellular metabolism (Baker *et al*. 1988; Bolli *et al*. 1988; Zweier *et al*. 1987). For example, ROS have been shown to have the ability to cause self-perpetuating chain reactions of lipid peroxidation (Singal *et al*. 1983) as well as modify Ca^{2+} handling (Hool, 2007) in cardiac myocytes. The major ROS known to mediate cellular damage and other adverse affects include superoxide, H_2O_2, and the hydroxyl radical. The superoxide anion and H_2O_2 are produced in the cell through the following general chemical reactions:

Reaction 1: $2O_2 + e^- \rightarrow 2{\cdot}O_2^-$ Generation of superoxide anion

Reaction 2: $2{\cdot}O_2^- + 2H+ \rightarrow H_2O_2 + O_2$ Generation of hydrogen peroxide

Reaction 3: $2H_2O_2 \rightarrow 2H_2O + O_2$ Decomposition into wáter

In neutrophils, the above general chemical reaction takes place through activation of the enzyme NADPH oxidase. NADPH oxidase leads to production of the superoxide radical anion via reduction of O_2 using an electron that is supplied by NADPH oxidase as shown below:

$$2O_2 + NADPH \rightarrow 2{\cdot}O_2^- + NADPH^+ + H^+$$

In aqueous solution, the superoxide anions created can subsequently form H_2O_2 through the following chemical reaction:

$$2{\cdot}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The hydroxyl radical can be produced during the Fenton Reaction, as shown below:

$Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$

There are many possible sources of ROS in a cell and are produced in the body as a natural byproduct of oxygen metabolism. Cells normally defend themselves against excess buildup of ROS through enzymes such as catalases and peroxidases which convert ROS into less damaging byproducts. In reperfusion injury, it has been suggested that ROS may be produced not only directly within cardiac myocytes, primarily in mitochondria, but also extracellularly by neutrophils, which infiltrate infarcted tissue (Chen *et al*. 2006).

Neutrophils are known to migrate to sites of ischemia in response to an inflammatory stimulus, and ROS are produced in normal functioning of the neutrophils during phagocytosis. Acute ischemia causes endothelium activation, increased permeability, and expression of adhesion molecules (Gueler, 2004). In this way, ischemic endothelial cells become primed for inflammatory cell infiltration upon reperfusion (Gueler, 2004). Neutrophils are thought to be among the early cellular components of the innate immune system that infiltrate and mediate local microvascular changes (Linferta *et al*. 2008). As a part of the humoral response to ischemic damage and subsequent reperfusion, neutrophils migrate to the site of injury caused by ischemia. These neutrophils when activated are a potential source of ROS as they begin phagocytosis of foreign particles or damaged tissue (Werns *et al*. 1985). The "respiratory burst" accompanying phagocytosis is characterized by activation of the enzyme NADPH oxidase (Werns *et al*. 1985). By oxidizing NADPH to NADP, NADPH oxidase produces

superoxide anions which can either spontaneously form H_2O_2 by picking up H^+ from other cellular compounds, or form H_2O_2 through catalysis via superoxide dismutases (Kanegasaki, 1986). Past experiments have demonstrated that neutrophils may be important in reperfusion injury. For example, Romson *et al* (1983) and Litt *et al* (1989) found that a depletion of leukocytes resulted in decreased infarct size and decreased incidents of myocardial failure. Interestingly, evidence suggests that the respiratory burst of neutrophils to release ROS into the extracellular space is not the only means by which neutrophils affect cardiac myocytes. It has been shown that neutrophils are capable of directly adhering to cardiac myocytes (Ward *et al*. 2006), following which they may introduce ROS to the intracellular environment as well.

Another potential source of intracellular ROS is mitochondria (Korshunov *et al*. 1997). In normal respiration, electrons transported through the electron transport chain (ETC) cause oxidation of NADH and $FADH_2$ and act on other metabolic pathways such as the beta-oxidation of fatty acids. However, *in vitro* studies have shown that the electrons in the ETC can leak and cause reduction of nearby oxygen atoms to superoxide. Furthermore, more electrons may leak depending on the cellular conditions. In a study by Korshunov *et al* (1997), ROS production was found to be highest when the electrochemical potential formed by the gradient of protons across the mitochondrial membrane was maximal.

While the mechanism of electron leak is not well known, evidence has suggested that ROS production in mitochondria is increased during ischemia/reperfusion (Chen *et al*. 2006). For example, one study examined the effects of reversible blockade of electron transport using amobarbital during reperfusion of ischemic cells. It was shown in the

study that blockade of ETC using amobarbital resulted in decreased ROS production as measured by preservation of NADH-FAD redox state and decreased myocardial damage as measured by preserved function and decreased infarct size. Blockade of the electron transport chain and partial uncoupling of respiration has been suggested as a possible means for prevention of reperfusion injury (Chen *et al*. 2006).

Recent studies have suggested the possibility of ROS-Induced ROS Release (RIRR) via induction and opening of the mitochondrial permeability transition (MPT) pore (Zorov *et al*. 2000; Zorov *et al*. 2006). Although a relatively new theory, it was suggested that this phenomenon may contribute to post ischemic pathologies and arrhythmias via further exacerbating the increased production and release of ROS found in ischemia-reperfusion (Zorov *et al*. 2000; Zorov *et al*. 2006).

ROS as mediators of reperfusion injury

A major mechanism proposed for the cause of reperfusion injury concerns the effects of the ROS produced immediately after ischemia-reperfusion. *In vitro* studies with isolated hearts following reperfusion have shown a rapid rise in oxygen free radicals following reperfusion (Zweier *et al*. 1987; Baker *et al*. 1988). In one study, imaging using electron spin resonance spectroscopy in an open-chest preparation of dogs subject to 15 minutes of coronary occlusion showed a burst of ROS formation peaking at 3 minutes after initiation of reperfusion (Bolli *et al*. 1988). ROS production, as measured by electron spin resonance, only took place during greater than 60% coronary occlusion (Bolli *et al*. 1988). Interestingly, ROS production continued for up to 1-3 hours after

reperfusion had begun suggesting a large window of time through which ROS could potentiate its effects (Bolli *et al*. 1988).

Although ROS include a number of possible compounds, H_2O_2 has been suggested as one of the main mediators in ROS-induced reperfusion injury (Duan & Moffat, 1992; Goldhaber & Liu, 1994). It has been shown that myocardial tissue concentration of H_2O_2 is significantly elevated in hearts exposed to ischemia-reperfusion (Slezak *et al*. 1995) and in failing hearts (Ide *et al*. 2000). Further evidence of H_2O_2 as a mediator of reperfusion injury was demonstrated by (Brown ***et al.*** 1988) when it was shown that the timing of ROS production and ventricular function were correlated. Brown *et al* (1988) showed that ventricular function rebounded immediately after reperfusion but after approximately 10 minutes began to decline. Since peak production of H_2O_2 occurred at 10 minutes and coincided with ventricular functional loss during reperfusion injury, it was suggested that H_2O_2 was the mediator of reperfusion injury. Furthermore, by infusing dimethylthiourea, a permeable O_2 metabolite scavenger, ventricular functional loss could be prevented (Brown ***et al.*** 1988). Subsequent studies enforced these findings by showing that inhibition of xanthine oxidase, one of the enzymes responsible for ROS formation also prevented loss of ventricular function (Brown ***et al.*** 1989).

While superoxide is a common ROS produced in cells and is the precursor to H_2O_2 formation, it has been suggested that its site of action is limited to the organelles in which it is produced as it has difficulty crossing cell membranes (Thannickal & Fanburg, 2000). H_2O_2 on the other hand is comparatively more stable and, therefore, has the ability to diffuse further and cause injury to a greater tissue mass. Furthermore, H_2O_2 has been

shown to be capable of reacting in many different ways with cardiac myocytes. For example, it has been found to cause lipid peroxidation of cell membranes (Rubin & Farber, 1984), alter energy metabolism (Spragg *et al*. 1985), activate second messenger systems (Molkentin *et al*. 1998; Bolli, 1998; Ward & Moffat, 1995), cause redox of cysteine residues and subsequent protein modification (Barford, 2004), alter ATP-sensitive K^+ currents (Goldhaber & Liu, 1994), alter Ca^{2+} channel function (Hool, 2007), alter Na^+ channel inactivation (Ward & Giles, 1997; Song *et al*. 2006), and cause increase in intracellular Ca^{2+} (Hayashi *et al*. 1989, Ward & Moffat, 1995).

CICR and other theories of Ca^{2+} influx

The predominate theory of excitation contraction coupling (ECC) involves influx of Ca^{2+} ions through L-type Ca^{2+} channels and subsequent Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR). In CICR theory, depolarization and subsequent propagation of a cardiac action potential causes voltage-dependent activation of L-type Ca^{2+} channels, which open to allow entry of Ca^{2+} into the cardiac myocyte. This entry of Ca^{2+} triggers opening of ryanodine receptors (RyRs) located on the SR and thus the release of stored Ca^{2+} from the SR (Fabiato, 1983). The increase in localized intracellular Ca^{2+} caused by SR Ca^{2+} release is important in the activation of contractile filaments resulting in cardiac cell contraction (Fabiato, 1983). Cardiac muscle relaxation then takes place after termination of Ca^{2+} release and cystolic Ca^{2+} is sequestered into the SR by the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) or extruded from the cell through the Na^+-Ca^{2+} exchanger (NCX) (Fabiato, 1983).

An alternate hypothesis providing a potential mechanism for Ca^{2+} entry into cardiac myocytes involves entry of Ca^{2+} ions through Na^{+} channels. This form of Ca^{2+} entry, termed "slip-mode conductance", has been shown to occur in certain *in vitro* conditions such as removal of extracellular Na^{+} (Cole *et al*. 1997). In that study Ca^{2+} was shown to permeate TTX and veratridine-sensitive voltage gated Na^{+} channels in guinea pig ventricular myocytes. However, it was suggested that influx of Ca^{2+} through Na^{+} channels likely does not occur under physiological ion concentrations (Cole *et al*. 1997).

Voltage-sensitive release mechanism (VSRM) is another mechanism through which an increase of $[Ca^{2+}]_i$ can be mediated through voltage induced Ca^{2+} release from the SR. Ferrier & Howlett (1995) demonstrated that, at holding potentials of either -65 mV or -55 mV, the magnitude of contraction increased with voltage, even at positive membrane potentials. This contraction was shown to operate independent of I_{Na} and abolished by low concentrations of ryanodine. Furthermore, contractions were not shown to be proportional to magnitude of inward I_{CaL}. These results suggested that voltage stimulation can potentially cause contraction independent of membrane depolarization, and I_{CaL} (Ferrier & Howlett, 1995). More recent evidence, however, suggests that this mechanism of Ca^{2+} release may be invalid as it was shown that, under similar experimental conditions, both unblock of L-type Ca^{2+} channels, and reverse mode operation of the NCX could occur (Griffiths & MacLeod, 2003).

All together, previous studies have provided evidence for contraction of cardiac myocytes and transient increases in $[Ca^{2+}]_i$ in the presence of known I_{CaL} blockers (Leblanc & Hume, 1990; Ferrier & Howlett, 1995). While other theories such as slip-mode conductance (Santana *et al*. 1998) and VSRM (Ferrier & Howlett, 1995) were

hypothesized as possible alternate methods of Ca^{2+} influx, they are controversial and not well characterized.

Intracellular Ca^{2+} handling and delayed afterdepolarizations

Ventricular tachyarrhythmias, such as ventricular fibrillation, are characteristic of patients with heart failure and are a major cause of death in these patients (Stevenson *et al*. 1993). One suggested mechanism for the development of arrhythmia is the development of delayed afterdepolarizations due to increased intracellular Ca^{2+}.

Delayed afterdepolarizations are spontaneous depolarizations and contractions of cardiac myocytes after repolarization has completed, during late phase 3 or phase 4 of the cardiac action potential. When diastolic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) becomes sufficiently high, the SR may become overloaded and spontaneously release Ca^{2+}. It is possible that this is spontaneous release of Ca^{2+} is triggered by increased diastolic Ca^{2+} concentrations, instead of sarcolemmal depolarizations and influx through the L-type Ca^{2+} channels (Thandroyen *et al*. 1991; Lakatta & Guarnieri, 1993). According to (Lakatta & Guarnieri, 1993), if the Ca^{2+} oscillations caused by spontaneous CICR are synchronized, afterdepolarizations in cardiac myocytes occur and may result in subsequent ventricular fibrillation.

Ca^{2+} overload may also cause ventricular tachyarrhythmias through other mechanisms. For example, in a review of literature by Kleber (1992) it was suggested that Ca^{2+} overload caused arrhythmia and ventricular fibrillation through uncoupling of the normal process of CICR due to mechanical damage of cells in Ca^{2+} overload. This

uncoupling of CICR can result in non-uniform conduction of the cardiac action potential, leading to development of arrhythmias (Kleber, 1992). Other theories of Ca^{2+} overload induced arrhythmia include the possibility that Ca^{2+} overload leads to heterogenous recovery of cardiac myocytes after depolarization (Aronson & Ming, 1993). An interesting finding by Kihara & Morgan (1991) was that ventricular fibrillation could also be the cause of Ca^{2+} overload, providing further indication of the pathology of Ca^{2+} overload. According to Kihara & Morgan (1991), Ca^{2+} overload can cause ventricular fibrillation which can result in further Ca^{2+} overload, creating a feedback loop in which intracellular Ca^{2+} handling progressively becomes more pathologic.

ECC and the possible targets of H_2O_2

The targets of H_2O_2 are not well known and are potentially numerous. One possible mechanism of H_2O_2 mediated arrhythmia is through modification of the redox state of a cell. The properties, such as inactivation and gating, of some ion channels and transporters may be affected by redox modulation (Kourie, 1998; Pessah *et al*. 2002; Waring, 2005). Since ROS such as H_2O_2 are produced in excess during ischemia-reperfusion, potentially changing the redox state of the environment, redox modulation is an especially significant possible mechanism.

Previously it was discussed that Ca^{2+} overload can be detrimental to cardiac function because it causes tachyarrhythmias. The ability of H_2O_2 to cause Ca^{2+} overload has been documented in previous experiments (Hayashi *et al*. 1987, 1989; Ward & Giles, 1997; Gen *et al*. 2001) but the underlying mechanism remain unclear. Possible

mechanisms of this H_2O_2 mediated effect range from changes in conductance of ion channels on the plasma membrane such as L-type Ca^{2+} channel, and Na^+ channels, to changes in activity of the ion transporters on the sarcoplasmic reticulum such as the RyRs and SERCA. In addition to pathways leading to increased intracellular Ca^{2+}, it is also possible that H_2O_2 could be acting directly on the contractile mechanisms of the cell to modulate contractility.

Modulation of Na^+ channels by ROS

(Duan & Moffat, 1992) conducted a study examining the effects of H_2O_2 on isolated guinea pig right ventricular cardiac myocytes and the mechanisms by which H_2O_2 induces cardiac arrhythmia. The results of the study showed that H_2O_2 caused prolongation of action potential duration (APD). Their results suggested that this prolongation may be causing increased Ca^{2+} entry into myocytes thus contributing to increased $[Ca^{2+}]_i$ and Ca^{2+} overload. A study by Ward & Giles (1997) examining the effects of H_2O_2 on action potentials in rat ventricular myocytes confirmed the phenomenon. Furthermore, in the study by Ward & Giles (1997) it was demonstrated that H_2O_2 induced a persistent Na^+ current that displayed altered inactivation kinetics. It was suggested by Ward & Giles (1997) that the persistent Na^+ current could be the cause of prolonged action potentials. Selective blocking of I_{Na} significantly attenuated the action potential prolonging effects seen in the presence of H_2O_2 (Ward & Giles, 1997) suggesting modulation of Na^+ conductance is a possible effect of H_2O_2. These results were consistent with studies by Beresewicz & Horackova (1991) who found that action

potential prolongation in the presence of H_2O_2 could be permanently abolished with tetrodotoxin (TTX), a Na^+ channel blocker.

A recent study by Song *et al* (2006) confirmed the findings of Ward & Giles (1997). In their study, guinea pig and rabbit isolated ventricular myocytes were exposed to 200 uM H_2O_2 while membrane voltages, cell shortening, and both intracellular Na^+ and Ca^{2+} were recorded. Similar to previous experiments, it was observed that H_2O_2 caused persistent I_{Na} and delayed afterdepolarizations that were again almost completely inhibited by TTX. Furthermore, it was found that H_2O_2 increased intracellular $[Na^+]$ and $[Ca^{2+}]$. A novel persistent I_{Na} blocker, Ranolazine, was used and block of I_{Na} resulted in decrease in APD and decrease in occurrence of afterdepolarizations. While it has been concluded, therefore, that persistent I_{Na} could cause prolonged APD and therefore increased intracellular Ca^{2+}, it is only one potential mechanism by which intracellular Ca^{2+} becomes elevated. It has also been suggested that increased intracellular $[Na^+]$ could cause reversal of the NCX and through this mechanism result in increased intracellular $[Ca^{2+}]$.

Reversal of the NCX

The Na^+-Ca^{2+} exchanger (NCX) is one of the major contributors to the relaxation phase of cardiac contraction and does so through extrusion of one Ca^{2+} ion through the sarcolemma in exchange for three Na^+ ions. Detubulation experiments have shown that the majority of NCX are localized in T-tubules and it has been suggested that some NCX are colocalized with RyRs (Scriven *et al*. 2000; Yang *et al*. 2002). Forward mode of the

NCX, in which Na^+ enters the cell and Ca^{2+} is extruded, is an important part of normal Ca^{2+} homeostasis in cardiac myocytes. However, mathematical modeling of the NCX has suggested that local levels of the two ions are the driving force for the NCX and determine the function of the NCX. Therefore, another feasible explanation for increased intracellular Ca^{2+} in ischemia reperfusion injury, which has been associated with increased intracellular $[Na^+]$, is the reversal of the NCX, bringing Ca^{2+} in and extruding Na^+. This increase in intracellular $[Ca^{2+}]$ caused by the NCX may have the potential to cause release of SR Ca^{2+} through CICR. Previous experiments have showed that triggering of SR Ca^{2+} release by the reversal of NCX is possible under certain ionic conditions such as removal of extracellular Na^+ (Litwin *et al.* 1998; Goldhaber *et al*. 1999).

Many other experiments suggest the existence of reverse mode NCX. Waaserstrom & Vites (1996) have demonstrated that a complete nifedipine-induced block of I_{CaL} only reduced contraction of rat ventricular myocytes by 50%. Ferrier & Howlett (1995) observed similar results in guinea-pig ventricular myocytes when contraction did not disappear after pharmacological block of I_{CaL}. It was therefore reasoned that another mechanism must be involved in the increase in intracellular Ca^{2+} which causes CICR and subsequent contraction. Waasterstrom & Vites (1996) demonstrated that during nifedipine block, influx of Na^+ during the action potential was capable of activating contraction. The contraction present after nifedipine block was found to be sensitive to Ni^{2+}, a NCX blocker. They concluded that the NCX operated in reverse to decrease elevated intracellular Na^+ and this consequently lead to contraction independent of I_{CaL} (Waasterstrom & Vites, 1996).

Mathematical modeling of the NCX has suggested local levels of intracellular Ca^{2+} and Na^{+} are the determinants in the predicted driving force for ions moving across the NCX (Sher *et al*. 2008). According to this theory, depending on extracellular and intracellular conditions of the cardiac cell, reversal is possible. Sher *et al* (2008) predicted that reversal of the NCX even occurs in normal contraction depending on local, global, and submembrane [Ca^{2+}]. The mathematical model supports the theory that the NCX can function to regulate local Ca^{2+} levels and cause influx of Ca^{2+} leading to CICR in pathological conditions (Sher *et al*. 2008).

Wagner *et al* (2003) demonstrated that failing cardiac myocytes were more susceptible to ROS-induced damage than healthy cardiac myocytes and hypothesized that it could be due to increased NCX expression in the failing myocytes. In native myocytes, ROS exposure causes hypercontracture, presumably due to increased intracellular [Ca^{2+}]. Using adenovirus mediated gene-transfer to overexpress NCX in rabbit ventricular cardiac myocytes, it was shown that overexpression of NCX caused enhanced hypercontracture, mirroring the effects found in native myocytes after ROS exposure. With the use of KB-R7943, a reverse mode blocker of NCX, hypercontracture was significantly reduced in cardiac myocytes with overexpression of NCX. It was concluded that since increased expression of NCX caused enhanced hypercontracture and block of NCX prevented it, increased intracellular [Ca^{2+}] could potentially be caused by reversal of the NCX.

Finally, in a study by Zeitz *et al* (2002) it was suggested that ROS such as the hydroxyl radical may also play a role in mediating reverse mode NCX. When intact contracting cardiac trabeculae from rabbits were exposed to ROS in the form of hydroxyl

radicals, it resulted in an increase in diastolic force. With the use of KB-R7943, diastolic function was preserved and no change in diastolic force was seen. The set of experiments were repeated on rat myocardium and similar results were found. Electron microscopy of cardiac myocytes did not show membrane damage to cells and thus it was concluded that the hydroxyl radical induced diastolic dysfunction was caused by increased Ca^{2+} due to reverse mode NCX.

Modulation of Ca^{2+} channels

The L-type Ca^{2+} channel is a key component of CICR, acting as a trigger for cardiac myocyte contraction and playing an important role in Ca^{2+} homeostasis. In a recent paper by Zima & Blatter (2006) it was suggested that the L-type Ca^{2+} channel contained 10 cysteine residues at the pore-forming subunit 1C that could undergo redox modification. Expression of the human 1C subunit in HEK293 cells found that thiol oxidizing agents (e.g. thimerosal) irreversibly decreased current through the human 1C subunit (Fearon *et al*. 1999). However, since results in ferret ventricular myocytes showed that use of 5.5'-dithio-bis(2-nitrobenzoic acid) (DTNB), an oxidizer of free SH groups, caused stimulation of Ca^{2+} current (Campbell *et al*. 1996), it is possible that the modulation of the L-type Ca^{2+} channel may be species specific.

Other previous experiments have also shown modification of activity of the L-type Ca^{2+} channel by ROS. The use of oxidizing agents, such as 4,4'-dithiodipyridine (DTDP) and thimerosal ethyl mercury sodium salt on rabbit smooth muscle to mimic ROS as an oxidizing agent, has shown inhibition of L-type Ca^{2+} channel activity

(Chiamvimonvat *et al*. 1995). Many previous studies, for example, (Cerbai *et al.* 1991; Matsuura & Shattock, 1991; Moghadam & Winlow, 1995) have reported decreased I_{CaL} as a result of ROS.

While I_{CaL} has mostly been reported to be inhibited by redox modulation, reports regarding ion channel function have been contradictory. For example, I_{CaL} has been found to be increased in some experiments (Sato *et al*. 1989). Coetzee & Opie (1992) reported a reduction in peak amplitude but slowing of inactivation of the L-type Ca^{2+} channel resulting in an overall increase in mean current. Although it is unclear why previous studies report such contradictory results, Ward & Giles (1997) reported that with perforated patch clamp techniques, H_2O_2 caused marked prolongation of APD but that with whole cell techniques H_2O_2 did not affect APD. It was speculated by Ward & Giles (1997) that such inconsistencies in observed channel function could be attributed to the electrophysiological recording techniques used. Specifically, whole cell patch clamp causes dialysis of the intracellular environment, which may alter processes involving second messengers. Indeed, it is widely known that L-type Ca^{2+} channels are highly modulated by second messengers such as cAMP. In contrast to whole cell technique, perforated patch recordings preserve the intracellular environment.

Modulation of Ca^{2+} handling in the SR

Gen *et al* (2001) has previously suggested that the mechanism through which Ca^{2+} overload caused by H_2O_2 takes place is through dysfunction of Ca^{2+} handling at the SR rather than the L-type Ca^{2+} channel or the NCX. Gen *et al* (2001) reported that H_2O_2

mediated an increase in intracellular Ca^{2+} and hypercontracture, but that these effects were independent of the L-type Ca^{2+} channel as nifedipine did not block increases in $[Ca^{2+}]_i$ caused by H_2O_2. It was shown that cell viability improved after perfusion with caffeine and caffeine attenuated the increase in intracellular Ca^{2+} associated with exposure to H_2O_2. Furthermore, it was shown that KB-R7943 and chelerythrine, a PKC inhibitor, did not attenuate the increase in $[Ca^{2+}]_i$ caused by H_2O_2. From these findings, it was concluded in this study that the H_2O_2 induced Ca^{2+} overload was caused by the SR, since caffeine attenuated the action of H_2O_2 and neither the L-type Ca^{2+} channel nor the NCX seemed to mediate the increase in $[Ca^{2+}]_i$ (Gen *et al.* 2001).

Of the Ca^{2+} channels/transporters another possible target of ROS and redox regulation is the RyR Ca^{2+} release channel. Kourie (1998) suggested that ROS mediated effects on the RyR through oxidation of SH groups. Support for this theory came from previous experiments showing that the addition of H_2O_2 on cardiac muscle RyR's resulted in a higher open probability of the channels (Boraso & Williams, 1994; Oba *et al*. 1996). In an experimental setup of sheep SR Ca^{2+} release channels incorporated into planar phospholipid bilayers, Boraso & Williams (1994) showed that H_2O_2 increased open probability of channels. Furthermore, single channel recordings showed that H_2O_2 affected neither conductance of the SR release channels, nor response to activating compounds, leading to the conclusion that it affected gating properties of the SR release channels. Boraso & Williams (1994) also tested whether the effects of H_2O_2 were mediated through redox modulation of the channels by adding reduced thiol groups such as DTT, and showed that addition of reducing groups resulted in reversal of H_2O_2 mediated changes in open probability.

The sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) is another potential target of redox modulation. It has been found DTNB inhibits SERCA activity whereas reducing agents such as DTT protect SERCA from inhibition (Scherer & Deamer, 1986; Morris & Sulakhe, 1997). Inhibition of SERCA Would result in increased intracellular Ca^{2+} through an inability of the cell to properly sequester Ca^{2+}. Furthermore, ROS have been found to inhibit Ca^{2+} uptake into the SR by interfering with the ATP binding site of SERCA (Xu *et al*. 1997). Since ATPase activity is coupled to the Ca^{2+} pump, inhibition of the ATPase leads to decreased Ca^{2+} pump rate (Zima & Blatter, 2006).

Summary

The mechanisms through which H_2O_2 mediates its arrhythmogenic properties are not well known and many possible mechanisms exist. Furthermore, arrhythmia could be a result of more than a single mechanism. Modulation of Na^+ channels causing an increased influx of Na^+, reversal of the NCX resulting in Ca^{2+} influx, modulation of Ca^{2+} channels causing increased influx of Ca^{2+}, and modulation of SR Ca^{2+} handling are all potential mechanisms of arrhythmia. Through our study, we hope to acquire a better understanding of H_2O_2 and the mechanism through which it induces arrhythmia.

HYPOTHESIS AND OBJECTIVES

Based on previous studies, the action of ROS such as H_2O_2 can mediate development of arrhythmia through a number of mechanisms. The preceding discussion of the literature summarizes some of the previously postulated mechanisms. While, many exist, it is still unclear how much each potential mechanism actually contributes to the development of arrhythmia. Our goal is therefore to examine the effects of H_2O_2 on freshly dissociated cardiac myocytes to increase our understanding of the underlying cellular mechanisms. For these studies, **the hypothesis is that H_2O_2-induced changes in electrophysiology are caused by reverse-mode function of the NCX.** Our objectives include to:

1. Examine effects of H_2O_2 on unloaded cell shortening, Ca^{2+} transients, action potentials and Ca^{2+} current.
2. Examine effects of H_2O_2 in the presence of L-type Ca^{2+} channel blocker Cd^{2+}.
3. Examine ability of the putatively reverse-mode selective NCX inhibitor, KB-R7943, to attenuate H_2O_2 mediated effects on electrophysiology of cardiac myocytes

One of the unique aspects of our study is that we are using perforated patch clamp technique in order to conduct our electrophysiological experiments. Many previous experiments are conducted using whole cell patch clamp technique. Furthermore, our study is conducted on freshly dissociated adult ventricular myocytes.

CHAPTER 2

Cell isolation

The Ward and Giles (1997) method of isolating cardiac myocytes was used to isolate right ventricular myocytes from male Spraque-Dawley rats (225-275g) using protocols approved by the Queen's University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. After decapitation the hearts were removed, set into cold buffer (4° C) and the aorta rapidly cannulated for retrograde perfusion at 37º C. Initially, Tyrode's solution (mM): NaCl 140; KCl 5.4; $MgCl_2$ 1; Na_2HPO_4 1; HEPES 5; glucose 10, pH adjusted to 7.4 with NaOH, and with 1 mM $CaCl_2$ added, was used to perfuse the heart at a rate of 10ml/min for 5 minutes. Afterward, nominally Ca^{2+}-free (absence of $CaCl_2$) Tyrode's solution was used to perfuse the heart for 5 minutes. Finally the heart was superfused for 7-8 minutes using Tyrode's solution with the addition of collagenase (0.02 mg/mL; Yakult Co. Ltd, Tokyo) and protease (0.004 mg/mL; Type XIV; Sigma).

The heart was then removed from the canula and the right ventricular free wall was dissected and minced in 10mL of Tyrode's solution containing collagenase (0.5 mg/mL), protease (0.1 mg/mL), bovine serum albumin (BSA; 2.5 mg/mL; Sigma) and $CaCl_2$ (50 μM). The minced right ventricle was gently agitated in a shaker bath at 37 º C for the rest of the isolation procedure. At 10 minutes, 5 mL of the digestion solution was removed and decanted. When dissociated cardiac myocytes were observed, aliquots were removed at 3 min intervals and stored in 3 mL of modified Kraft-Brühe **(**KB) solution. Modified KB solution was used as the medium to store cells, and was pH adjusted to 7.2

with KOH containing (in mM): potassium glutamate 100; potassium aspartate 10; KCl 25; glucose 20; KH_2PO_4 10; Hepes 5; $MgSO_4$ 2; taurine 20; creatine 5; and EGTA 0.5; with 1mg/mL BSA.

Unloaded cell shortening

A grass SD9 stimulator was used at a rate of 1 Hz to field stimulate cardiac myocytes to induce contractions. An edge detection device (Crescent Electronics, USA) was used to monitor the video signal received from the microscope. Output from the edge detection device was acquired and calibrated for cell length using a pClamp 9.0 / Digidata 1320 data acquisition system. Cells were allowed to contract in normal buffer for 5 minutes to stabilize before making recordings.

Perforated patch clamp

A microprocessor-controlled multiple stage puller (model P97, Sutter Instruments) was used to pull borosilicate glass electrodes and a microscope with an attached heated filament (MF-200 micro-forge (World Precision Instruments) was used to polish the electrodes. A stock solution of Amphotericin B (Sigma) was made by adding 3 mg of Amphotericin into 30 uL DMSO (0.108 M). An internal solution pH-adjusted to 7.2 with KOH was used to fill the pipettes (mM): KCl 20; K-Aspartate (L-Aspartic Acid) 110, EGTA 10, HEPES 10, $MgCl_2$ 1, K_2ATP 5, $CaCl_2$ 1, NaCl 10.

Cells were placed onto the cover slip of a superfusion chamber located on the stage of the inverted microscope (Nikon TE300) and allowed to settle for 10 minutes. Under control conditions, they were then superfused with standard Tyrode's solution containing 1 mM $CaCl_2$. Prior to experimentation, 2.0 uL of stock amphotericin was added to 500 uL of internal solution and sonicated briefly to prepare an internal amphotericin solution. This solution was kept in a syringe protected from light and remade every hour. The tip of the glass electrodes were dipped in amphotericin-free internal solution to enhance seal formation and back filled with amphotericin-containing internal solution. When filled with internal solution, pipette resistances ranged from 1 to 3 MΩ. Recording glass electrodes were lowered to the surface of the myocytes by fine manipulation until electrodes were touching the myocytes and pipette resistance was observed to increase. Suction was then applied on the cells to create a high resistance seal (>2 GΩ). During the process of sealing, cells were voltage clamped at a holding potential of -80 mV. After seal formation, the glass pipette was not moved until the pipette access resistance decreased to below 20 MΩ, and cells that did not reach below 20 MΩ were discarded. After the access resistance reached below 20 MΩ, cells were gently lifted off the plate to allow the myocytes to contract freely.

Action potential recordings

Action potentials were recorded using the perforated patch clamp technique already described. Action potentials were elicited under current clamp at a rate of 1 Hz, using a 5ms, 700pA current injection.

L-Type Ca^{2+} current recordings

L-Type Ca^{2+} current (I_{CaL}) recordings were made under voltage-clamp using the perforated patch technique described. Na^{+} current was inhibited using a combination of lidocaine, and voltage inactivation. I_{CaL} was elicited at a rate of 0.1 Hz from a holding potential of -80 mV with a 1s ramp to -40 mV (to voltage inactivate Na^{+} currents) followed by a 300 ms step to 0 mV to activate $I_{Ca,L}$. The internal pipette solution used to measure current contained (in mM): CsOH 110; aspartate free acid 110; CsCl 10; $MgCl_2$ 1; NaCl 10; HEPES 10; MgATP 4, EGTA 1; pH adjusted to 7.2 using CsOH. The superfusate used as the external bath solution contained (in mM): NaCl 10; CsCl 3; $CaCl_2$ 1; KCl 5.4; Na_2HPO_4 1; HEPES 5; Glucose 10; $MgCl_2$ 1; lidocaine 250 μM; pH adjusted to 7.4 with CsOH. Currents are expressed relative to cell capacitance and reported as current density (pA/pF).

Intracellular Ca^{2+} transient recordings

Cardiac myocytes were incubated with 2.5-3.0 μM Fluo-3-AM (Invitrogen/Molecular Probes, Carlsbad, CA) in 1ml of Ca^{2+} free Tyrode's solution. Following 30 minutes, the cells were superfused with standard Tyrode's solution containing 1mM Ca^{2+} to remove extracellular dye. An oil immersion 100X objective (Nikon) and type A oil (Cargille Laboratories, Cedar Grove, NJ) were used to visualize

and record fluorescence. Cardiac myocytes were excited at 480 nm using a Model 1600 Power Supply (Opti Quip), a model 720 lamp house with 150 W Xenon bulb, and a DX-100 optical switch (Solamere Tech Group). A microfluorimeter was used to record emitted fluorescence at 510 nm and the data was inputted into pClamp 9.0/digidata 1320 data acquisition system. Cardiac myocytes were stimulated to contract using a Grass SD9 field stimulator at a rate of 1 Hz. Before making recordings, cells were allowed to equilabrate in normal buffer for at least 5 minutes.

Caffeine pulse experiments

Prior to experimentation, isolated rat myocytes loaded with fluo-3 as described above. Cells were then plated onto a cover slip and field at a rate of 1 Hz for 5 minutes so that cells could equilibrate and attain a stable level of Ca^{2+} flux before recordings were made. To trigger a caffeine-induced Ca^{2+} transient, the stimulator was turned off and the Tyrode's solution superfusion stopped. Using a perfusion bypass system, unrestricted flow of Tyrode's solution containing 100 mM caffeine was superfused onto plated cells. Fluorescence was recorded until the signal returned to baseline levels and became stable. The caffeine superfusion was then stopped and normal superfusion including electrical stimulation resumed. After 10 minutes of superfusion the process was repeated and a second caffeine pulse was elicited and recorded. Fluorescence measurements were recorded using a pClamp 9.0 / Digidata 1320 data acquisition system. All treatments occurred following the first application of caffeine to allow each myocyte to serve as its own control. Data for SR Ca^{2+} content was taken as area under the fluorescence curve

and expressed as fractional fluorescence, which is percent fluorescence with respect to the caffeine pulse elicited at t=0.

Statistical Analysis

All statistical analysis was done using GraphPad Prism 4.0 software. As most data sets reflect small sample size and the data were transformed for analysis, non-parametric analysis was utilized. When appropriate, data were analyzed using the Kruskal-Wallis test (non-parametric ANOV) followed by Dunns test for post-hoc analysis. When only two groups were to be compared, the Mann-Whitney test was used. Data Data were analyzed with a confidence interval of 95% and considered significantly different if $p < 0.05$.

CHAPTER 3

The global goal of this project was to further characterize the underlying mechanisms by which H_2O_2 mediates both changes in contractile function and action potential waveform. Specifically, we are interested in the ability of H_2O_2 to induce afterdepolarizations, positive inotropy, and contractility in the presence of L-type Ca^{2+} channel blockers. Our methods of investigation included unloaded cell shortening, intracellular Ca^{2+} transient fluorescence, caffeine-induced Ca^{2+} fluorescence, action potential recordings, and L-type Ca^{2+} current recordings. Right ventricular cardiac myocytes were used for all of the studies as the right ventricle is considered a homogeneous cell population.

Effects of H_2O_2 on action potential waveforms

To confirm previous reports of H_2O_2-induced action potential prolongation, we recorded right ventricular action potential waveforms in the absence and presence of H_2O_2. Figure 1 illustrates representative traces to demonstrate the concentration-dependent effects of H_2O_2 on action potential waveforms. Both concentrations examined prolonged the action potential waveform yet their effects are dramatically different. At 100 μM H_2O_2, APD90 (the time it takes for a myocyte to repolarize by 90%) is significantly increased at all time points after t=6 minutes, compared to control recordings (Figure 2). APD50 (the time it takes for a myocyte to repolarize by 50%) is similarly increased, and is significantly higher than control at t=10 minutes (Figure 2). However, at 200 μM H_2O_2, prolongation also occurs, but is much more dramatic. Under

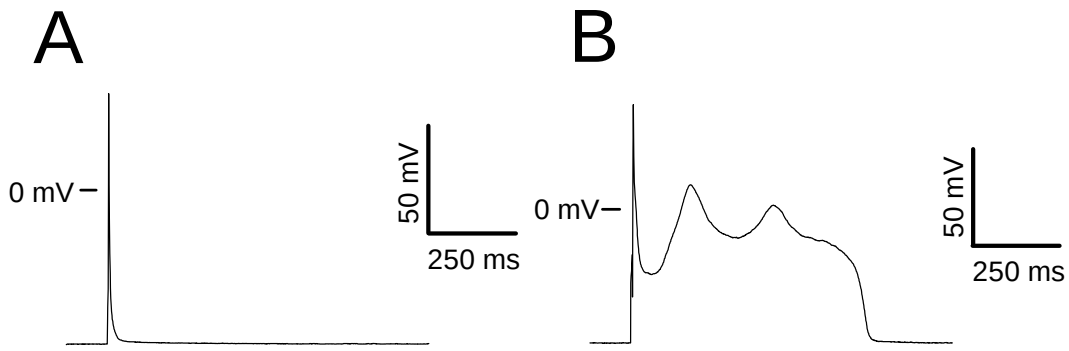

Figure 1. H_2O_2-induced delayed afterdepolarizations. Representative action potential traces showing action potentials recorded in the presence of 100 μM H_2O_2 (A), and 200 μM H_2O_2 (B).

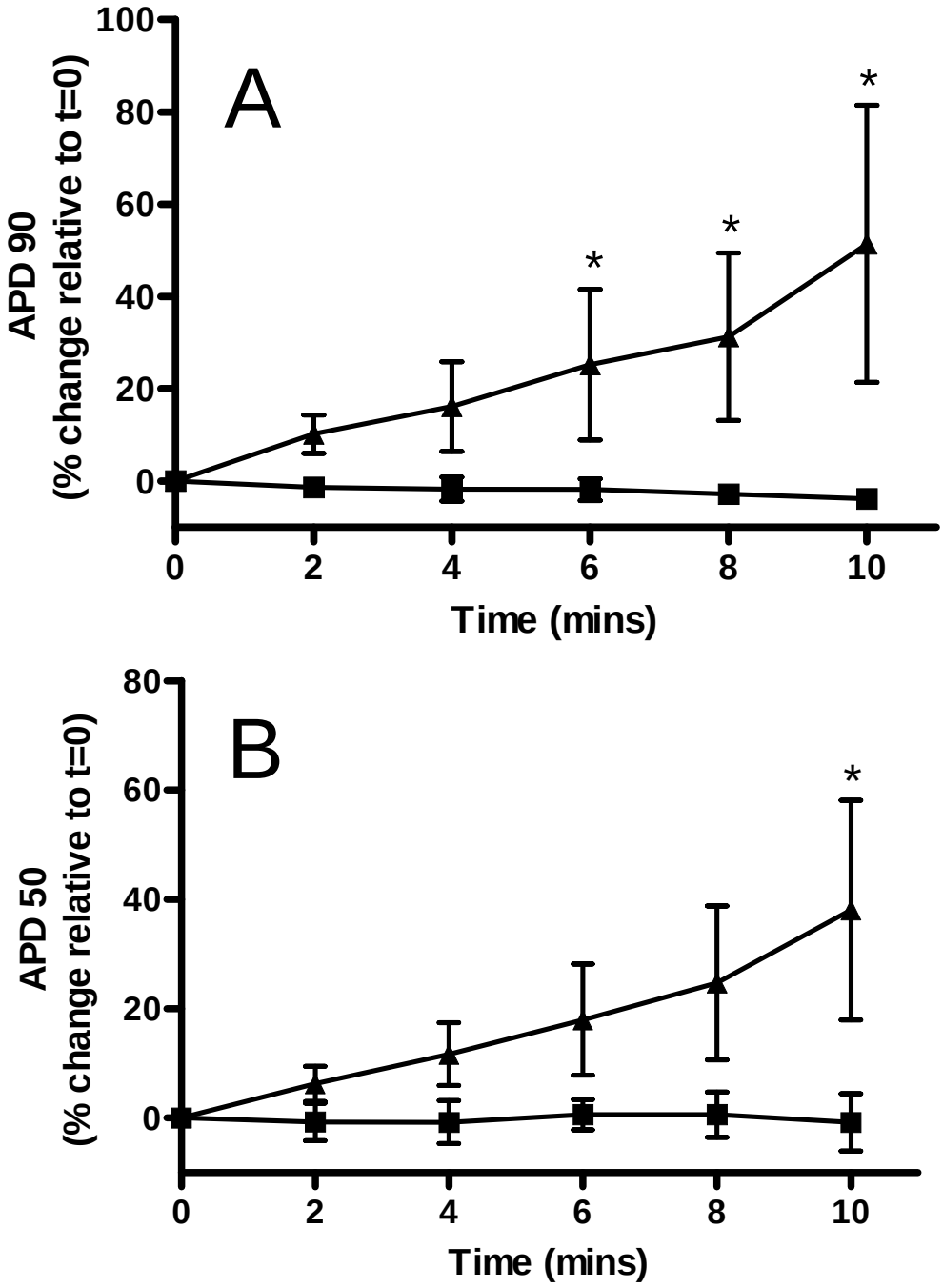

Figure 2. Effect of H_2O_2 on APD90 and APD50. APD90 of myocytes superfused with 100 µM H_2O_2 (▲; n=5) were compared to control cardiac myocytes (■; n=6) in part A. In part B, the experiment was repeated and the comparison made using APD50 (▲; n=5, ■; n=6). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

these conditions, 10 minutes superfusion with H_2O_2 increased APD90 values by 3400 ± 1400% (n=4) relative to pre-treatment values (Figure 3). Furthermore, this value is an underestimation, as in some recordings the action potential did not repolarize prior to the next stimulation and was assigned a maximum time value of 1 second.

In the presence of 200 μM H_2O_2, APD90's dramatically increased and were consistently characterized by afterdepolarizations (Figure 4). Afterdepolarizations occurred at distinct time intervals and prior to repolarization, corresponding to the re-opening of calcium channels during the formation of early afterdepolarization (Marban et al, 1986; January and Riddle, 1989). Consequently, these afterdepolarizations are also associated with aftercontractions and multiphasic intracellular calcium transients as represented in Figure 4.

Effects of H_2O_2 on contractility and Ca^{2+} transients

It has previously been shown that H_2O_2 has marked electrophysiological and contractile effects on rat ventricular myocytes (Ward & Giles, 1997). Positive inotropy is evident in our recordings of unloaded cell shortening. It can be seen in Figure 5 that following 10 minutes of superfusion with 100 μM H_2O_2, unloaded cell shortening was increased by 80 ± 20% (n=6). This was significantly different from the control group, which demonstrated a slight decrease in unloaded cell shortening by 20 ± 10% (n=5) over the same time period. The time course of the H_2O_2-induced positive inotropic effect is rapid with H_2O_2 significantly increasing unloaded cell shortening at all time points after two minutes of superfusion (Figure 5).

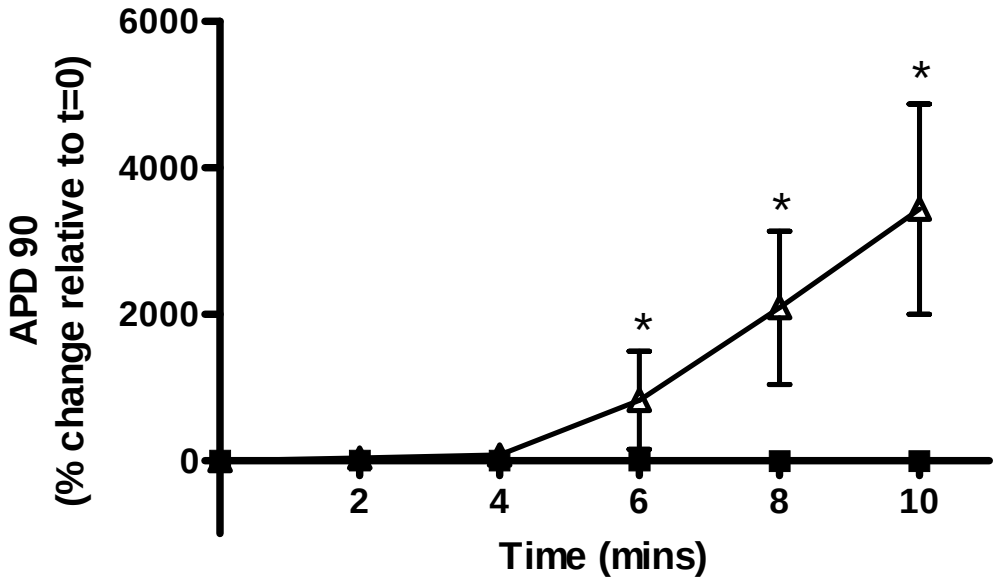

Figure 3. Effect of 200 µM H_2O_2 on APD90. Ventricular myocytes were superfused with 200uM H_2O_2 (△; n=4) and compared to control cardiac myocytes (■; n=6) in part. Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

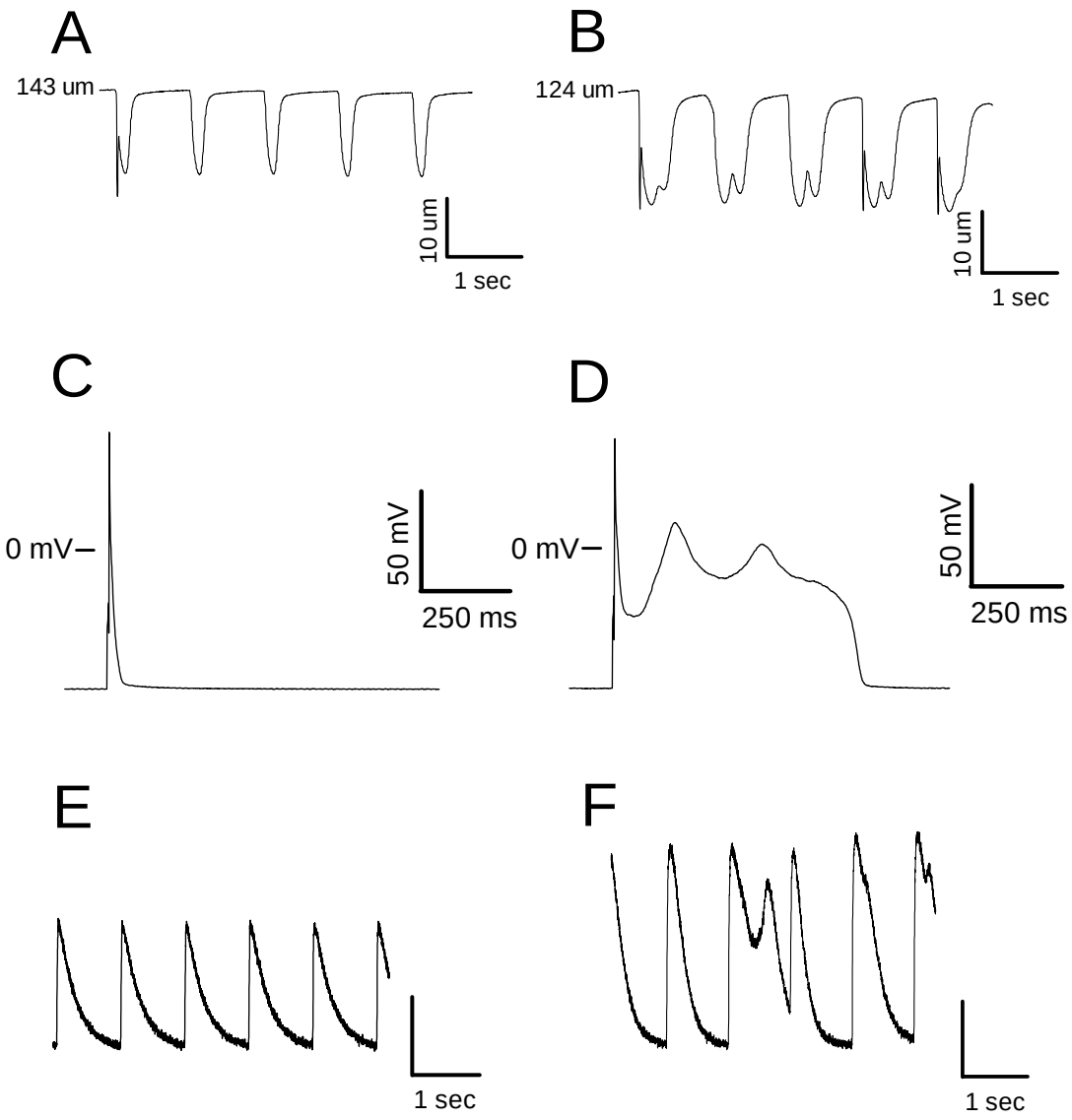

Figure 4. H_2O_2-induced delayed afterdepolarizations. Representative traces showing unloaded cell shortening recorded in the absence of H_2O_2 (A), and after 10 minutes of superfusion with H_2O_2 (B). Representative traces showing action potentials recorded in the absence of H_2O_2 (C) and after 10 minutes of superfusion with H_2O_2 (D). Representative traces showing intracellular Ca^{2+} transients recorded in the absence of H_2O_2 (E), and after 10 minutes of superfusion with H_2O_2 (F).

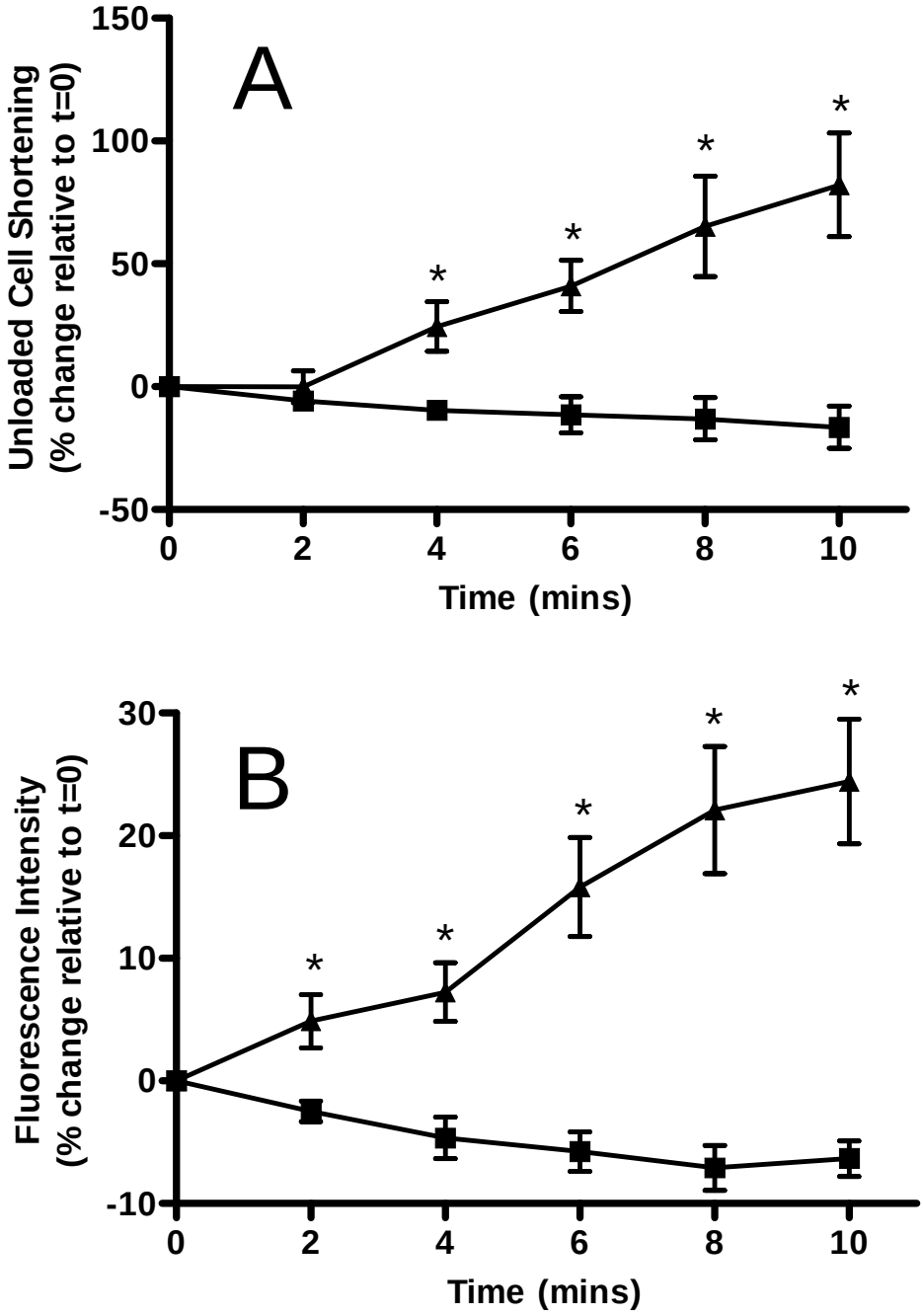

Figure 5. Effect of H_2O_2 on cell shortening and intracellular Ca^{2+} transients. Myocytes superfused with 100 μM H_2O_2 (▲; n=5) were compared to control cardiac myocytes (■; n=6) in part A. In part B, the experiment was repeated using calcium transients (▲; n=8, ■; n=6). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

The H_2O_2-induced positive inotropic effect was paralleled by increases in intracellular Ca^{2+} transients (Figure 5). Following 10 minutes exposure to 100 μM H_2O_2, peak Ca^{2+} transient amplitudes significantly increased by 24 ± 14% (n=8) relative to pretreatment values. In control myocytes, a slight decline in peak Ca^{2+} transient amplitude by 6 ± 4% (n=6) was observed over the same time period, relative to t=0 minutes after 10 minutes superfusion. The H_2O_2 induced increases in Ca^{2+} transient amplitude were significantly different from control Ca^{2+} transients for all time points including and after 2 minutes of superfusion ($p<0.05$; Figure 5).

Similiar to action potential recordings, afterdepolarizations were recorded after superfusion with H_2O_2 in unloaded cell shortening and fluorescent Ca^{2+} transients. Figure 4 shows that superfusion with 100 μM H_2O_2 elicits afterdepolarizations as confirmed by the extra-systole following the field stimulated contractions in unloaded cell shortening recordings. Afterdepolarizations developed later in the time course, sometimes after 10 minutes of superfusion. Afterdepolarizations are also present in Ca^{2+} transient recordings as indicated by the extra, smaller, Ca^{2+} transient peak following the normal rhythm of field stimulated contraction (Figure 4).

Increased diastolic Ca^{2+} fluorescence

Ca^{2+} overload is linked to the formation of delayed afterdepolarizations. While H_2O_2 was observed to cause increased peak Ca^{2+} transients during systole, it also affected baseline diastolic intracellular Ca^{2+} fluorescence, indicating possible Ca^{2+} overload.

Figure 6 shows representative data illustrating the increase in baseline that is observed during intracellular Ca^{2+} transient recordings of myocytes superfused with 100 μM H_2O_2.

Effects of Cd^{2+} on H_2O_2-induced electrophysiological changes

In order to confirm that the aftercontractions were due to repetitive L-type Ca^{2+} channel activity, select experiments were repeated in the presence of Cd^{2+}, an inorganic ion that selectively blocks this channel. Representative traces of these experiments are shown in Figure 7. As expected, the afterdepolarizations normally associated with H_2O_2 were attenuated. However, pretreatment with Cd^{2+} did not abolish the increase in APD observed with superfusion of H_2O_2. Prolongation of APD90 with superfusion of 100 μM H_2O_2, after pretreatment with Cd^{2+}, can be seen as early as t=2 minutes (Figure 8). At t=10, APD90 of the H_2O_2 group pretreated with Cd^{2+} increased significantly by 30 ± 10% compared to a minor increase of 2 ± 1% in the Cd^{2+} alone group (Figure 8, $p<0.05$). While mean APD50's also increased, as shown in Figure 8, the increases were not significant ($p<0.05$). The effect was mirrored with superfusion of 200 μM H_2O_2 in Cd^{2+} pretreated cells, as APD90 increased by 1800 ± 900% (Figure 3).

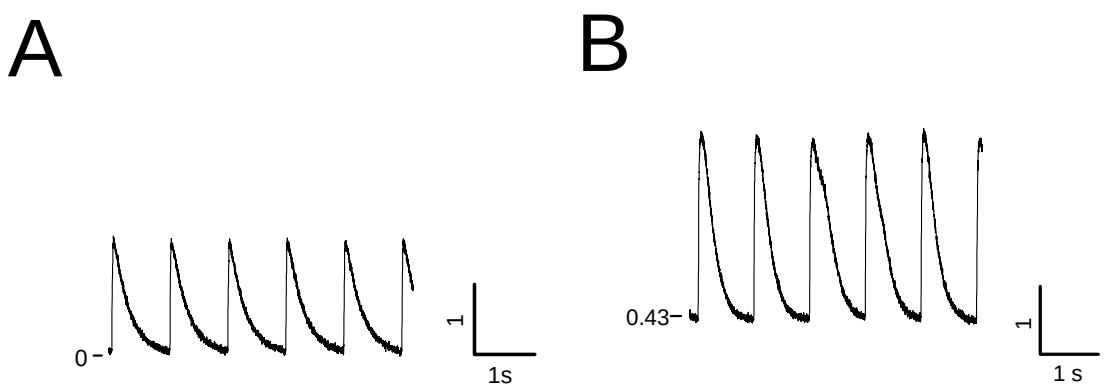

Figure 6. H_2O_2-induced increase in diastolic Ca^{2+} fluorescence. A representative tracing showing systolic and diastolic Ca^{2+} before superfusion with H_2O_2 (A), and after 10 minutes of superfusion with 100 μM H_2O_2 (B). Diastolic Ca^{2+} fluorescence is represented by troughs after each peak.

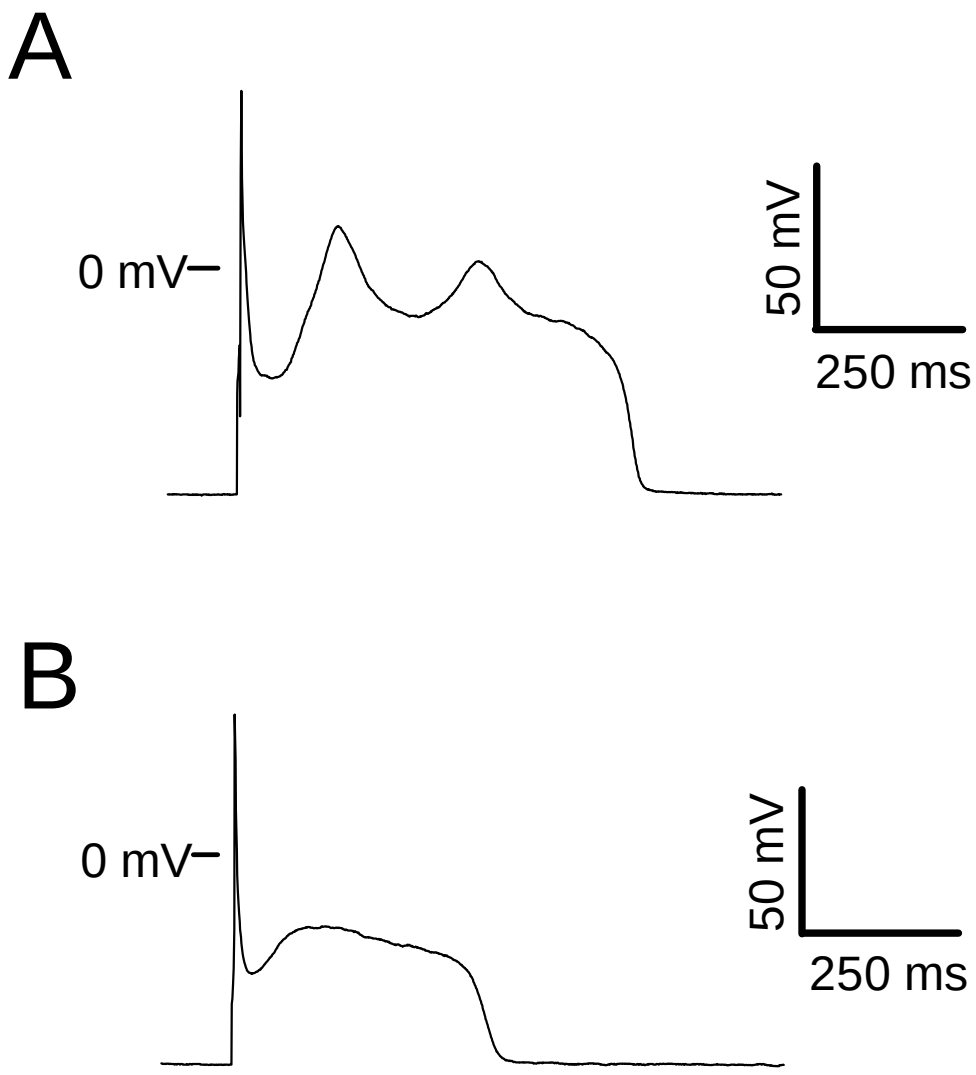

Figure 7. Effect of H_2O_2 on action potentials in the presence of Cd^{2+}. A representative action potential recording in the presence of 200 μM H_2O_2 (A), and another after Cd^{2+} pretreatment and continued superfusion (B).

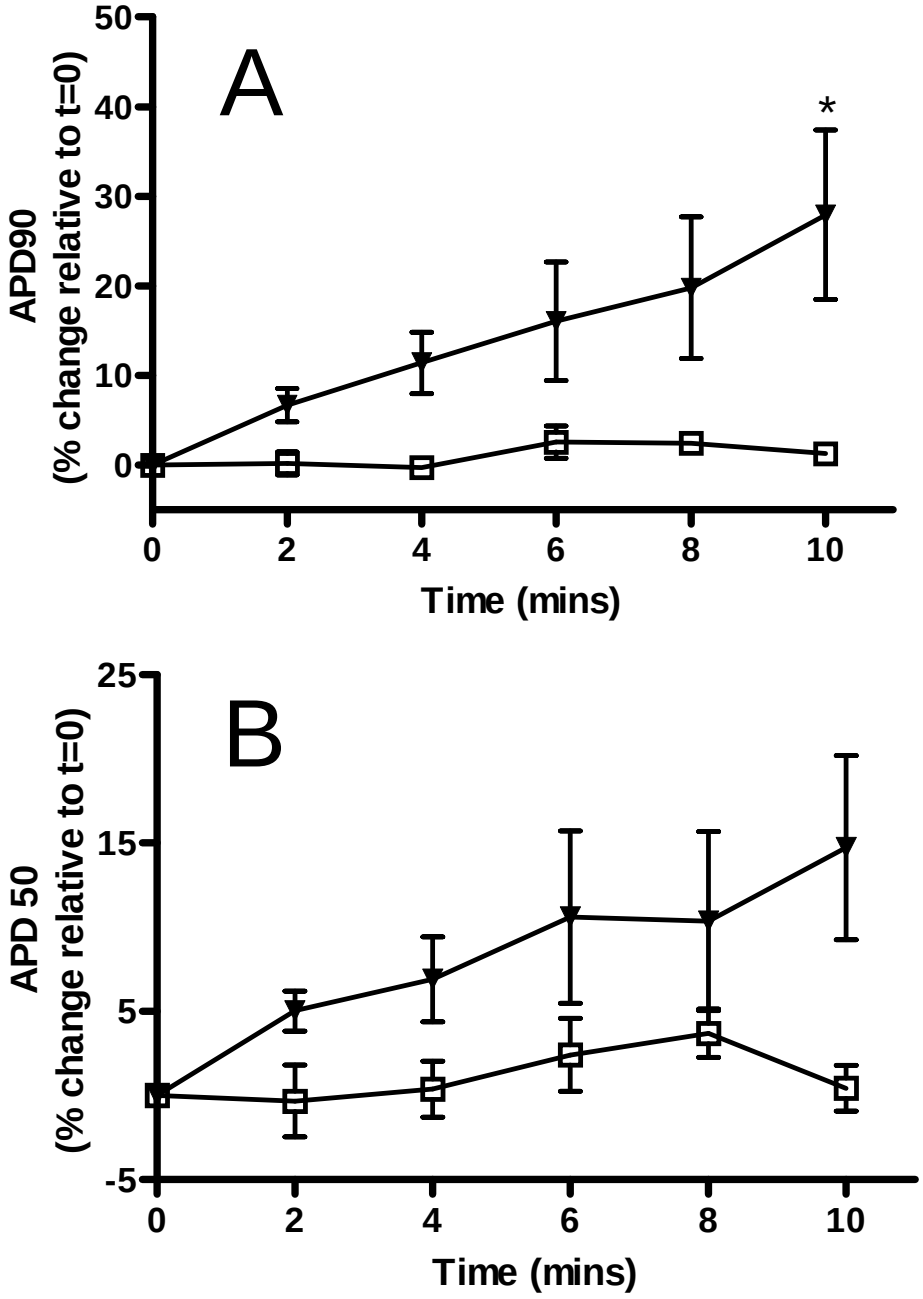

Figure 8. Effect of H_2O_2 on APD90 and APD50 of Cd^{2+} pretreated myocytes. In part A, APD90 of myocytes pretreated with Cd^{2+} and superfused with 100 μM H_2O_2 (▼; n=5) were compared to Cd^{2+} alone (□; n=5). In part B, the experiment was repeated and the comparison made using APD50 (▼; n=5, □; n=5). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

Effects of Cd^{2+} pretreatment on contractility

The purpose of this set of experiments was to further study the effects of Cd^{2+} on H_2O_2-induced electrophysiological changes. Figure 9 illustrates the consequences of Cd^{2+} block of unloaded cell shortening in comparison to vehicle superfused myocytes. In the control (vehicle) group, after 10 minutes of superfusion, unloaded cell shortening was measured at 80 ± 10% (n=5) of t=0 minute values. However, in the presence of Cd^{2+} unloaded cell shortening was significantly inhibited to 4 ± 2% (n=5; $p<0.05$) of pretreatment values. This effect is paralleled in Ca^{2+} transient recordings, as increases of intracellular Ca^{2+} transient were also blocked by Cd^{2+} (Figure 9). Following 10 minutes superfusion, peak intracellular Ca^{2+} transient amplitude in the Cd^{2+} group decreased to 4 ± 2% (n=5) of pretreatment values, which was significantly less in comparison to the control group (90 ± 10%; n=6; $p<0.05$).

Interestingly, with superfusion of H_2O_2 to myocytes pretreated with Cd^{2+}, unloaded cell shortening increased despite the presence of Cd^{2+} throughout the entire experiment (Figure 10). For these experiments, myocytes were superfused with 50 μM Cd^{2+} for 5 minutes prior to addition of 100 μM H_2O_2, then continually superfused with Cd^{2+} for the remainder of the experiment to ensure continuous Cd^{2+} block of Ca^{2+} channels. Figure 11 demonstrates that, under such conditions, H_2O_2 still induced an increase of unloaded cell shortening to 15 ± 3 % (n=5) of pre-H_2O_2 values compared to the Cd^{2+} alone group (4 ± 2 %; n=9; $p<0.05$), despite the presence of the Ca^{2+} channel blocker. A similar pattern of results was observed during recording of intracellular Ca^{2+} transient amplitudes. Myocytes superfused with H_2O_2 after preconditioning with Cd^{2+} had increased peak Ca^{2+} transient fluorescence as seen in Figure 11, again despite the

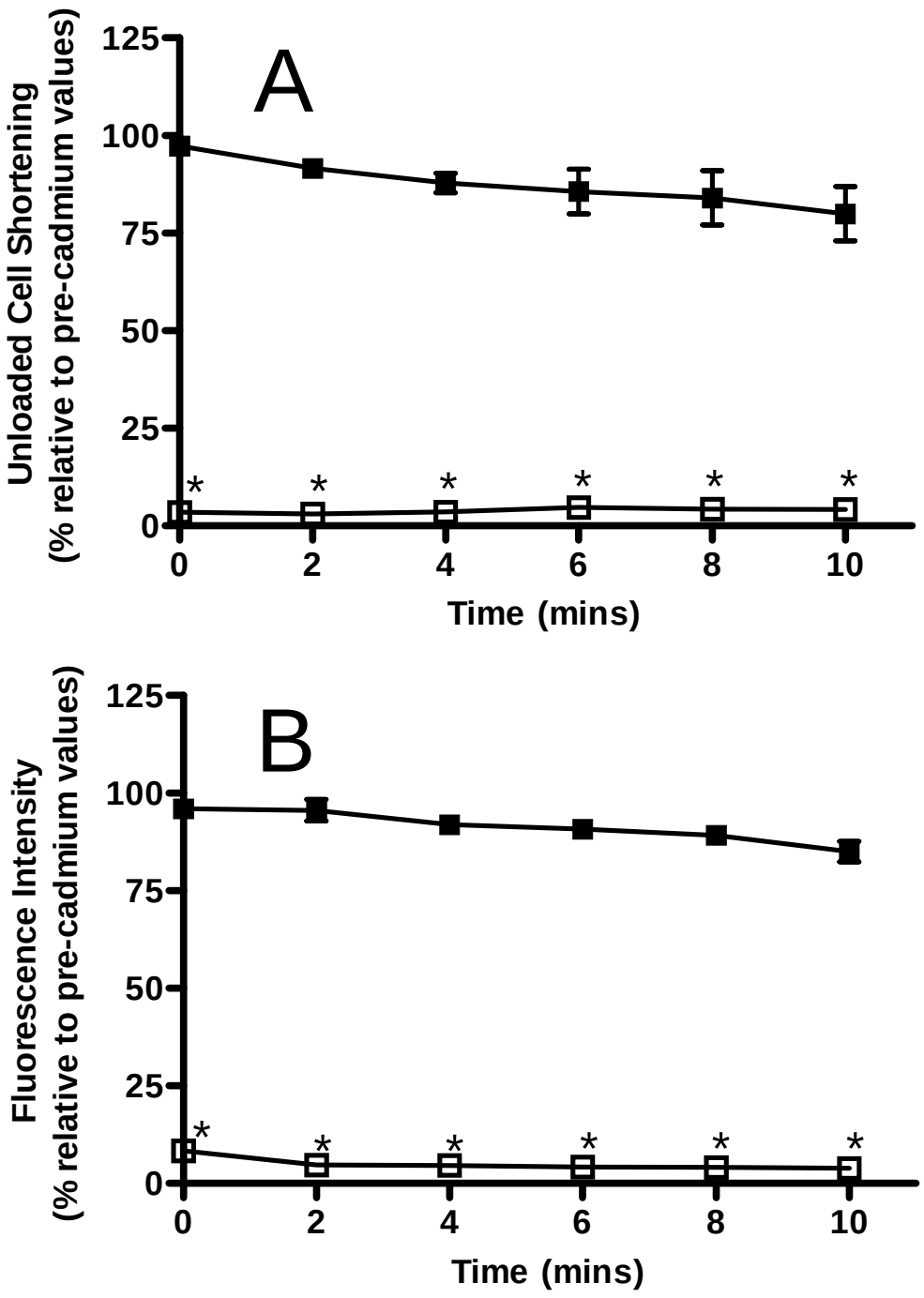

Figure 9. Effect of Cd^{2+} on cell shortening and intracellular Ca^{2+}. In part A, myocytes were superfused with either 50 μM Cd^{2+} (□; n=5) or vehicle (■; n=5). The experiment was repeated in part B using calcium transient recordings (□; n=6, ■; n=5). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

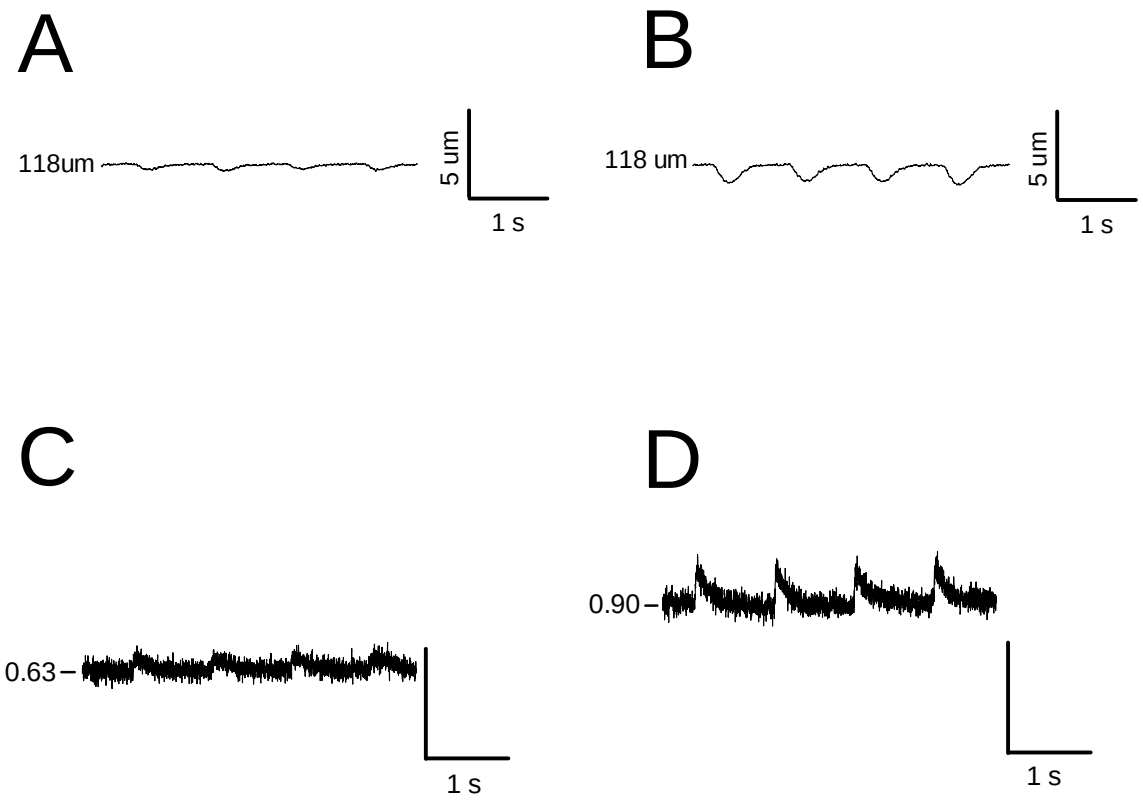

Figure 10. Representative cell shortening and intracellular Ca^{2+} in Cd^{2+}-pretreated myocytes in the presence and absence of H_2O_2. Representative unloaded cell shortening tracings of a myocyte in the presence of Cd^{2+} (A), and the same cell after 10 minutes of perfusion with both Cd^{2+} and H_2O_2 (B). Representative calcium transients tracings of a myocyte in the presence of Cd^{2+} (C), and the same cell after 10 minutes of perfusion with both Cd^{2+} and H_2O_2 (D).

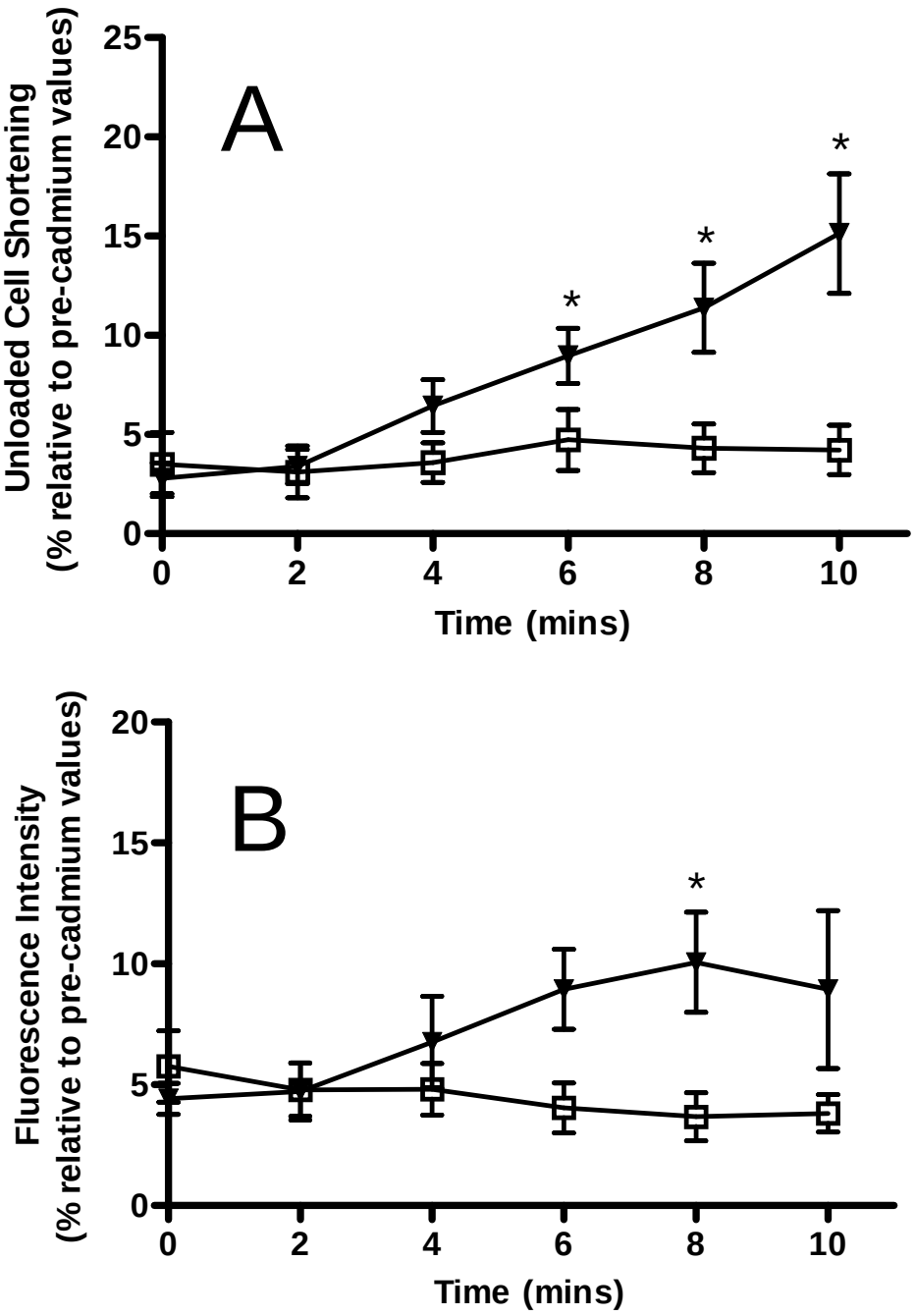

Figure 11. Effect of H_2O_2 on cell shortening and intracellular Ca^{2+} in Cd^{2+}-pretreated myocytes. In part A, Cd^{2+} pretreated myocytes were superfused with 100 μM H_2O_2 (▼; n=9) or Cd^{2+} alone (□; n=4). The experiment was repeated in part B using calcium transient recordings (▼; n=5, □; n=8). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

presence of Cd^{2+} throughout the entire time course of the experiment. By 8 minutes of H_2O_2 exposure, these Ca^{2+} transients were significantly increased to 6 ± 3 % (n=4) in contrast to the slight decrease of 2 ± 1 % (n=8) in the absence of H_2O_2 ($p<0.05$).

Effects of H_2O_2 on L-type Ca^{2+} conductance

One possible explanation for the increase of cell shortening and calcium transients despite Cd^{2+} block is that H_2O_2 compromised the ability of Cd^{2+} to block L-type Ca^{2+} channels. To test this, we used Ca^{2+} current recordings of myocytes in the conditions in which we characterized the increase of cell shortening and calcium transients. A comparison between the same myocytes before and after the addition of 50 μM Cd^{2+} shows that pretreatment and subsequent superfusion with Cd^{2+} successfully blocks I_{CaL} as shown in Figure 12. With the addition of H_2O_2, there is no significant increase in the amount of inward current at or around a voltage of 0mV, where peak inward current occurs (Figure 13). However, inward current did significantly increase at a stimulation voltage of 20mV.

In control recordings of Ca^{2+} current, peak inward current occurred at a voltage of 0 mV and were stable over the entire 10 minutes of control superfusion (Figure 14). In the presence of H_2O_2, however, slight but significant increases in inward current at -20 mV and -10 mV were observed (Figure 15). At -20 mV, inward current increased to -6 ± 1 pA/pF (n=4) which significantly differed from control values of -3 ± 1 pA/pF (n=4; $p<0.05$). Furthermore, peak amplitude of inward current occurred at -10 mV in the H_2O_2 superfused group instead of at 0 mV and peaked at -10 ± 2 pA/pF (n=4), while -10 mV

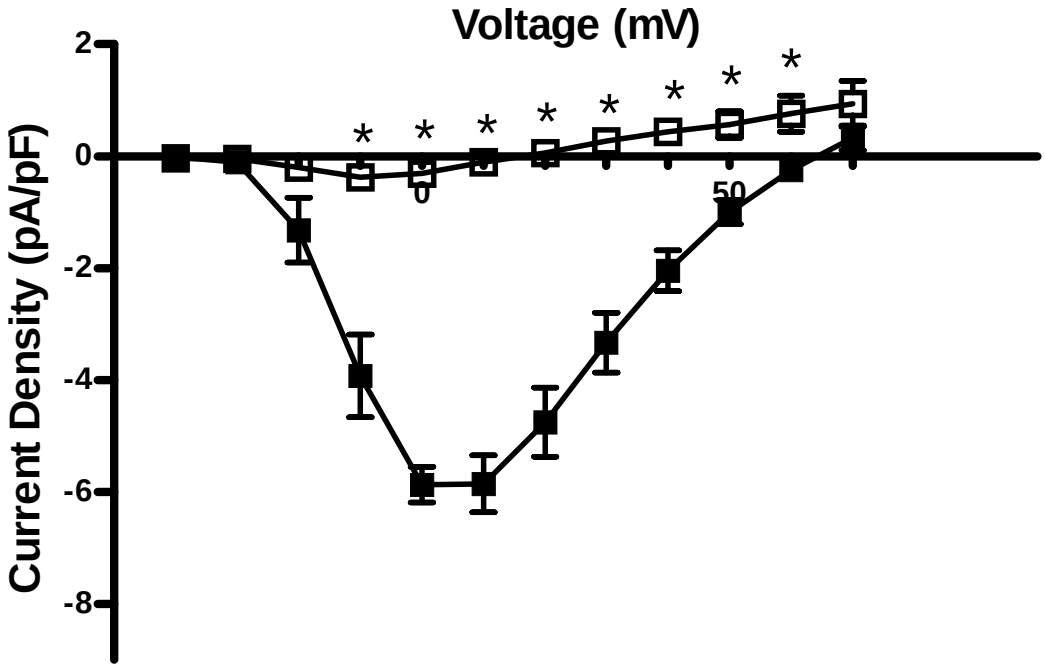

Figure 12. Effect of Cd^{2+} on L-type Ca^{2+} currents. Current-voltage relationships were recorded at t=0 prior to Cd^{2+} superfusion (■; n=5) and again following superfusion of Cd^{2+} at t=10 minutes (□; n=5). Data are expressed as current density (pA/pF) and represent the mean ± standard error of the mean. (*) indicates data significantly different from recordings prior to Cd^{2+} superfusion.

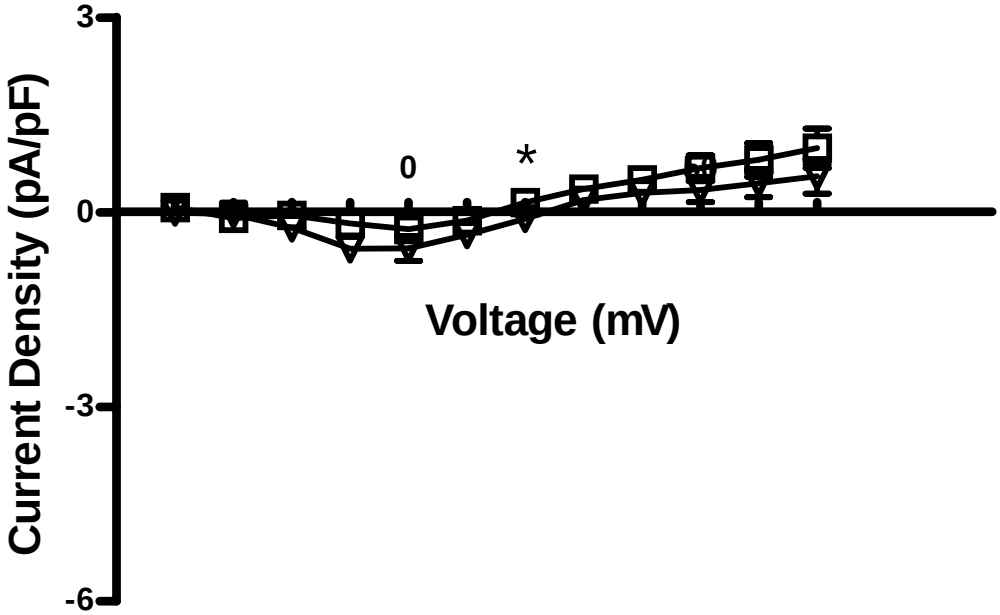

Figure 13. Effect of H_2O_2 on L-type Ca^{2+} currents of Cd^{2+}-pretreated myocytes. Current-voltage relationships of Cd^{2+}-pretreated myocytes were recorded at t=0 prior to superfusion of 200 μM H_2O_2 (□; n=4) and again following superfusion of H_2O_2 at t=10 (▽; n=4). Data are expressed as current density (pA/pF) and represent the mean ± standard error of the mean. (*) indicates data significantly different from recordings of Cd^{2+}-pretreated myocytes prior to superfusion of H_2O_2.

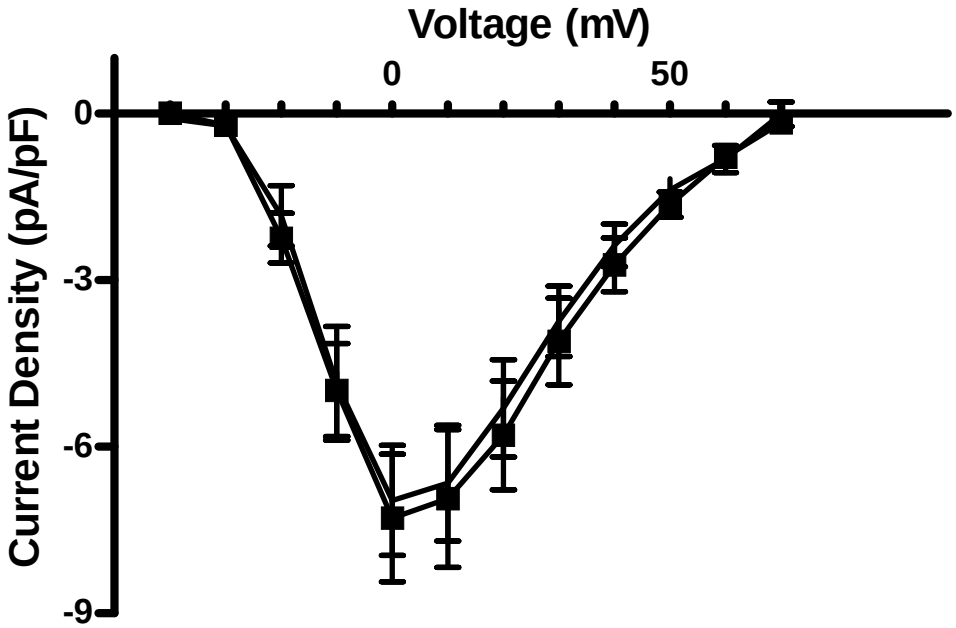

Figure 14. L-type Ca^{2+} currents of control myocytes at t=0 and t=10. Current-voltage relationships of control myocytes were recorded at t=0 (| ; n=7) and again following superfusion at t=10 (n; n=7). Data are expressed as current density (pA/pF) and represent the mean ± standard error of the mean.

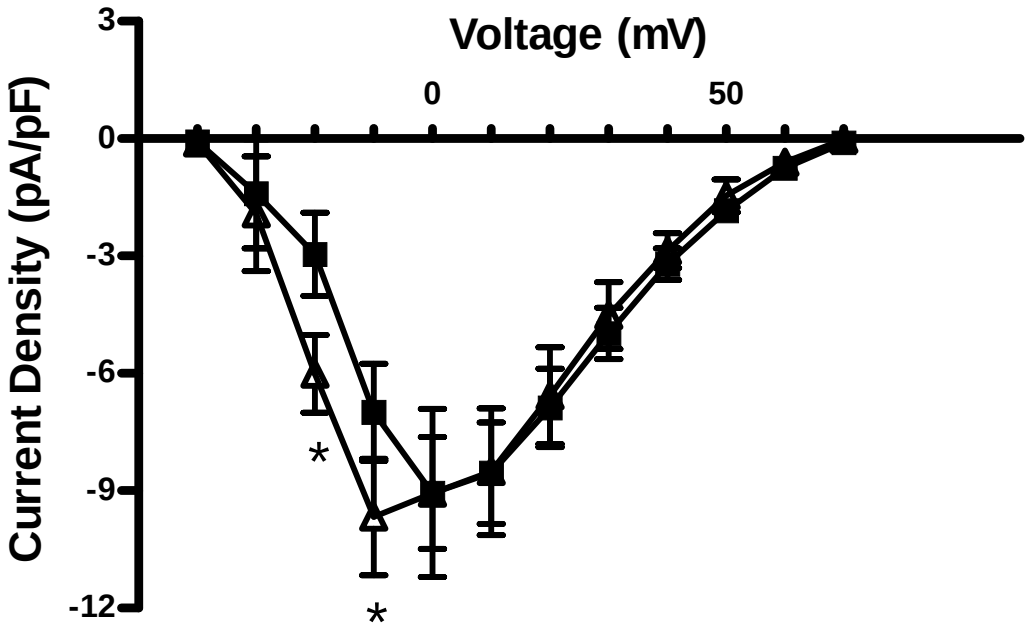

Figure 15. Effect of H_2O_2 on L-type Ca^{2+} currents currents. Current-voltage relationships were recorded at t=0 prior to superfusion of 200 μM H_2O_2 (■; n=4) and again following superfusion of H_2O_2 for 10 minutes (△; n=4). Data are expressed as current density (pA/pF) and represent the mean ± standard error of the mean. (*) indicates data significantly different from recordings at t=0 prior to superfusion of H_2O_2.

control current was significantly less at -7 ± 2 pA/pF (n=4; $p<0.05$).

Effects of H_2O_2 on SR load

The positive inotropic effects of H_2O_2 in the presence of Cd^{2+} block could be a result of H_2O_2 directly affecting SR calcium handling. To investigate this possibility, we tested whether H_2O_2 affects SR Ca^{2+} load, using a rapid solution exchanger and control buffer containing 100mM caffeine. As can be seen in Figure 16, time control values were measured at 100 ± 10% (n=6) at t=10 minutes, indicating that after 10 minutes SR load remained constant. Following superfusion with 100 μM H_2O_2 for 10 minutes, caffeine-induced calcium transients were measured at 90 ± 10% (n=7) of the control caffeine pulse. When compared to control at t=10 minutes, caffeine-induced calcium transients elicited in myocytes superfused with 100 μM H_2O_2 were not significantly different ($p<0.05$), suggesting that H_2O_2 did not affect SR Ca^{2+} load.

10 μM KB-R7943 blocks H_2O_2-induced effects

Reverse-mode action of the NCX has previously been shown to occur (Litwin *et al* 1998; Goldhaber *et al*, 1999) and has been postulated to be a possible mechanism through which extracellular Ca^{2+} can enter cardiac myocytes independent of L-type Ca^{2+} channels. To study this potential mechanism, KB-R7943 was used as a putatively selective blocker of the reverse-mode NCX. We hypothesized that use of KB-R7943 would abolish H_2O_2-induced increases in contractility in the presence of Ca^{2+}. Indeed,

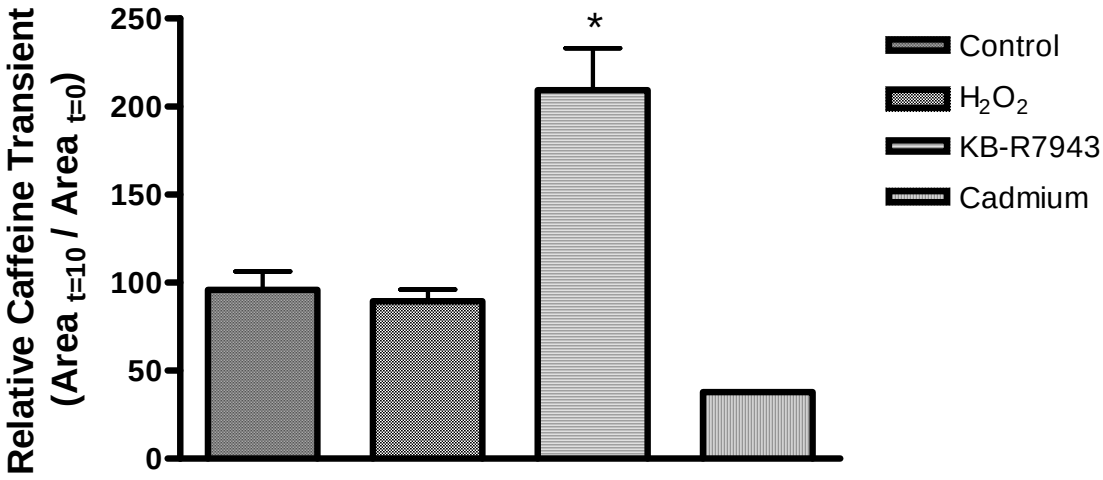

Figure 16. SR calcium load. Transient unloading of the SR was elicited using 100mM caffeine under the conditions listed in the table legend. Data are represented as the percentage of the area under the caffeine-induced Ca^{2+} transient at t=10 compared to the same myocyte at t=0. Control (n=6), 100 μM H_2O_2 (n=7), KB-R7943 10 μM (n=5), Cd^{2+} 50 μM (n=2). Data represent the mean ± standard error of the mean and (*) indicates data significantly different from control ($p<0.05$).

10 μM KB-R7943 blocked H_2O_2-induced increases in unloaded cell shortening in the presence of Cd^{2+} as shown in Figure 17. In Cd^{2+} pretreated cells, at t=10 minutes, H_2O_2-induced increases of unloaded cell shortening to 15 ± 3% (n=9; $p<0.05$) in the absence of KB-R7943, which was significantly different from Cd^{2+} alone group which measured at 4 ± 2% (n=5) (Figure 11). However, when 10 μM KB-R7943 was added to the superfusion solution, it inhibited H_2O_2-induced increases in contraction of Cd^{2+} pretreated cells as seen in Figure 17. In the presence of KB-R7943, cell shortening was significantly attenuated with values of 4 ± 1% (n=5), which is significantly decreased compared to the H_2O_2 with Cd^{2+} treatment.

The ability of KB-R7943 to block the enhancement of contraction caused by H_2O_2 in Ca^{2+} pretreated myocytes is mirrored in intracellular Ca^{2+} transient recordings. Superfusion of 10 μM KB-R7943 and H_2O_2 to Ca^{2+} pretreated myocytes caused intracellular Ca^{2+} transient amplitude to decrease by 9 ± 2% (n=5) at t=10 minutes, compared to pre-H_2O_2 values. This was significantly different from in the absence of KB-R7943 (n=6; $p<0.05$) in which intracellular calcium transients increased by 7 ± 4% (Figure 17).

Interestingly, 10 μM KB-R7943 greatly affects the ability of the cardiac myocytes to initiate action potentials. Figure 18 shows a representative action potential recorded in the presence 10 μM KB-R7943. It can be seen that, following the addition of 10 μM KB-R7943, the ability to stimulate action potentials is impaired. By increasing stimulation voltage, action potentials could potentially be re-elicited, but with continued superfusion with 10 μM KB-R7943, these action potentials were also affected. Since reverse-mode NCX function is not characterized as a significant component of the normal action

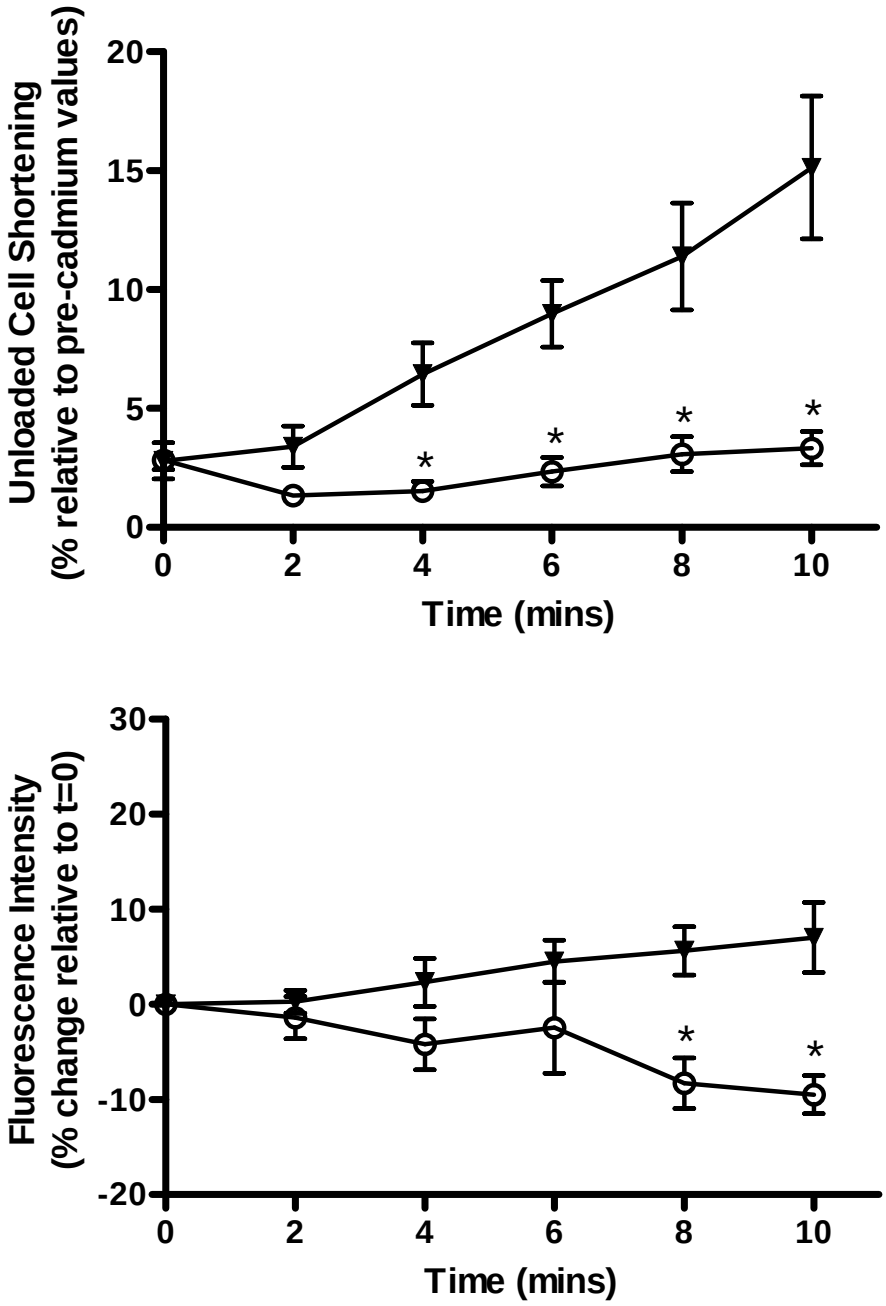

Figure 17. Effect of H_2O_2 on Cd^{2+}-pretreated myocytes in the presence of 10 µM KB-R7943. The effect on unloaded cell shortening of H_2O_2 on Cd^{2+}-pretreated myocytes in the presence of 10 µM KB-R7943 (❍; n=5) is compared to in the absence of KB-R7943 (▼; n=9) in part A. In part B, the experiment is repeated using calcium transient recordings (❍; n=5, ▼; n=4). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from Cd^{2+}-pretreated group in the absence of KB-R7943 at the same time points.

potential, these effects of KB-R7943 provide evidence that KB-R7943 may not be selectively blocking reverse-mode NCX.

The effects of KB-R7943

When 1 μM KB-R7943 alone is added into the superfusion, unloaded cell shortening is increased. Figure 19 shows that in the presence of 1 μM KB-R7943, significant increases in cell shortening occur, including and after t=6 minutes, compared to control ($p<0.05$). Following 10 minutes superfusion with KB-R7943 alone, unloaded cell shortening increased by 50 ± 20% (n=5, $p<0.05$), while in control it decreased slightly by 10 ± 10 % (n=5; $p<0.05$).

In caffeine pulse experiments, KB-R7943 at 10 μM was found to cause an increase in SR Ca^{2+} load (Figure 16). Control measurements of caffeine-induced Ca^{2+} transients, relative to t=0 minutes, were 100 ± 10% (n=6), indicating the SR load did not change after 10 minutes. However measurements of superfusion with 10 μM KB-R7943 for 10 minutes showed dramatic increases in SR load to 210 ± 30% (n=5; $p<0.05$). The results indicating that SR load doubled in the presence of 10 μM KB-R7943 provide further evidence of non-selective block of reverse-mode NCX using KB-R7943.

APD recorded in the presence of a concentration of 1 μM KB-R7943 showed significant changes, compared to control, beginning as early as 4 minutes of superfusion. While APD90 decreased slightly in control myocytes by 4 ± 2% (n=6), APD90 in the presence of 1 μM KB-R7943 significantly increased by 80 ± 10% (n=3; $p<0.05$) as seen in Figure 20. APD50 increased even more dramatically in the presence of KB-R7943 and

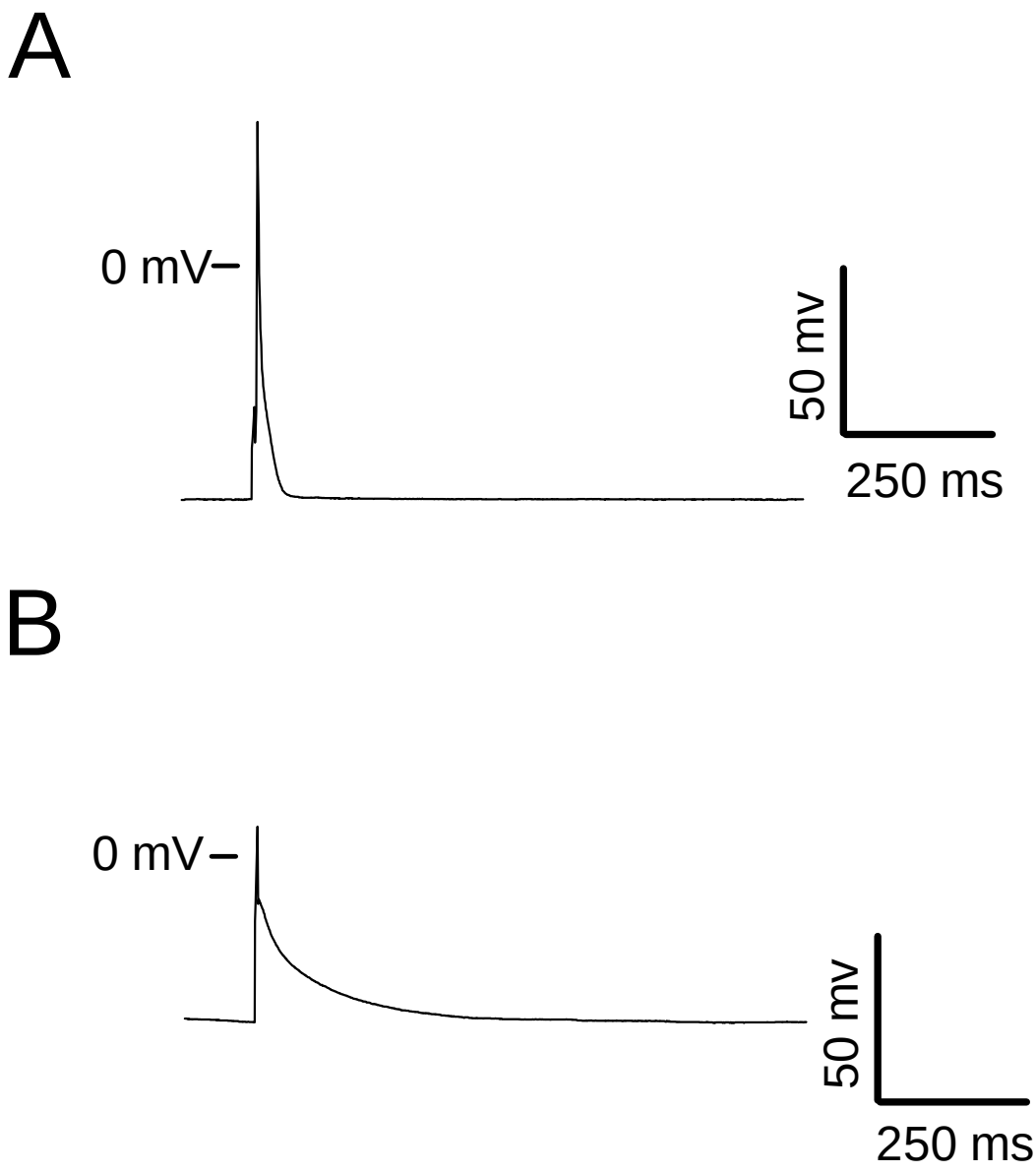

Figure 18. Action potential in the presence of KB-R7943. A representative tracing showing an action potential recorded in a myocyte before (A) and after superfusion of 10 μM KB-R7943 (B).

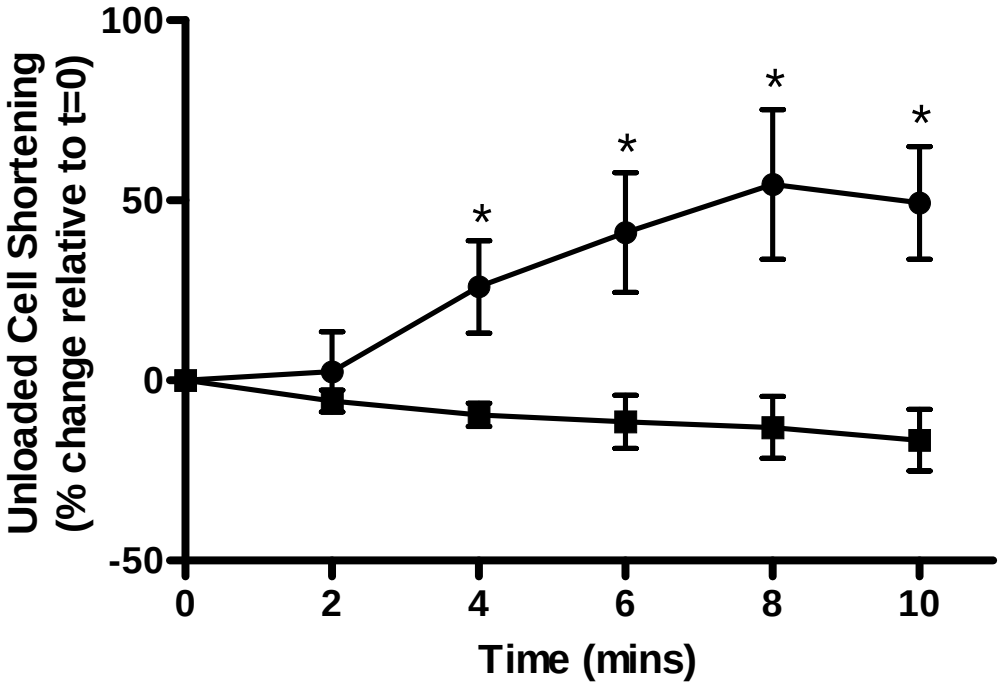

Figure 19. Effect of KB-R7943 alone on cell shortening. The effect of superfusion with 1 µM KB-R7943 (●; n=5) compared to control cardiac myocytes (■; n=5). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

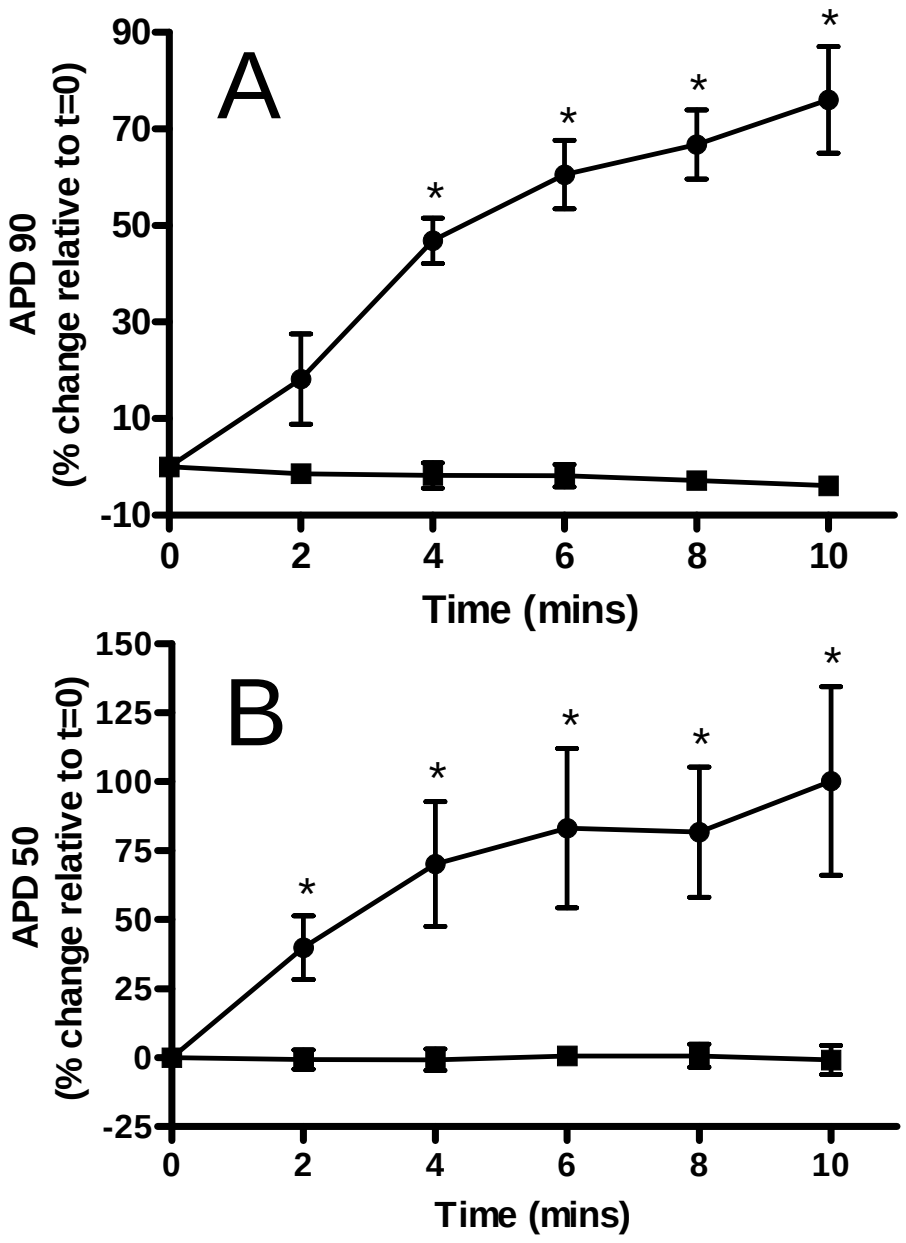

Figure 20. Effect of KB-R7943 on APD90 and APD50. Part A shows the effect of superfusion with 1 μM KB-R7943 (●; n=3) compared to control cardiac myocytes (■; n=5) on APD90. Part B shows the same experiments and the corresponding APD50s (●; n=3; ■; n=5). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control group at the same time points.

differences compared to control were found to be significant at all time points following 2 minutes (Figure 20). At t=10 minutes, control APD50 differed from t=0 by -1 ± 5% (n=5) but in the KB-R7943 group increased by 100 ± 34% (n=3; $p<0.05$). The APD prolongation effect of KB-R7943 is a possible indicator of non-selective reverse-mode block of the NCX.

Effects of lower concentrations of KB-R7943

We used 1 μM KB-R7943 in an attempt to block reverse-mode NCX while minimizing action potential degradation seen with the use of 10 μM KB-R7943. However, 1 μM KB-R7943 did not block the H_2O_2 mediated recapitulation of contraction after pretreatment with Cd^{2+}. In the presence of 1 μM KB-R7943 unloaded cell shortening significantly increased in comparison to use of 10 μM KB-R7943, as seen in Figure 21. Following 10 minutes of superfusion with both H_2O_2 and 1 μM KB-R7943 in Cd^{2+} pretreated myocytes, unloaded cell shortening increased to 16 ± 6% (n=4) at t=10 minutes. The same experiment conducted with 10 μM KB-R7943 resulted in a significantly smaller percentage of cell shortening measured at 4 ± 1% (n=5; $p<0.05$) after 10 minutes of superfusion.

At 1 μM, KB-R7943 also did not block H_2O_2-mediated positive inotropy in the absence of Cd^{2+} pretreatment. Figure 22 shows cells superfused with 1 μM KB-R7943 and 200 μM H_2O_2 significantly differed from control at all time points after t=2 minutes. Differences peaked at t=10 minutes when unloaded cell shortening decreased by 20 ± 10% (n=5) in control, but increased by 80 ± 10% (n=3; $p<0.05$) when superfused with

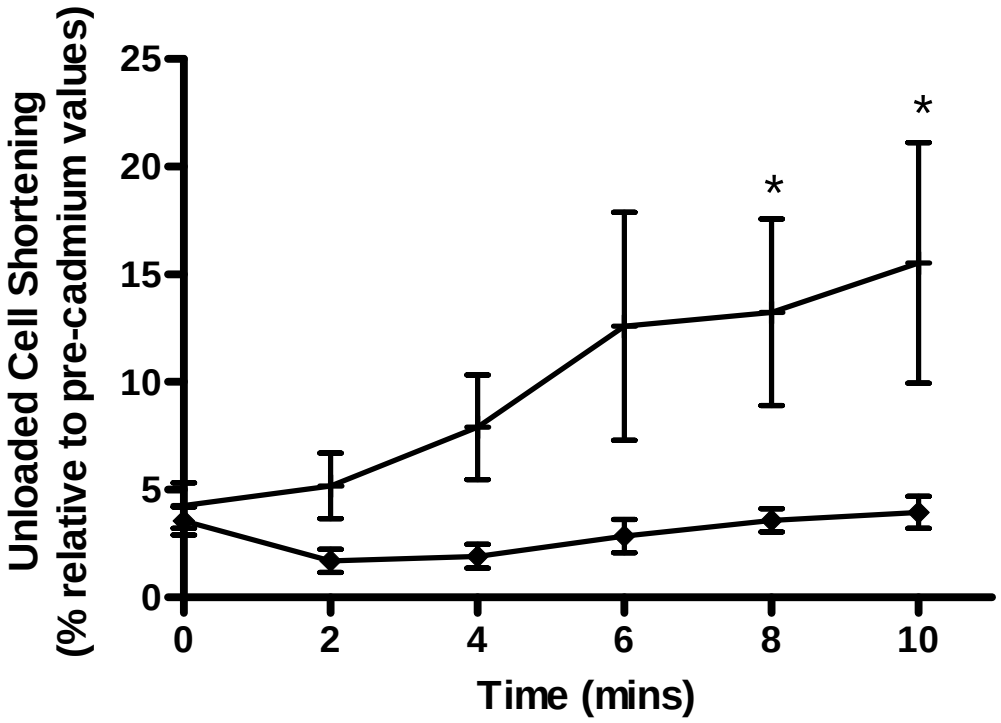

Figure 21. Ability of 1 µM KB-R7943 to inhibit increase of contraction compared to 10 µM **KB-R7943.** The effect on unloaded cell shortening of 200 µM H_2O_2 on Cd^{2+}-pretreated myocytes in the presence of 1 µM KB-R7943 (+; n=4) is compared to in 10 µM KB-R7943 (◆; n=5). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from Cd^{2+}-pretreated group in the presence of 10 µM KB-R7943 at the same time points.

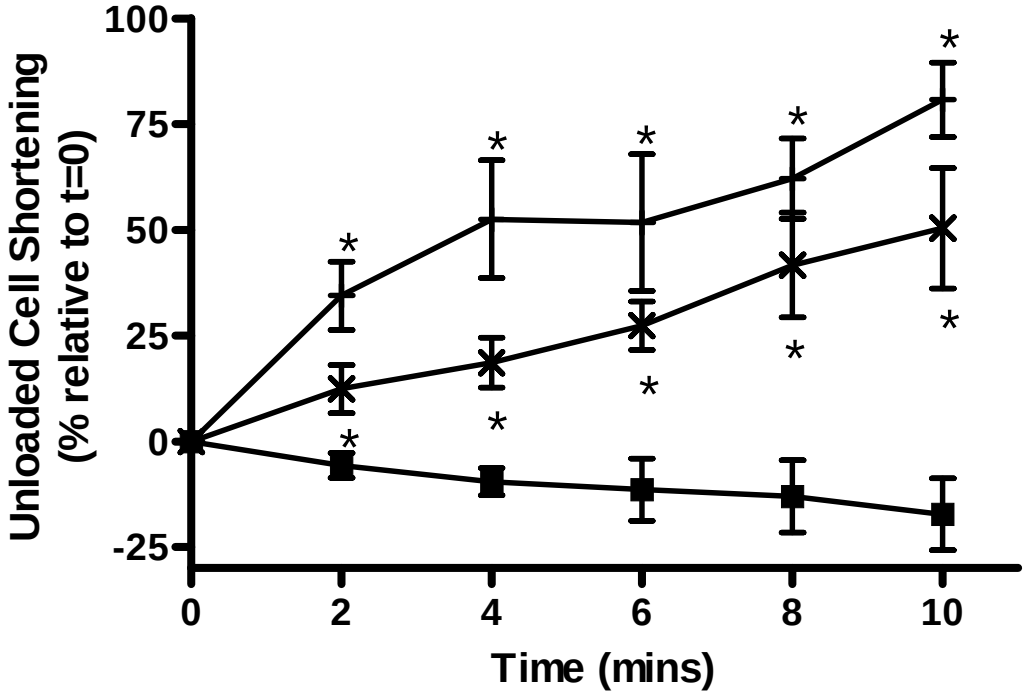

Figure 22. Ability of 1 µM and 0.1 µM KB-R7943 to inhibit H_2O_2-induced positive inotropy. The effect on unloaded cell shortening of 200 µM H_2O_2 in the presence of 1 µM KB-R7943 (+; n=3) and 0.1 µM KB-R7943 (X; n=4) is compared to control (■; n=5). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from control at the same time points.

both H_2O_2 and 1 μM KB-R7943. This effect was mirrored in recordings of cell shortening using 0.1 μM KB-R7943 and 200 μM H_2O_2 when at t=10 minutes, unloaded cell shortening increased by 50 ± 14% (n=4) and again significantly differed from control at all time points after t=2 minutes (Figure 22; $p<0.05$). Indeed, it can be seen in Figure 23 that positive inotropy in the presence of 0.1 μM and 1 μM KB-R7943 mimics that of H_2O_2 alone, and neither 1 μM KB-R7943 nor 0.1 μM KB-R7943 significantly inhibited H_2O_2 mediated positive inotropy except at t=2 minutes.

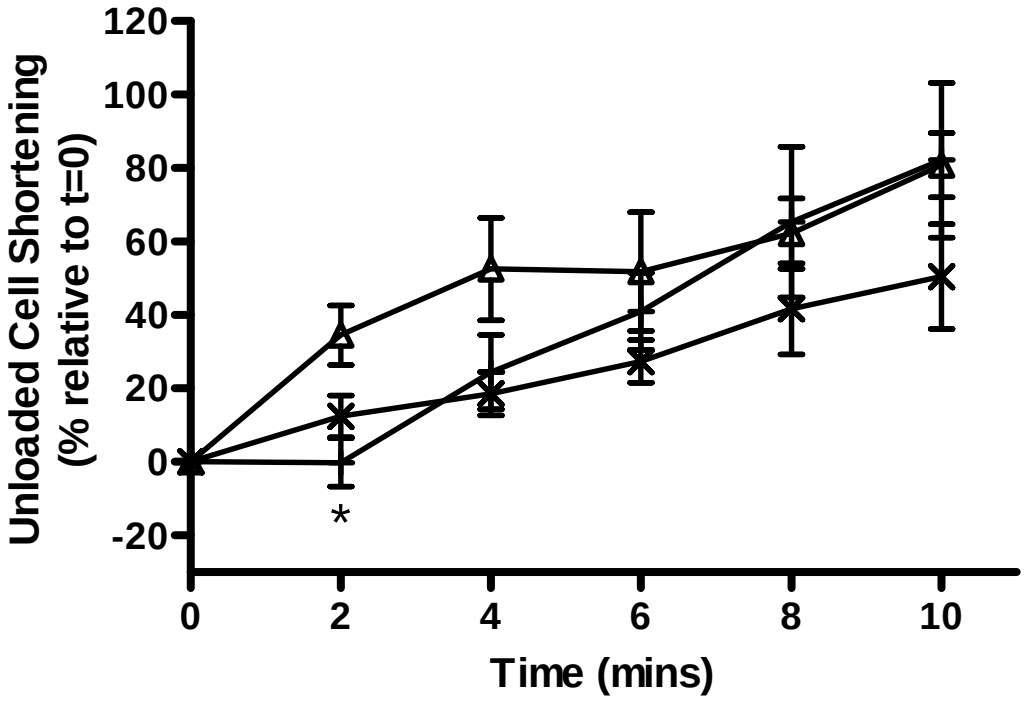

Figure 23. Ability of 1 µM and 0.1 µM KB-R7943 to inhibit H_2O_2-induced positive inotropy compared to H_2O_2 alone. The effect on unloaded cell shortening of 200 µM H_2O_2 in the presence of 1 µM KB-R7943 (+; n=3) and 0.1 µM KB-R7943 (x; n=4) is compared to H_2O_2 alone (△; n=6). Data points represent the mean ± standard error of the mean. (*) indicates data significantly different ($p<0.05$) from H_2O_2 alone group at the same time points.

CHAPTER 4

Summary of results

This study demonstrated a number of different effects of H_2O_2 on isolated rat ventricular cardiac myocytes including action potential prolongation, induction of afterdepolarizations, and positive inotropy even in the presence of calcium channel inhibition.

Previous experiments have shown that H_2O_2 causes action potential prolongation. Our results confirmed this finding, as superfusion with H_2O_2 resulted in increased action potential duration as measured by APD90 and APD50. Furthermore, when action potentials remained depolarized beyond a certain amount of time, approximately 300ms, the action potential waveforms where characterized by formation of afterdepolarizations that were attenuated with the use of L-type Ca^{2+} channel blocker, Cd^{2+}. When we tested the concentration-dependent effects of H_2O_2 by using 200 μM H_2O_2, we demonstrated that increases in APD and presence of afterdepolarizations were more pronounced with use of higher concentrations of H_2O_2.

Unloaded cell shortening and intracellular Ca^{2+} transient recordings were used to confirm the ability of H_2O_2 to induce increases in contractility. For both experimental methods, superfusion of cardiac myocytes with H_2O_2 resulted in a progressively greater degree of contractility. Interestingly, when experiments were repeated in the presence of Cd^{2+}, we found that H_2O_2 was able to increase contractility, even in the presence of Cd^{2+}. We found that pretreatment and continued superfusion with Cd^{2+} did not attenuate the positive inotropic effects of H_2O_2 suggesting that H_2O_2-induced effects were not entirely

mediated by I_{CaL}. When we used recordings of I_{CaL} to ensure that H_2O_2 did not reverse Cd^{2+} block of I_{CaL}, we showed that at peak currents blockade of I_{CaL} was not affected. Therefore, it was concluded that H_2O_2 mediated effects such as increase in contractility were at least in part caused by alternative mechanisms such as modification of SR Ca^{2+} handling or reversal of the NCX.

To investigate whether SR Ca^{2+} handling was altered, we measured SR Ca^{2+} load using the paired caffeine pulse method. However, it was found that superfusion with H_2O_2 only resulted in a small, non-significant decrease in SR load compared to control. These results suggest that H_2O_2 does not affect SR Ca^{2+} loading, and are contradictory to some published literature that indicates an effect of ROS on SERCA and RyRs. For example, H_2O_2 has previously been shown to increase open probability of RyRs (Boraso & Williams, 1994; Oba *et al*. 1996). Furthermore, ROS are strong oxidizers and it has previously been shown that oxidizing agents inhibit SERCA activity while reducing agents protect SERCA from inhibition (Scherer & Deamer, 1986; Morris & Sulakhe, 1997).

We investigated the possibility of reverse-mode NCX as an alternative mechanism of Ca^{2+} influx. KB-R7943 preferentially inhibits reverse-mode NCX (Watano *et al*. 1999), and it has previously been suggested that the action of KB-R7943 would be especially pronounced in pathological cases such as intracellular $[Na^+]$ loading. Since H_2O_2 has been shown to induce persistent I_{Na} (Ward & Giles, 1997), which could increase intracellular $[Na^+]$, KB-R7943 seemed an ideal candidate to use as a selective reverse-mode blocker of the NCX. Furthermore, it has been suggested that KB-R7943 does not affect cardiac myocytes under physiological conditions because inhibition of reverse-

mode operation of the NCX may not contribute to normal physiology (Weber et al. 2002). However, the results from our experiments using the novel NCX reverse-mode blocker were inconclusive and indicated that KB-R7943 acted in unpredictable ways suggesting non-selective block of the reverse-mode NCX.

While in our initial experiments, it appeared that 10 μM KB-R7943 attenuated the H_2O_2 mediated contraction in the presence of Cd^{2+}, it was later observed that 10 μM KB-R7943 detrimentally affected the ability of cardiac myocytes to initiate normal action potentials, which could have led us to misinterpret our findings. Our results show that 10 μM KB-R7943 affected the ability of the myocytes to depolarize, with depolarization ending at lower voltages than control. Thus, 10 μM KB-R7943 attenuated H_2O_2-induced effects by preventing normal action potential firing of the cardiac myocytes, but not necessarily by blocking NCX reverse-mode.

We tested other concentrations of KB-R7943 since use of different concentrations ranging from 10 μM of KB-R7943 to 0.1 μM KB-R7943 have been cited (Watano *et al*. 1996; MacDonald & Howlett, 2008). However, H_2O_2-mediated affects such as increased contractility in the presence of Cd^{2+} were not blocked when KB-R7943 concentration was reduced from 10 μM to 1 μM. Furthermore, even at 1 μM KB-R7943, action potentials were affected, resulting in prolonged APD. We concluded that the specificity of KB-R7943 was questionable because our experiments using 1 μM KB-R7943 alone showed positive inotropy and prolonged action potential duration. A possible explanation for these unexpected effects of KB-R7943 is that KB-R7943 caused blockade of both forward mode and reverse mode NCX. At lower concentrations of 0.1 μM KB-R7943, H_2O_2 mediated recapitulation of contraction was again not affected, similar to results

recorded using 1 μM KB-R7943. Thus we concluded that our original experiments using 10 μM KB-R7943 showing abolition of H_2O_2 mediated affects were inconclusive.

We were not able to test our hypothesis that H_2O_2 mediated effects were due to reversal of the NCX because KB-R7943 was not observed to have non-selective effects. However our experiments provided evidence that H_2O_2 enhanced I_{CaL} via increased peak currents at voltages of -20 mV and -10 mV. Since I_{CaL} has been shown to be inhibited by redox modulation (Cerbai *et al.* 1991; Matsuura & Shattock, 1991; Moghadam & Winlow, 1995), the role of the L-type Ca^{2+} channel in H_2O_2 mediated increase in intracellular Ca^{2+} has been suggested as mostly non-important. In our study, however, rat ventricular myocytes showed an increased peak current at voltages of -20 mV and -10 mV. Furthermore, our results indicate a possible shift in voltage activation of peak current since peak current occurred at -10 mV instead of 0 mV. The resulting increases in peak current and shift in activation could have led to greater overall inward flow of I_{CaL}.

Lastly, in our recordings of I_{CaL}, we found evidence in support of enhanced I_{CaL} as one of the mechanisms through which H_2O_2 causes positive inotropy and Ca^{2+} overload. Though enhanced I_{CaL} contradicts many previous experiments which have reported a decrease in I_{CaL} (Cerbai *et al.* 1991; Matsuura & Shattock, 1991; Moghadam & Winlow, 1995), it is possible that it is a species specific phenomenon or that the effect is only apparent using perforated patch clamp technique, which prevents dialysis of intracellular second messengers. Finally, although enhanced I_{CaL} may help to explain Ca^{2+} overload and positive inotropy, it does not explain the results of our experiments using Cd^{2+} pretreatment. H_2O_2 was not seen to mediate changes in I_{CaL} in the presence of Cd^{2+} pretreatment. Therefore, enhanced peak I_{CaL} does not explain the ability of H_2O_2 to cause

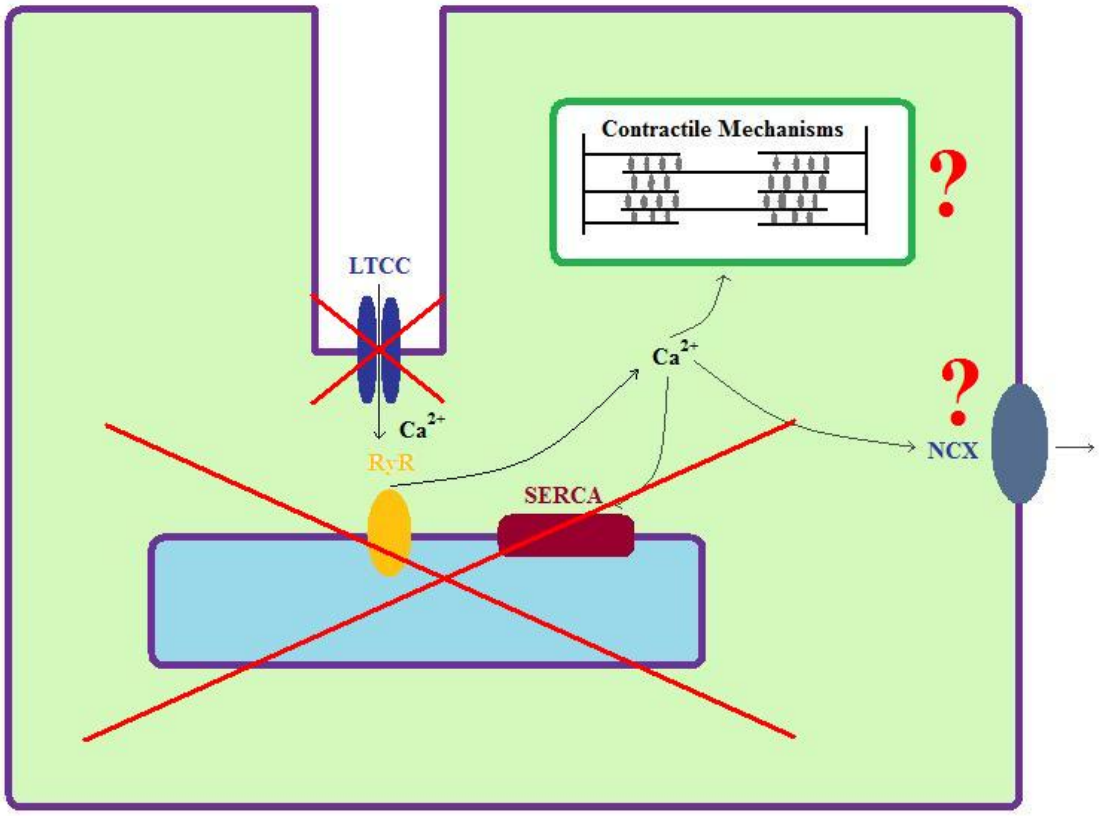

Figure 24. Potential mechanisms of H_2O_2 action. Our results provided evidence that the effects of H_2O_2 in the presence of Cd^{2+} were neither mediated by I_{CaL} nor mediated by the SR. It is possible that the mechanism of action was through affecting the contractile properties of the cell or through causing reverse-mode operation of the NCX.

positive inotropy after Cd^{2+} pretreatment, and this phenomenon is likely occurring through a different mechanism. H_2O_2 likely also mediates other changes in electrophysiology such as reverse of the NCX, or modification of contractile mechanisms.

H_2O_2-induced afterdepolarizations

Afterdepolarizations are indicative of arrhythmic potential and in our experiments H_2O_2 induced afterdepolarizations were shown in unloaded cell shortening, Ca^{2+} transients, and action potential recordings. Our results suggest that the mechanism through which afterdepolarizations developed could be prolongation of action potential duration, increased intracellular [Ca^{2+}], or a combination of both. APD90 and APD50 of cells in the presence of 100 μM H_2O_2 were significantly increased, and the prolongation was drastically more pronounced in the presence of 200 μM H_2O_2. In recordings of action potentials it could be seen that afterdepolarizations developed if action potential prolongation reached a critical time of approximately 300ms. The effect was mirrored in both cell shortening and Ca^{2+} transient recordings.

One possible mechanism for the development of afterdepolarizations is reopening of L-type Ca^{2+} channels. Our results show that action potentials had to be prolonged for afterdepolarizations to occur. Furthermore, afterdepolarizations consistently developed only after a critical period of prolongation of APD90 suggesting that they may be caused by reopening of the L-type Ca^{2+} channels. Previous experiments have showed that early afterdepolarizations form in conditions of prolonged action potential plateau and are due to recovery from inactivation and subsequent activation of L-type Ca^{2+} channels (Marban

et al. 1986; January & Riddle, 1989). It is possible that the L-type Ca^{2+} channels were undergoing a change from an inactivated state to a closed state in the 300ms time window. We also showed that Cd^{2+} attenuated the afterdepolarizations caused by H_2O_2 suggesting that the mechanism of afterdepolarizations was at least in part due to reopening of Ca^{2+} channels.

Another possible mechanism for development of afterdepolarizations is H_2O_2-induced Ca^{2+} overload. Increased intracellular [Ca^{2+}] is among the most commonly reported ROS-induced effects in cardiac myocytes (Hayashi *et al*. 1987; Hayashi *et al*. 1989; Ward & Giles, 1997; Gen *et al*. 2001) and it has previously been shown that afterdepolarizations may form through spontaneous release of SR due to increased intracellular [Ca^{2+}] (Thandroyen *et al*. 1991; Lakatta & Guarnieri, 1993). In our experiments, using intracellular calcium transients, we noticed a progressively increasing diastolic $[Ca^{2+}]_i$ in myocytes superfused with H_2O_2 indicating possible Ca^{2+} overload. Many mechanisms could contribute to increasing intracellular [Ca^{2+}]. The results from our experimentation suggest that an increase in intracellular Ca^{2+} could have been a result of enhanced I_{CaL}, slowed inactivation of L-type Ca^{2+} channels, or secondary activation of L-type Ca^{2+} channels.

H_2O_2-induced action potential prolongation

Positive inotropy was consistently found in our recordings of unloaded cell shortening and peak intracellular Ca^{2+} transients. A possible mechanism for the observed positive inotropy is H_2O_2-mediated prolongation of action potential duration.

Physiological cardiac action potentials consist of a plateau phase which is characterized by inward movement of current through L-type Ca^{2+} channels. The mechanisms that underlie L-type channel inactivation and deactivation are complex, and involve interaction between time and voltage-dependant gating as well as Ca^{2+} induced inactivation (DeFelice, 1993; McDonald *et al.* 1994). Since voltage-dependent inactivation (VDI) may play a significant role in L-type Ca^{2+} conductance (Cens *et al*. 2006; Findlay *et al*. 2008), a prolonged action potential could result in delayed inactivation of the channel. A delay in inactivation could also result in increased intracellular Ca^{2+} both directly by increasing Ca^{2+} influx through I_{CaL}, and therefore enhanced triggering of CICR. Previous studies have shown increased release of Ca^{2+} from intracellular sources as a result of prolonged action potentials (Wood *et al*. 1969; Morad & Goldman, 1973; Allen, 1977; Wolfhart, 1979). Bouchard *et al* (1995) demonstrated that a marked prolongation of the action potential can result in a large, time-dependent positive inotropic effect. Experiments conducted by Bouchard *et al* (1995) using simulated prolonged action potentials through the action potential clamp technique, showed that prolonged depolarization increased overall amplitude of the Ca^{2+} influx. Bouchard *et al* (1995) measured a net Ca^{2+} influx increase of twice that of cells with normal APDs during action potential prolongation, even though peak I_{CaL} decreased.

H_2O_2-induced positive inotropy after I_{CaL} block

One of our most intriguing findings involved the use of Cd^{2+} in an attempt to block H_2O_2-induced positive inotropic effects. With recordings of action potentials, we

showed that Cd^{2+} prevented afterdepolarizations and concluded that this was through blockade of the L-type Ca^{2+} channel. However, Cd^{2+} did not have the same effect on contractility and intracellular calcium transient fluorescence in the presence of H_2O_2. Instead, H_2O_2 caused increases in both unloaded cell shortening and intracellular calcium transients despite Cd^{2+} pretreatment. Initially, we hypothesized that H_2O_2 may be affecting the ability of Cd^{2+} to block I_{CaL}. However, our results demonstrated that in the presence of H_2O_2 there was no significant change in peak I_{CaL}, and thus H_2O_2 did not induce re-opening of the blocked L-type Ca^{2+} channel. We concluded that increased contraction occurred via another mechanism independent of the I_{CaL} such as reversal of the NCX.

H_2O_2-induced changes in I_{CaL}

Interestingly, our results show that H_2O_2 altered I_{CaL} properties in an unexpected way. Previous studies regarding the effect of ROS on I_{CaL} have been contradictory but have mostly suggested an inhibitory effect (Cerbai *et al.* 1991; Matsuura & Shattock, 1991; Moghadam & Winlow, 1995). For example, L-type Ca^{2+} channels are known to undergo redox modulation, and in a study by Fearon *et al* (1999), the oxidizing agents thimerosal and p-chloromercuribenzene sulphonic acid, caused inhibition of I_{CaL}. Furthermore, Fearon *et al* (1999) found that use of reducing agent 1,4-dithiothreitol had no effect on Ca^{2+} channels when used alone, but reversed the inhibitory effect of thimerosal and p-chloromercuribenzene sulphonic acid. These past studies implicate a decreased I_{CaL} as a result of redox modulation.

On the other hand, evidence has suggested that even though peak amplitudes of I_{CaL} are reduced, overall influx of I_{CaL} is enhanced (Coetzee & Opie, 1992). Coetzee & Opie (1992) reported a reduction in peak amplitude of I_{CaL}, but slowing of inactivation of the L-type Ca^{2+} channel, resulting in an overall increase of mean current in guinea pig ventricular myocytes. This effect was suggested to be caused by the increased time constant of inactivation of I_{CaL}, which was measured to increase by 53% after 30 min (Coetzee & Opie, 1992).

In contrast to previous studies (Cerbai *et al.* 1991; Matsuura & Shattock, 1991; Coetzee & Opie, 1992; Moghadam & Winlow, 1995; Fearon *et al.* 1999), I_{CaL} was not found to be decreased in our experiments. Instead, from our results it appears that I_{CaL} may have been slightly increased as evidenced by the increased Ca^{2+} influx at voltages of -20 mV and -10 mV. Furthermore, voltage activation of the L-type Ca^{2+} channel may be shifted toward more negative potentials, as evidenced by the shift of peak current from 0 mV in control to -10 mV in H_2O_2, and inactivation of Ca^{2+} channels in the presence of H_2O_2 may be prolonged. Prolonged I_{CaL} is consistent with previous studies (Coetzee & Opie, 1992) and maybe the mechanism through which positive inotropy occurs. However, our findings also suggest positive inotropy caused by H_2O_2 may be mediated in part by an increase in I_{CaL}, and not only because of prolonged inactivation.

An alternative mechanism for increased Ca^{2+} influx is the secondary activation of L-type Ca^{2+} channels due to APD prolongation. Secondary activation has previously been described in guinea-pig heart cells. Rose *et al* (1992) showed that the measured Ca^{2+} flux through L-type Ca^{2+} channels in guinea pig heart cells during depolarizing steps as long as 180 ms could not be described by channel inactivation kinetics. Since channel

inactivation could not explain the extra current density that was measured in experimentation it was suggested that inactivation in L-type Ca^{2+} channels must have been reversible, and that reactivation of the channels, termed secondary activation, could have occurred to account for the extra current density that was measured in experimentation (Rose *et al*. 1992).

Effects of H_2O_2 on SR calcium content

Gen *et al* (2001) suggested that H_2O_2 mediated Ca^{2+} overload was caused by changes in Ca^{2+} handling or release from the SR rather than changes in I_{CaL} or NCX. Similar to our results, their experiments demonstrated that H_2O_2 caused increased intracellular Ca^{2+} and hypercontracture but that nifidepine, a blocker of I_{CaL}, did not abolish H_2O_2 mediated changes. Furthermore, Gen *et al* (2001) found that KB-R7943 also did not attenuate increases in intracellular Ca^{2+}, concluding that therefore H_2O_2 induced changes in SR Ca^{2+} handling.

H_2O_2 is a strong oxidizer and known to cause redox modulation of SR ion transporters such as the RyR and SERCA. Previous experiments have reported altered Ca^{2+} release and handling by the SR due to modification of sulfhydryl groups by reactive disulfide compounds such as 2,2-DTDP (Zaidi *et al*. 1989). These compounds caused oxidation of free SH sites via a thiol-disulfide exchange reaction resulting in enhanced efflux of Ca^{2+} from the SR. H_2O_2 has, for example, been shown to cause increased open probability of SR Ca^{2+} release channels during single-channel recordings under voltage clamp of SR Ca^{2+} release channels incorporated into planar phospholipid conditions

(Boraso & Williams, 1994). Boraso & Williams (1994) also found that use of dithiothreitol, a sulfhydryal-protecting compound, reduced H_2O_2 induced changes in open probability of SR Ca^{2+} release channels. While H_2O_2 may cause increased RyR release of Ca^{2+} there is also evidence that it affects SERCA function.

SERCA plays a central role in regulation of cardiac muscle relaxation and has previously been reported to be inhibited by redox modulation. Experiments by Shcerer & Deamer (1986) recorded a decline in Ca^{2+} ATPase activity induced by using oxidants such as peroxydisulfate and H_2O_2. The effects were not linked to lipid peroxidation since lipid antioxidants did not prevent the decline in SR function. It was concluded in the study that rises in intracellular calcium due to oxidants such as H_2O_2 was due to oxidation of sulfhydryl groups in SERCA. In another study, reducing agents such as DTT protected the inhibition of SERCA by oxidizing agents (Morris & Sulakhe, 1997).

Other studies that measured inhibited SERCA function suggested that the location of inhibition was the ATP binding site on the Ca^{2+} ATPase. An experimental model exposing hydroxyl radical to isolated SR vesicles containing Ca^{2+} ATPase from rabbit cardiac muscle showed that the hydroxyl radical completely inhibited Ca^{2+} ATPase activity and SR Ca^{2+} uptake.

Inhibition of SERCA by ROS would likely lead to decreased filling of the SR. This would be recorded in caffeine pulse experiments as decreased caffeine pulse areas measured using intracellular Ca^{2+} fluorescence. However, our results did not show a significant decrease in SR calcium load. Though ROS mediated modification of SR Ca^{2+} ion transporters has been widely reported, our results do not show evidence of modified

Ca^{2+} handling. However, it is important to note that our experiments did not directly measure the effects of ROS on RyR and SERCA.

Reversal of the NCX

In normal myocyte physiology, the primary role of the NCX is thought to be removal of Ca^{2+} through forward mode extrusion of one Ca^{2+} ion in exchange for three Na^+ ions. Thus it plays a major role in the relaxation phase of cardiac contraction. However, the possibility of reversal of the NCX resulting in extrusion of three Na^+ ions in exchange for influx of one Ca^{2+} ion has been proposed in pathophysiological cases such as increased intracellular [Na^+]. Experiments conducted using the absence of extracellular Na^+ to provide a favorable electrochemical driving force for reversal showed that, in these conditions, reversal of the NCX was possible (Litwin *et al.* 1998; Goldhaber *et al*. 1999). Furthermore, mathematical models of the NCX predict that the electrochemical driving forces in the case of a ion imbalance, such as buildup of [Na^+], could provide enough driving force to cause reversal of the NCX (Sher *et al.* 2008). In previous experiments (Ward & Giles, 1997; Song *et al*. 2006) it was found that H_2O_2 caused cells to develop persistent I_{Na}. It is feasible that this persistent I_{Na} could result in abnormal buildup of $[Na^+]_i$.

While persistent I_{Na} is one mechanism by which $[Na^+]_i$ could have increased, another mechanism is increased activity of the Na^+/H^+ exchanger following reperfusion. Previous experiments have shown that Na^+ concentrations are higher in ischemic cardiac cells due to decreased intracellular pH and increased activity of the Na^+/H^+ exchanger

(Tani & Neely, 1989, Pike *et al.* 1990, Park *et al*. 1999). Park *et al* (1990) showed that under ischemic conditions the Na^+/H^+ exchanger was inactive, contributing to buildup of H^+ and consequent cell acidosis. Following reperfusion, it was found that the Na^+/H^+ exchanger activated and $[Na^+]_i$ increased rapidly. The rapid rise in $[Na^+]$ due to Na^+/H^+ exchanger activity is a possible driving mechanism for reversal of the NCX.

KB-R7943 as a selective NCX reverse mode blocker

To test our hypothesis that the H_2O_2-mediated positive inotropy in the presence of Cd^{2+} was caused by reversal of the NCX, we used the putatively selective NCX reverse mode blocker KB-R7943. Watano *et al* (1996*)* reported the effects of a novel compound termed No. 7943 and showed that under certain in vitro conditions and concentrations, No. 7943 suppressed the inward NCX current, but did not affect the outward exchange current. Since the publication of these results, interest in the compound quickly developed, and many studies were done using KB-R7943 as a selective reverse mode blocker. However, more recent publications regarding KB-R7943 have had conflicting results.

In 1999, the same group Watano *et al* (1999) published results showing that KB-R7943 did not selectively block reverse-mode NCX under bi-directional ionic conditions. Instead KB-R7943 was shown to have an IC50 of approximately 1 μM for both forward-mode and reverse-mode of the NCX. The published results concluded that selectivity of KB-R7943 for the reverse-mode NCX depended on the extracellular and intracellular conditions of the cell. In vitro experiments showed that under uni-directional ionic

conditions favoring NCX reverse-mode, KB-R7943 selectively blocked reverse-mode NCX (Watano *et al*. 1996).

Since the publishing of the results by Watano *et al*. (1999) many studies were conducted examining the pharmacology of KB-R7943 and related benzyloxyphenyl analogues. One such study conducted by Lee *et al* (2004) reported that the inhibitory potency of SEA0400, a recently developed NCX inhibitor related in chemical structure to KB-R7943, strongly depended on the intracellular Na^+ concentration. In the study by Lee *et al* (2004) the inhibitory effect of SEA0400 was greatly reduced in the absence of $[Na^+]_i$. In accordance with their results, Lee *et al* (2004) suggested that the targets of the NCX inhibitors may be the intracellular Na^+ dependent inactive state of the NCX.

Recent experiments using benzyloxyphenyl analogues including KB-R7943, SN-6, and SEA0400 have shown that these compounds exert protective effects against ischemia-reperfusion injury (Hagihara *et al*. 2005; Yoshitomi *et al*. 2005; Motegi *et al*. 2007; MacDonald & Howlett, 2008). The mechanism of action responsible for this in all the preceding studies has been blockade of the reverse-mode of the NCX. Interestingly, in a study by MacDonald & Howlett (2008), KB-R7943 was found to be cardioprotective at lower concentrations of 0.1 uM. At a concentration of 0.1 uM KB-R7943 was shown to abolish afterdepolarizations, and hypercontracture, while at higher concentrations, KB-R7943 exacerbated detrimental effects such as post-ischemic contractile dysfunction.

Our results indicate that KB-R7943 has non-selective actions at concentrations including and above 1 μM. Our initial experiments using 10 μM KB-R7943 demonstrated that it abolished positive inotropy in Cd^{2+} pretreated cells superfused with

H_2O_2. However, at 10 μM KB-R7943 was seen to detrimentally affect physiological action potential formation and cause increased loading of the SR. These effects suggest that KB-R7943 may have blocked bi-directional function of the NCX at a concentration of 10 μM. At lower concentrations of 1 μM KB-R7943, prolonged action potential duration was observed, suggesting that even at 1 μM, it continued to exert bi-directional block of the NCX. At 0.1 μM and 1 μM KB-R7943, recapitulation of contraction due to H_2O_2 in Cd^{2+} pretreated cells was not affected. Our results do not exclude NCX reverse mode function as a possible mechanism through which recapitulation of contraction occurs in the presence of H_2O_2. We were unable to provide evidence for NCX reverse mode as a possible mechanism because we concluded that the non selective effects of KB-R7943 could potentially have masked the effects NCX reverse mode blockade.

CHAPTER 5

Cardiac arrhythmias can be benign and asymptomatic. However, depending on the severity, they can also be life-threatening, inducing the occurrence of sudden cardiac death. Previous studies have demonstrated detrimental effects such as cardiac tachyarrhythmia linked to the production of ROS in cardiac myocytes. While ROS have been implicated in reperfusion injury, and many previous studies have characterized multiple different effects of ROS on cardiac tissue, the electrophysiological effects of ROS such as H_2O_2 are still not well studied. H_2O_2 has been shown to be an important mediator of reperfusion injury (Duan & Moffat, 1992; Goldhaber & Liu, 1994) and may play an even bigger role than other ROS considering its relative stability and ability to diffuse.

Our study provides evidence for the ability of H_2O_2 to cause a number of different electrophysiological changes including novel evidence that it can cause contraction and increases in calcium transient fluorescence in the presence of L-type channel blockade by Cd^{2+}. We also found that H_2O_2 could alter L-type Ca^{2+} channel conductance causing increased I_{CaL}. Furthermore, we demonstrated that H_2O_2 did not affect SR Ca^{2+} load, which is in contrast to many other studies which suggest the SR, and associated ion transporters, as the cause of ROS-induced arrhythmia (Boraso & Williams, 1994; Oba *et al*. 1996; Gen *et al*. 2001). Since we report that H_2O_2 did not affect the ability of Cd^{2+} to block of I_{CaL} but was still able to cause increase in unloaded cell shortening and intracellular Ca^{2+} transients in the presence of Cd^{2+}, we hypothesized that H_2O_2 was causing increases in Ca^{2+} via an I_{CaL}-independent pathway. We further hypothesized that such a pathway could be the reverse-mode operation of the NCX. However, while we

attempted to use KB-R7943 as a selective reverse-mode inhibitor of the NCX as a method of investigating H_2O_2's effects on the NCX, KB-R7943 did not appear to act selectively. Therefore, we could not determine whether reverse-mode NCX was a mechanism through which H_2O_2 mediated its' effects. Nevertheless, our findings are consistent with such a hypothesis even in the absence of a direct means of addressing this issue.

Our results demonstrating that H_2O_2 altered I_{CaL} were not unusual. However, most previous experiments showed that ROS caused I_{CaL} to be decreased (Cerbai *et al.* 1991; Matsuura & Shattock, 1991; Moghadam & Winlow, 1995). On the other hand, one previous experiment reported a decreased peak current, but an increase in overall I_{CaL} due to slowed inactivation (Coetzee & Opie, 1992). This may be an interesting avenue of research to pursue. From our experiments it is unclear whether it is the conductance or the inactivation kinetics of the L-type Ca^{2+} channel that has changed. Future experiments could be directed at further studying the properties of Ca^{2+} channels in the presence of H_2O_2.

Our experiments using superfusion of H_2O_2 in the presence of Cd^{2+} pretreatment and superfusion showed indirect evidence that an I_{CaL}-independent pathway was responsible for some the H_2O_2-induced electrophysiological changes. Currently, newer, more specific selective reverse-mode NCX inhibitors are being developed. Studies are being performed using these novel reverse-mode NCX inhibitors as drugs to help regulate pathologic Ca^{2+} handling. These NCX inhibitors have been shown to help prevent some of the detrimental effects associated with ischemia reperfusion injury, and are reported to function only in pathological situations as they are inactive during normal physiological

function of cardiac myocytes (Iwamoto, 2007). Furthermore, they are being modified in hopes of enhancing selectivity. Perhaps in the near future, development of more specific NCX reverse-mode inhibitors will allow us to confirm whether reverse-mode NCX was indeed a mechanism of H_2O_2 action.

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