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CARDIAC ISCHEMIA AND REPERFUSION: CELLULAR PHYSIOLOGY AND PHARMACOLOGICAL INTERVENTION

by

William E. Louch

Submitted in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

October 2001

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

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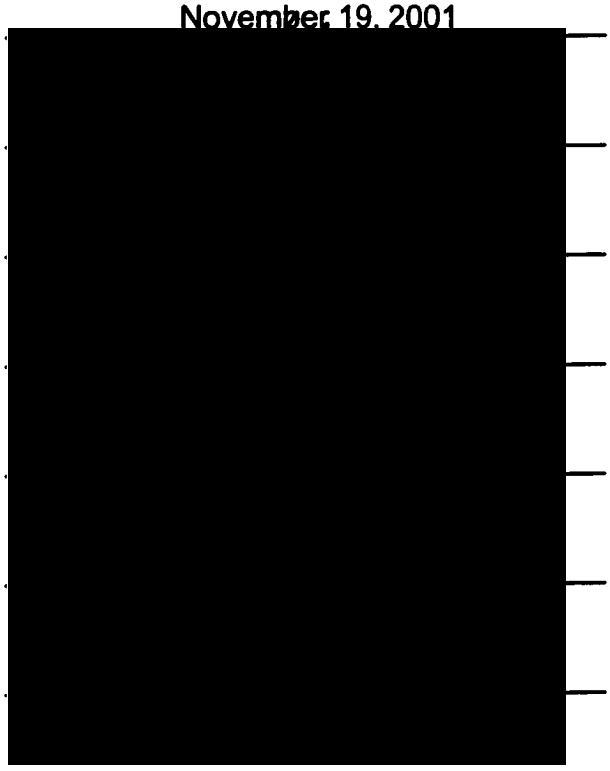

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This thesis is dedicated to my parents, Bill and Fay,

and to my brothers, Jeff, Mark, and David,

for their love, support, and questionable sense of humour.

"I am a scientist – I seek to understand me
All of my impurities and evils yet unknown
I am a journalist – I write to you to show you
I am an incurable
And nothing else behaves like me."

- Robert Pollard
of Guided by Voices

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ABSTRACT

Exposure to ischemia and reperfusion can trigger protracted contractile depression in the myocardium, despite normal electrical activity and the absence of irreversible damage. This condition is known as myocardial stunning. Although it is believed that stunning is associated with reduced responsiveness of the myofilaments to Ca^{2+}, it is unclear whether altered Ca^{2+} homeostasis in late reperfusion also might contribute to this condition. This question was addressed by examining EC coupling in an isolated myocyte model of stunning. The cellular model was developed by demonstrating that isolated myocytes exposed to 30 min of simulated ischemia exhibited contractile depression in reperfusion that was not associated with alterations in action potential configuration. Stunned myocytes exhibited normal Ca^{2+} transients, supporting the view that reductions in myofilaments responsiveness are a key component of the physiology of stunning. Stunned myocytes also exhibited normal diastolic $[Ca^{2+}]_i$ and SR Ca^{2+} content, but $I_{Ca\text{-}L}$ was reduced. Contractions elicited by the VSRM and CICR were similarly depressed in late reperfusion, suggesting that the gain of CICR may be increased in stunning. Increased Ca^{2+} influx, possibly by the $NaCa_{ex}$ may compensate for post-ischemic reductions in $I_{Ca\text{-}L}$ to maintain a normal Ca^{2+} transient in reperfusion. The results support the view that altered Ca^{2+} homeostasis could contribute to contractile depression in stunning. In a second series of experiments, the actions of the AT_1 receptor antagonist, losartan, were examined in the cellular model of stunning. Losartan treatment during ischemia protected against stunning by a mechanism that appeared to be independent of AT_1 blockade. Since losartan also inhibited I_{Ti} in early reperfusion, it was hypothesized that losartan may protect against Ca^{2+} overload. Losartan was, in fact, observed to attenuate increases in diastolic $[Ca^{2+}]_i$ during ischemia and prevent overshoot of Ca^{2+} transients in early reperfusion. Losartan also increased the magnitude of Ca^{2+} transients in late reperfusion. SR Ca^{2+} stores appeared to be increased in losartan-treated cells without effects on diastolic $[Ca^{2+}]_i$, an action that could explain the protective actions of losartan in ischemia and reperfusion. These results suggest that losartan may increase SR Ca^{2+} stores, possibly by increasing activity of the SR Ca^{2+} ATPase, by an action independent of AT_1 blockade.

LIST OF ABBREVIATIONS

°C	-	degrees Celsius
AI	-	angiotensin I
AII	-	angiotensin II
AIII	-	angiotensin III
AIV	-	angiotensin IV
ACE	-	angiotensin converting enzyme
A/D	-	analog-to-digital
ADP	-	adenosine diphosphate
APD	-	action potential duration
AT_1 receptor	-	angiotensin II type-1 receptor
AT_{1A} receptor	-	angiotensin II type-1 subtype-A receptor
AT_{1B} receptor	-	angiotensin II type-1 subtype-B receptor
AT_2 receptor	-	angiotensin II type-2 receptor
ATP	-	adenosine triphosphate
Ca^{2+}	-	calcium ion
$[Ca^{2+}]_i$	-	intracellular calcium ion concentration
CaMK	-	calcium calmodulin-dependent protein kinase
cAMP	-	cyclic 3',5'-adenosine monophosphate
cGMP	-	cyclic 3',5'-guanosine monophosphate
CICR	-	calcium-induced calcium release
dSEVC	-	discontinuous single-electrode voltage clamp
EC coupling	-	excitation-contraction coupling
ECG	-	electrocardiogram
$FADH_2$	-	flavin adenine dinucleotide
g	-	grams
H^+	-	hydrogen ion
$[H^+]_i$	-	intracellular hydrogen ion concentration
$[H^+]_o$	-	extracellular hydrogen ion concentration
Hz	-	hertz
$I_{Ca\text{-}L}$	-	L-type calcium current
I_{TI}	-	transient inward current
K^+	-	potassium ion
$[K^+]_o$	-	extracellular potassium ion concentration
kg	-	kilograms
kHz	-	kilohertz
LVP	-	left ventricular blood pressure
MΩ	-	megaohms
mg	-	milligrams
min	-	minutes
ml	-	milliliters
mm	-	millimeters
mM	-	millimolar
mm Hg	-	millimeters of mercury

ms	-	millisecond
mV	-	millivolts
n	-	number of myocytes examined
nA	-	nanoamps
Na^+	-	sodium ion
$[Na^+]_i$	-	intracellular sodium ion concentration
$NaCa_{ex}$	-	sodium-calcium exchanger
NADH	-	nicotinamide adenine dinucleotide
nM	-	nanomolar
NMDA	-	N-methyl-D-aspartate
NMR	-	nuclear magnetic resonance
OAP	-	oscillatory afterpotential
P_i	-	inorganic phophate
PO_2	-	partial pressure of oxygen
pH_i	-	intracellular pH
pH_o	-	extracellular pH
PKA	-	protein kinase A
PKC	-	protein kinase C
RAS	-	renin-angiotensin system
RMP	-	resting membrane potential
s	-	seconds
SR	-	sarcoplasmic reticulum
T-tubule	-	transverse tubule
V_C	-	command potential
V_M	-	membrane potential
VSRM	-	voltage-sensitive release mechanism
µM	-	micromolar
µm	-	micrometer

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PUBLICATIONS

Portions of this thesis have been previously published.

Manuscripts

1. **Louch WE**, Ferrier GR, and Howlett SE (2000) Losartan Improves Recovery of Contraction and Inhibits Transient Inward Current in a Cellular Model of Cardiac Ischemia and Reperfusion. J Pharmacol Exp Ther **295**:697-704.

2. **Louch WE**, Ferrier GR, and Howlett SE. Stunning is Associated with Reduced L-type Ca Current, but Action Potentials, SR Stores, and Ca Transients are Normal in Isolated Ventricular Myocytes. Am J Physiol (submitted).

Abstracts

1. Louch WE, Ferrier GR, and Howlett SE (1999) Losartan Reduces Induction of Transient Inward Current and Attenuates Post-Ischemic "Stunning" In Simulated Ischemia and Reperfusion in Guinea Pig Ventricular Myocytes. Biophys J **76**:A460.

2. Louch WE, Ferrier GR, and Howlett SE (2000) Single Isolated Guinea Pig Ventricular Myocytes Exhibit "Stunning" in Response to Simulated Ischemia and Reperfusion. Biophys J **78**:376A.

3. Louch WE, Ferrier GR, and Howlett SE (2001) Losartan Preserves Normal Diastolic $[Ca]_i$ and Ca Transients During Ischemia and Reperfusion in Isolated Cardiac Myocytes. Biophys J **80**:596a.

INTRODUCTION

1. Historical Overview

Sudden cardiac death is a major public health concern. It is believed to account for between 11 and 15% of all deaths worldwide, and an even larger proportion of mortality in Western society (World Health Organization, 1985). Most cases of sudden cardiac death are associated with ischemic heart disease. Thus, an important goal of current research is to elucidate the pathogenetic mechanisms of ischemic heart disease to improve treatment strategies.

Our understanding of ischemic heart disease has been made possible by literally thousands of years of investigation. Accounts of angina and sudden cardiac death have been found in translations of hieroglyphs from ancient Egypt. Advice to the physicans of the time is inscribed on the famous Ebers papyrus (1550 BC):

> "When you examine a man for illness in his cardia, he has pains in his arm, in his breast, on the side of his cardia; it is said thereof ... it is death which approaches him. Then you shall prepare for him stimulating herbal remedies: fruits of pea and bryony; let them be boiled in beer and be drunk by the man."
>
> - Leibowitz (1970)

Although the Egyptians had a clear appreciation for the consequences of heart disease, no understanding of the mechanisms underlying this condition is historically documented until the writings of the ancient Greek scholars. Hippocrates (400 BC) suggested that a number of disease states might result from obstruction of the blood vessels (Harris, 1973). However, Hippocrates' theory was entirely hypothetical and not based on

anatomical dissection. Galen (130-200 AD), another Greek physician, later provided the first anatomical description of the coronary arteries, and correctly proposed that these vessels supply the heart muscle with blood. Although Galen did not directly suggest that coronary occlusion can lead to sudden cardiac death, he did state that "When the heart is deprived of respiration it dies instantaneously" (Harris, 1973).

From these early times, conceptual knowledge of the pathogenesis of heart disease remained surprisingly unchanged until the Renaissance period. In 1586, Petrus Salius Diversus was the first to explicitly state that sudden cardiac death results from obstruction of the coronary arteries (East, 1958). This view was later supported with anatomical evidence from Lorenzo Bellini (1683) who observed narrowing and calcification of the coronary arteries in heart disease patients (Leibowitz, 1970). In later years, the disease characterized by the narrowing of the coronary arteries came to be known as atherosclerosis (East, 1958).

The condition that results from insufficient blood flow is referred to as ischemia. The consequences of cardiac ischemia depend on the extent and duration of coronary artery occlusion, but can include angina, myocardial infarction, and cardiac arrhythmias which are the cause of sudden cardiac death (Goldstein, 1974). Although ischemia produces a number of deleterious effects, the re-establishment of blood flow, called reperfusion, can also have harmful consequences. Myocardial reperfusion may occur spontaneously or following therapeutic interventions, and can result in reperfusion arrhythmias and reversible contractile depression (stunning) (for review see Maxwell and Lip, 1997). Despite continuing research efforts, the mechanisms of ischemia / reperfusion injury remain poorly understood.

The overall objective of this thesis is to examine changes in cellular physiology associated with cardiac ischemia and reperfusion, and to identify possible mechanisms for pharmacological intervention. In this study, particular attention will be given to an examination of stunning. In the first part of this thesis, the pathophysiology of this condition will be investigated in ischemia and reperfusion by developing a cellular model of stunning. The second part of this thesis will use this cellular model to examine the effects of inhibition of the renin-angiotensin system during ischemia and reperfusion.

The following chapters of this introduction will summarize current understanding of cellular alterations that occur during myocardial ischemia and reperfusion. This review will also consider remaining questions concerning the mechanisms of both myocardial stunning and therapies that attenuate this condition. First, however, the cellular mechanisms responsible for cardiac contraction under normoxic conditions will be reviewed.

2. Excitation-Contraction (EC) Coupling

In each cardiac myocyte, contraction is triggered by electrical excitation of the cell membrane, a process known as excitation-contraction (EC) coupling (Brady, 1964). Intracellular Ca^{2+} is of central importance in EC coupling. Early experiments by Ringer (1883) demonstrated that Ca^{2+} is required for the heart to contract. It was later shown that Ca^{2+} is not only necessary for contraction, but actually causes contraction of the cardiomyocyte by binding to the myofilaments (Ebashi and Endo, 1968). It is now widely accepted that the contraction / relaxation cycle results from a rapid rise and fall in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) known as the Ca^{2+} transient (Eisner, 2000). In

order to explain the mechanisms which regulate the Ca^{2+} transient and EC coupling, a description of the cellular structures involved in these processes is necessary.

A. The Sarcolemma

The cell membrane (sarcolemma) is of vital importance to Ca^{2+} homeostasis as it contains ion channels and exchangers which control Ca^{2+} fluxes into and out of the cell. It is no coincidence, then, that EC coupling begins with electrical excitation of the sarcolemma by the cardiac action potential. The action potential propagates as a wave of depolarization along the surface of the cell. Invaginations of the sarcolemma into the interior of the muscle fiber called transverse tubules (T-tubules) improve the capacity of the action potential to stimulate the myocyte (Bers, 2001). As will be explained in the following sections, many sites that play important roles in cardiac EC coupling are located along the T-tubules.

B. Voltage-Gated Ca^{2+} Channels / The Na^{+}-Ca^{2+} Exchanger

During the action potential, voltage-gated Ca^{2+} channels open and Ca^{2+} enters the cardiac myocyte. In cardiac muscle, Ca^{2+} influx occurs through two types of Ca^{2+} channels, L-type and T-type (Trautwein and Cavalie, 1985). These channels differ considerably in their activation and inactivation characteristics. L-type Ca^{2+} channels are activated at potentials positive to -30 mV, while T-type channels are activated positive to –60 mV (Hess, 1988; Triggle, 1998). As well, although both types of channels exhibit voltage-dependent inactivation, L-type channel inactivation is also Ca^{2+}-dependent and much slower than that of T-type channels (Hess, 1988; Triggle, 1998). Another

important difference between these two channel types is that L-type channels are found in much higher densities in adult cardiomyocytes, and exhibit a much larger conductance than T-type channels (Hess, 1988). Therefore, it is believed that L-type Ca^{2+} current ($I_{Ca\text{-}L}$) is a more important contributor to cardiac EC coupling than T-type Ca^{2+} current (Sipido et al., 1998). In adult ventricular cells, L-type channels are predominantly localized in the T-tubules near other important proteins involved in EC coupling (Scriven et al., 2000).

Another sarcolemmal protein which is important for Ca^{2+} homeostasis is the Na^{+}-Ca^{2+} exchanger ($NaCa_{ex}$). The $NaCa_{ex}$ is an electrogenic, energy-independent pump that moves three Na^{+} ions across the sarcolemma in exchange for one Ca^{2+} ion in the opposite direction (Pitts, 1979; Reeves and Hale, 1984). Although the $NaCa_{ex}$ is believed to normally extrude Ca^{2+} from the cell, the direction of exchange has been shown to reverse at positive potentials, bringing Ca^{2+} into the cell (Schuttler et al., 1991; Sham et al., 1992). Like the L-type Ca^{2+} channel, the $NaCa_{ex}$ exchanger is largely localized along the T-tubules (Scriven et al., 2000).

C. The Sarcoplasmic Reticulum and Ca^{2+}-Induced Ca^{2+} Release

Ca^{2+} entering the cell through L-type Ca^{2+} channels and by the $NaCa_{ex}$ can directly act on the myofilaments to initiate contraction, and may contribute to the Ca^{2+} transient (Schuttler et al., 1991; Sham et al., 1992; Eisner, 2000). However, the small rise in $[Ca^{2+}]_i$ resulting from Ca^{2+} influx during the action potential does not account for the large magnitude of the Ca^{2+} transient. Instead, this dramatic increase in $[Ca^{2+}]_i$ is largely the result of Ca^{2+} release from the sarcoplasmic reticulum (SR) (Langer, 1997). The SR,

which is the major site of Ca^{2+} storage in the cell, is located adjacent to the T-tubule network and is bounded by a membrane that is independent of the sarcolemma (Bers, 2001). Ca^{2+} is released from the SR through specialized Ca^{2+} release channels, known as ryanodine receptors. In an elegant series of experients, Fabiato (1985 a,b,c) demonstrated that in myocytes skinned of their sarcolemma, a puff of Ca^{2+} directed at the SR triggered a much larger release of Ca^{2+} from the SR. Fabiato called this process Ca^{2+}-induced Ca^{2+} release (CICR). More recent experiments have shown that Ca^{2+} influx via L-type channels, T-type channels, and the $NaCa_{ex}$ may trigger contractions and Ca^{2+} transients due to CICR (Barcenas-Ruiz and Wier, 1987; Hancox and Levi, 1995; Sipido et al., 1998). Thus, the process of CICR amplifies the rise in $[Ca^{2+}]_i$ which results from Ca^{2+} influx during the action potential.

D. The Voltage-Sensitive Release Mechanism

For many years, it was believed that CICR was the predominant mechanism of EC coupling in the heart. Recently, however, another important mechanism of EC coupling has been described. Ferrier and Howlett (1995) have demonstrated that contractions persist when macroscopic $I_{Ca\text{-}L}$ is blocked. Further, they observed that Ca^{2+} transients and contractions can be graded by membrane potential and not the magnitude of $I_{Ca\text{-}L}$. These findings suggest that changes in membrane voltage may directly trigger SR Ca^{2+} release, and thus, this new mechanism of cardiac EC coupling has been named the voltage-sensitive release mechanism (VSRM) (for review see Ferrier and Howlett, 2001).

Many studies previous to those of Ferrier and Howlett likely had failed to identify the VSRM because they used conditions that inhibited the VSRM in their experiments. In order for the VSRM to be activated, experiments must be performed at 37°C (Ferrier and Howlett, 1995). As well, in voltage-clamp studies, cells must either be impaled with high resistance electrodes or voltage-clamped with patch electrodes containing 8-bromo-cAMP (Ferrier et al., 1998). In order to observe the VSRM in isolation of CICR, voltage steps to potentials more negative than that required for $I_{Ca\text{-}L}$ activation are necessary. In guinea pig ventricular myocytes, the VSRM is selectively activated between potentials of -65 mV and -40 mV (Howlett et al., 1998).

E. Ca^{2+} Pumps

Following activation of contraction, Ca^{2+} must be removed from the cytosol to return $[Ca^{2+}]_i$ to diastolic levels and maintain homeostasis. Several cellular mechanisms are responsible for sustaining cellular Ca^{2+} homeostasis. The $NaCa_{ex}$ extrudes Ca^{2+} from the cell when acting in the forward direction (Reeves and Hale, 1984). However, two different types of energy-dependent Ca^{2+} pumps, or Ca^{2+} ATPases, are also present in the cell (Langer, 1997). The first is located in the sarcolemma and actively pumps Ca^{2+} out of the cell, albeit to a lesser extent than the $NaCa_{ex}$ (Bers, 2001). Another Ca^{2+} ATPase is located in the SR and is responsible for recycling released Ca^{2+} back into the SR. These Ca^{2+} ATPases will be given more attention in later chapters as they play an important role in the pathogenesis of ischemia.

F. Regulators of Intracellular Na^+ Concentration

It is important to note that $[Na^+]_i$ and $[Ca^{2+}]_i$ are interrelated because of the presence of the bi-directional $NaCa_{ex}$. Thus, alterations in $[Na^+]_i$ can indirectly affect EC coupling. A number of proteins are involved with Na^+ homeostasis. During the upstroke of the action potential, Na^+ enters the cell via Na^+ channels, and, during repolarization, K^+ exits via K^+ channels. Thus, an ionic imbalance exists at the end of the action potential. The Na^+-K^+ ATPase actively reverses this imbalance by removing three Na^+ from the cytosol in exchange for two K^+ (Stein, 1990). $[Na^+]_i$ is also passively regulated by the Na^+-H^+ exchanger, which extrudes one H^+ in exchange for one Na^+ (Frohlich and Karmazyn, 1997), and by the Na^+-HCO_3^- cotransporter, which simultaneously brings 1 Na^+ and 2 HCO_3^- into the cell (Aiello et al., 1998). As will be discussed in later sections, the Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter play important roles in maintaining intracellular pH (Grace et al., 1993).

G. The Myofilaments

The myofilaments are the end effector of EC coupling since they respond to increases in $[Ca^{2+}]_i$ to cause contraction of the myocyte (Bers, 2001). The myofilaments are composed of alternating thick and thin filaments, which are aligned in parallel. The thin filaments are composed of actin, tropomyosin, and a troponin complex. The troponin complex, in turn, is made up of three subunits: troponin T, which binds tropomyosin; troponin C, which binds Ca^{2+}; and troponin I, which binds troponin T and C, and can also bind to actin (Flicker et al., 1982). The thick filaments are composed of myosin. The myosin molecule has two globular heads which are capable of interacting

with actin. This interaction is inhibited by the position of the troponin-tropomyosin complex when the myocyte is at rest (Zot and Potter, 1987). In this situation, troponin I is weakly associated with troponin C but strongly associated with actin. However, when $[Ca^{2+}]_i$ increases, troponin C binds Ca^{2+} causing troponin I to interact more strongly with troponin C than actin (Flicker et al., 1982). This causes a conformational change which repositions the troponin-tropomyosin complex so that it no longer blocks the interaction of myosin and actin (Langer, 1997). With the hydrolysis of ATP, the myosin molecule then moves along the actin chain to shorten the myofilament. This process is known as the sliding filament theory and results in the contraction of the myocyte (Huxley and Simmons, 1971).

H. Summary

This chapter has reviewed the function of a number of key cellular structures involved in EC coupling. These are shown in Figure 1. Contraction in cardiomyocytes is caused by Ca^{2+} acting on the myofilaments. The rise in $[Ca^{2+}]_i$ that triggers contraction partly results from Ca^{2+} entry via Ca^{2+} channels and the $NaCa_{ex}$, however, Ca^{2+} is also released from the SR by CICR and the VSRM. During relaxation, Ca^{2+} is recycled into the SR by a Ca^{2+} ATPase, and extruded from the cell by the $NaCa_{ex}$ and a sarcolemmal Ca^{2+} ATPase. Proteins that regulate $[Na^+]_i$ homeostasis can also contribute to Ca^{2+} homeostasis due to the presence of the $NaCa_{ex}$. These proteins include Na^+ channels, the Na^+-K^+ ATPase, the Na^+-H^+ exchanger, and the Na^+-HCO_3^- cotransporter. The next chapter will discuss alterations in EC coupling that occur during ischemia, with particular attention given to effects on the cellular structures discussed above.

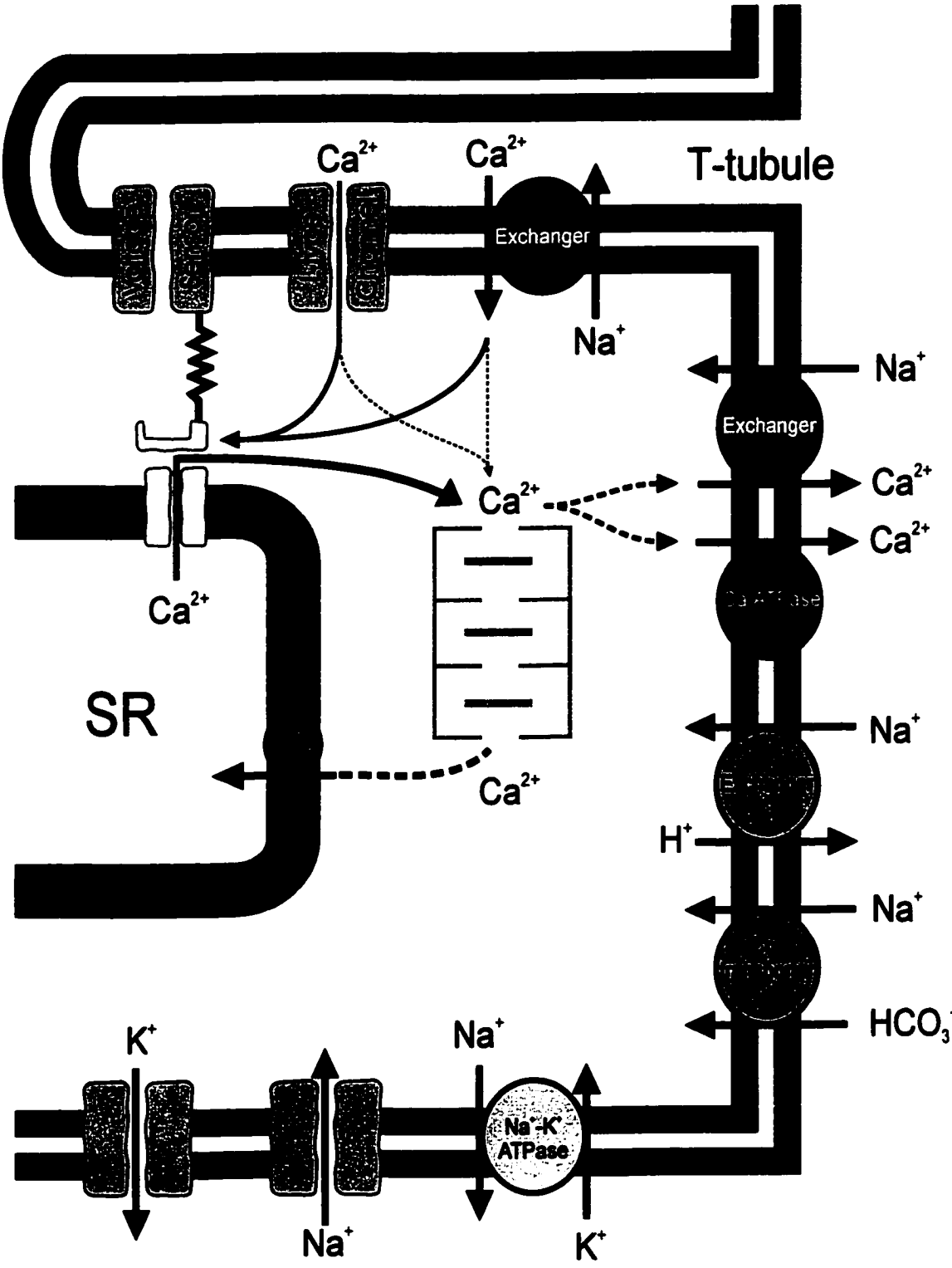


Figure 1

Figure 1. Key cellular structures involved in EC coupling.

The sarcolemma of the cardiomyocyte contains a number of proteins involved in EC coupling. Some of these proteins are located in T-tubules, which are invaginations of the sarcolemma into the interior of the muscle fibre. Contraction of the cardiomyocyte results from Ca^{2+} binding to the myofilaments. During the action potential, Ca^{2+} entering the cell via L-type Ca^{2+} channels and the $NaCa_{ex}$ may act directly on the myofilaments. However, the small increase in $[Ca^{2+}]_i$ that results from Ca^{2+} influx also triggers a large release of Ca^{2+} from the SR through Ca^{2+} release channels (ryanodine receptors). This process is known as Ca^{2+}-induced Ca^{2+} release (CICR). SR Ca^{2+} release can also be directly elicited in response to membrane depolarization by the voltage-sensitive release mechanism (VSRM). During relaxation, Ca^{2+} is recycled into the SR by a Ca^{2+} ATPase. Ca^{2+} is also extruded from the cell by the $NaCa_{ex}$ and a sarcolemmal Ca^{2+} ATPase. The Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter contribute to EC coupling by altering $[Ca^{2+}]_i$ via the $NaCa_{ex}$. During the action potential, Na^+ influx occurs via Na^+ channels and K^+ efflux occurs via K^+ channels, creating an ionic imbalance that is actively reversed by the Na^+-K^+ ATPase.

3. Myocardial Changes During Ischemia

Current understanding of the pathophysiology of ischemia has largely been made possible using models of ischemia. The first animal model of cardiac ischemia was described by Pierre Chirac in 1698 (Leibowitz, 1970). In his experiments on *in vivo* dog hearts, Chirac artificially produced myocardial ischemia by ligation of a coronary artery. In response to the ligation, he observed what is probably the most obvious consequence of cardiac ischemia, an almost complete cessation of contractile function. Another cardinal symptom of ischemia was described in this model by Fred Smith (1918) who observed profound changes in the electrocardiogram following coronary ligation. Thus, it has been known for a long time that ischemia produces dramatic effects on myocardial electrical and contractile function. However, the mechanisms responsible for these changes have proven to be much less obvious. This chapter will discuss what is currently known of the complex physiological alterations that contribute to contractile failure and electrical abnormalities in myocardial ischemia.

A. Biochemical Changes During Ischemia

According to the classic definition, ischemia occurs when the oxygen supply to the heart becomes insufficient to meet the demands of oxidative metabolism (Jennings, 1970). Disruption of oxidative metabolism can lead to significant disruptions in cellular function. In order to understand the biochemical implications of ischemia, we must first briefly review cellular respiration under normoxic conditions. Under aerobic conditions, NADH and $FADH_2$ are produced by glycolysis and by oxidation of glucose and fatty acids in the citric acid cycle. Then, in the electron transport chain, electrons are passed

from NADH and $FADH_2$ through a series of proteins. Oxygen is the final electron acceptor, and is reduced to form water. The energy provided by this process is used to drive protons out of the mitochondria against the electrochemical gradient. Re-entry of protons into the mitochondrial matrix through the F_0F_1 complex generates ATP (Nahrwold and Cohen, 1975).

During ischemia, the electron transport chain is stopped since oxygen is not available as the final electron acceptor. Instead, pyruvate is reduced to form lactic acid without the formation of ATP. Surprisingly, however, ATP levels remain relatively unchanged for the initial 10-15 min of ischemia (Ferrari et al., 1998). This is the result of several protective mechanisms which are activated in the early moments of ischemia. Cellular contractions are markedly reduced almost immediately upon ischemia which greatly reduces ATP demand (Lee and Allen, 1991). As well, ATP continues to be produced in small amounts by anaerobic glycolysis, a process that is stimulated in early ischemia (Jennings et al., 1993). Glycolysis is also facilitated during ischemia by increased activity of transmembrane glucose transporters, which compensates for decreased intracellular glucose levels (Young et al., 1997).

During prolonged periods of ischemia, ATP production eventually declines as glycolysis is inhibited by intracellular acidosis (Dennis et al., 1991). Thus, there is accumulation of the products of ATP hydolysis: ADP, H^+, and inorganic phosphate (P_i). Chemical thermodynamics dictate that an increase in [ADP] and [P_i] decreases the energy released by ATP hydrolysis (Fiolet et al., 1984). Therefore, ischemia is associated with decreased energy availability as described by a reduction in [ATP] / [ADP]. Inhibition of oxidative metabolism and the decrease in [ATP] / [ADP] ratio have important

consequences on cellular physiology during ischemia. As will be discussed below, these consequences involve changes in several intracellular ($[X]_i$) and extracellular ($[X]_o$) ion concentrations including: $[K^+]_o$, $[H^+]_i$ and $[H^+]_o$, $[Na^+]_i$, and $[Ca^{2+}]_i$.

B. Alterations in Ion Concentrations During Ischemia

i) Increase in $[K^+]_o$

In cardiomyocytes, $[K^+]_i$ is normally maintained near 130 mM and $[K^+]_o$ is approximately 4 mM. This concentration gradient is maintained by the Na^+-K^+ ATPase which compensates for changes in $[K^+]_i$ and $[Na^+]_i$ during the action potential. However, this equilibrium is upset during ischemia, and $[K^+]_o$ is increased to approximately 8 mM (Wilde and Aksnes, 1995). This rise in $[K^+]_o$ partly results from reduced activity of the Na^+-K^+ ATPase caused by the decrease in [ATP] / [ADP] (Daly et al., 1984). As well, oxygen-derived free radicals, which are produced during ischemia, have also been shown to inhibit the Na^+-K^+ ATPase (Shattock and Matsuura, 1993). Thus, the increase in $[K^+]_o$ observed during ischemia is believed to be partly the result of decreased K^+ influx by the Na^+-K^+ ATPase.

Enhanced K^+ efflux may also contribute to the increase in $[K^+]_o$ observed during ischemia. ATP-sensitive K^+ channels are activated by the low ATP levels associated with ischemia (Nichols and Lederer, 1991). As well, Na^+-activated K^+ channels have been shown to be activated by high $[Na^+]_i$ produced during ischemia (Kameyama et al., 1984). Activation of these two types of K^+ channels in ischemia results in K^+ efflux. The resulting increase in $[K^+]_o$ causes positive feedback since most K^+ channels increase their

conductance at elevated $[K^+]_o$ (Carmeliet, 1999). Thus, a large number of K^+ channels contribute to K^+ loss in ischemia once the rise in $[K^+]_o$ has been initiated.

The cellular mechanisms that lead to elevated $[K^+]_o$ are believed to play a role in arrhythmogenesis during ischemia. $[K^+]_o$ elevation contributes to ischemia-induced depolarization, which in turn, increases the likelihood of triggered cardiac arrhythmia (Ferrier, 1977; Janse and Wit, 1989). As well, activation of ATP-sensitive K^+ channels has been shown to be responsible for abbreviation of the action potential in ischemia (Noma, 1983, Grover and Garlid, 2000). Shortening of action potential duration (APD) is believed to underlie re-entrant arrhythmias (Bekheit et al., 1990). Thus, alterations in K^+ homeostasis may contribute to the generation of both triggered and re-entrant arrhythmias during ischemia.

ii) Intracellular and Extracellular Acidosis

Under normal conditions, intracellular pH (pH_i) is approximately 7.2 while extracellular pH (pH_o) is 7.4. However, if protons were in electrochemical equilibrium across the cell membrane, it would be predicted that pH_i should be close to 6.0. Therefore, pH_i must be actively maintained by pumping H^+ out of the cell and/or OH^- (HCO_3^-) into the cell (Grace et al., 1993). This task is accomplished by the concerted action of the Na^+-H^+ exchanger and the Na^+-HCO_3^- cotransporter, which act to decrease intracellular pH. As well, elimination of CO_2 reduces $[H^+]_i$ by driving the following reaction to the right:

$$HCO_3^- + H^+ \Rightarrow H_2CO_3 \Rightarrow CO_2 + H_2O$$

During ischemia, $[H^+]_i$ regulation is disrupted resulting in intracellular acidosis. It has been reported that intracellular pH can drop as low as 6.1 following 15 min of ischemia (Mohabir et al., 1991). This increase in $[H^+]_i$ during ischemia partly results from increased proton generation. Ischemia is associated with net ATP hydrolysis, which increases $[H^+]_i$ (Dennis et al., 1991). Lactate$^-$ accumulation also increases $[H^+]_i$ (Dennis et al., 1991), as does production of ATP by glycolysis instead of by oxidative metabolism (Katsura et al., 1992). Intracellular acidosis during ischemia is also caused by deficient removal of protons. CO_2 accumulates in ischemia and inhibits the removal of protons by the reaction shown above (Johnson et al., 1995). As well, proton extrusion via the Na^+-H^+ exchanger and the Na^+-HCO_3^- cotransporter is decreased during late ischemia, as $[Na^+]_i$ increases (Anderson et al., 1991). However, activity of the Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter in early ischemia is believed to cause extracellular acidosis (Katsura et al., 1992).

Acidosis during ischemia has a number of important consequences for EC coupling. It is widely believed that acidosis contributes to contractile depression in ischemia (Orchard and Kentish, 1990). However, the mechanisms responsible for this action are complicated since acidosis affects virtually every step in the EC coupling pathway. With only a few exceptions, most plasma membrane channels are inhibited by intracellular and/or extracellular acidosis (Carmeliet, 1999). Observations that $I_{Ca\text{-}L}$ is reduced during acidosis suggest that trigger Ca^{2+} for CICR might be decreased in ischemia (Sperelakis, 1990). Acidosis also inhibits activity of the SR Ca^{2+} release channel and the $NaCa_{ex}$ (Rousseau et al., 1986; Doering and Lederer, 1993; Xu et al., 1996), and decreases the Ca^{2+}-binding sensitivity of the myofilaments (Lee and Allen,

1991). In later sections, a more detailed consideration of the effects of acidosis on EC coupling in ischemia will be presented, after other important consequences of ischemia have been discussed.

iii) Na^+ Loading

A number of membrane-bound proteins are involved with cellular Na^+ homeostasis, including: Na^+ channels, the $NaCa_{ex}$, the Na^+-K^+ ATPase, the Na^+-H^+ exchanger, and the Na^+-HCO_3^- cotransporter. Under normal conditions, these proteins collectively maintain $[Na^+]_i$ at between 6–10 mM in ventricular myocytes (Bers, 2001). During ischemia, however, $[Na^+]_i$ is markedly increased. Indeed, studies using NMR have measured intracellular Na^+ levels as high as 20-25 mM after 20 min of ischemia (van Echteld et al., 1991; Pike et al., 1993).

The increase in $[Na^+]_i$ observed during ischemia is largely the result of decreased Na^+ extrusion by the Na^+-K^+ ATPase (Donoso et al., 1992). As mentioned in the previous discussion of alterations in $[K^+]_o$, activity of the Na^+-K^+ ATPase is inhibited by oxidative stress and the decrease in [ATP] / [ADP] ratio caused by ischemia (Daly et al., 1984, Shattock and Matsuura, 1993). Elevations in $[Na^+]_i$ are also believed to result from increased Na^+ influx. The Na^+-H^+ exchanger contributes significantly to the rise in $[Na^+]_i$ in response to intracellular acidosis (Anderson et al., 1991; Pike et al., 1993). In addition, inward Na^+ leak is triggered in ischemia by accumulation of lysophosphatidylcholine, a membrane-lipid metabolite that causes Na^+ channels to open at sub-threshold potentials (Burnashev et al., 1991; Undrovinas et al., 1995). Thus, it is believed that Na^+ loading during ischemia results from both reduced Na^+ extrusion and increased Na^+ influx. As

will be discussed in the following section, alterations in $[Na^+]_i$ have significant implications for Ca^{2+} homeostasis in ischemia.

iv) Changes in $[Ca^{2+}]_i$

Under normal conditions, diastolic $[Ca^{2+}]_i$ is approximately 100 nM and systolic $[Ca^{2+}]_i$ is generally between 500 and 1000 nM (Bers, 2001). However, the conditions of ischemia cause dramatic alterations in Ca^{2+} homeostasis. Perhaps the most consequential of these changes is a marked increase in diastolic $[Ca^{2+}]_i$, which can reach values close to 1000 nM following 15-20 min of global ischemia (Marban et al., 1990; Carrozza et al., 1992). Systolic $[Ca^{2+}]_i$ also increases during ischemia, although there is disagreement as to the extent of this elevation. Many studies have reported that the ischemic increase in systolic $[Ca^{2+}]_i$ is proportionally smaller than the increase in diastolic $[Ca^{2+}]_i$, suggesting that the magnitude of Ca^{2+} transients is reduced in ischemia (Smith and Allen, 1988; Wagner et al., 1990; Nishida et al., 1993; Schumacher et al., 1998; Arutunyan et al., 2001; Seki et al., 2001). However, a few studies have observed no change in the size of Ca^{2+} transient (Lee et al., 1998; Carrozza et al., 1992; Camacho et al., 1993), while others have reported an increase (Mohabir et al., 1991; Lee and Allen, 1992). Thus, it is well established that ischemia produces an increase in intracellular Ca^{2+} levels, but it remains unclear whether ischemia is associated with changes in the magnitude of Ca^{2+} transients. In the remainder of this thesis, the condition that results from elevations in diastolic and/or systolic $[Ca^{2+}]_i$ will be referred to as Ca^{2+} overload.

A number of mechanisms contribute to Ca^{2+} overload in ischemia. In the early moments of ischemia, intracellular acidosis is believed to initiate the rise in $[Ca^{2+}]_i$ as

protons displace Ca^{2+} from intracellular binding sites (Gambassi et al., 1993). As ischemia continues, further $[Ca^{2+}]_i$ elevation results from both increased Ca^{2+} influx and decreased Ca^{2+} efflux. Elevated levels of $[Na^+]_i$ are thought to promote Ca^{2+} influx by the reverse mode of the $NaCa_{ex}$ (Smith and Allen, 1988; Haigney et al.,1994). As well, the ability of the $NaCa_{ex}$ to extrude Ca^{2+} from the cell is compromised during ischemia as a result of increased $[Na^+]_i$ and intracellular acidosis (Philipson et al., 1982; Smith and Allen, 1988; Doering and Lederer, 1993). Reduction in [ATP] / [ADP] also contributes to reduced Ca^{2+} efflux during ischemia by decreasing activity of the sarcolemmal Ca^{2+} ATPase (Vrbjar et al., 1993). A similar reduction in activity of the SR Ca^{2+} ATPase causes decreased recycling of Ca^{2+} into stores and further elevates $[Ca^{2+}]_i$ (Griese et al., 1988). Thus, Ca^{2+} overload during ischemia results from altered activity of a number of the proteins involved with Ca^{2+} homeostasis.

Changes in $[Ca^{2+}]_i$ play a critical role in determining myocardial injury during ischemia (for review see Silverman and Stern, 1994). Indeed, it has been suggested that $[Ca^{2+}]_i$ may determine whether the cell injury caused by ischemia is reversible or irreversible with reperfusion (Shen and Jennings, 1972). Some of the cellular damage associated with Ca^{2+} overload results from activation of Ca^{2+}-dependent enzymes. For example, Ca^{2+}-dependent proteases have been shown to degrade components of the cytoskeleton (Papp et al., 2000), and to trigger apoptosis (Chen et al., 1998). As well, increased $[Ca^{2+}]_i$ activates phospholipase A2, which degrades membrane-phospholipids to form a number of compounds which are believed to contribute to ischemic myocardial dysfunction (Sargent et al., 1992). As will be discussed in the next chapter, cellular

damage is also indirectly mediated by Ca^{2+} overload through the generation of oxygen-derived free radicals.

Calcium overload in ischemia has a number of consequences for the activity of ion channels and exchangers involved in Ca^{2+} homeostasis. Elevated $[Ca^{2+}]_i$ enhances inactivation of $I_{Ca\text{-}L}$ (McDonald et al., 1994), which is believed to contribute to shortening of the action potential during ischemia (Linz and Meyer, 1997). Ca^{2+} overload also increases activity of the SR Ca^{2+} release channel, which can lead to spontaneous Ca^{2+} release (Fabiato and Fabiato, 1972; Lappe and Lakatta, 1980). This release of Ca^{2+} activates the myofilaments to cause aftercontractions, and also activates the $NaCa_{ex}$ (Lederer and Tsien, 1976). The extrusion of 1 Ca^{2+} in exchange for 3 Na^+ by the $NaCa_{ex}$ causes transient inward current (I_{TI}), and an oscillatory after potential (OAP) (Ferrier, 1977). In multicellular preparations, OAPs can lead to triggered arrhythmias (Ferrier, 1977). Re-entrant arrhythmias are also facilitated by Ca^{2+} overload (de Mello et al., 1975). The gap junction channel, which is the electrical connection between adjacent myocytes, exhibits decreased permeability in the presence of elevated $[Ca^{2+}]_i$ (Weingart, 1977). The result is an electrical uncoupling of neighbouring cells, improper impulse conduction, and increased risk of re-entrant arrhythmias (ter Keurs et al., 2001). In summary, Ca^{2+} overload during ischemia is of considerable clinical relevance as it contributes to cell injury and death, and cardiac arrhythmogenesis.

C. Generation of Oxygen-Derived Free Radicals:

One final pathogenetic consequence of ischemia that should be addressed is the accumulation of free radicals. Free radicals are reactive molecules with unpaired

electrons. During ischemia, several different types of radicals derived from oxygen are believed to cause oxidative damage to cellular structures. The least reactive of these radicals is the superoxide anion, $O_2^{-\bullet}$, which is produced by donation of an extra electron to the oxygen molecule (Ferrari et al., 1991). In the presence of superoxide dismutase, superoxide anion is converted to hydrogen peroxide (H_2O_2) which has greater reactivity. However, the cell is exposed to even greater danger when superoxide combines with nitric oxide to form peroxynitrate ($NO_3^{-\bullet}$), which is highly reactive (Kristian and Siesjo, 1998). As well, H_2O_2 and $O_2^{-\bullet}$ can combine to form the highly reactive hydroxyl radical, $OH^{\bullet}$ (Kukreja and Hess, 1992). Ischemia is associated with both increased production and deficient removal of $O_2^{-\bullet}$, H_2O_2, $NO_3^{-\bullet}$, and $OH^{\bullet}$ (Ferrari et al., 1991).

During ischemia, Ca^{2+} overload damages the mitochondria, allowing high energy electrons to leak out of the electron transport chain (Parr et al., 1975). Although oxygen availability is reduced in ischemia, enough residual oxygen is present to accept these electrons and increase production of $O_2^{-\bullet}$, H_2O_2, $NO_3^{-\bullet}$, and $OH^{\bullet}$ (Ferrari et al., 1991; Kukreja and Hess, 1992). Free radicals are also generated in ischemia by metabolism of arachidonic acid, which is released during ischemia by degradation of membrane phospholipids (Prasad and Kalra, 1988; Sargent et al., 1992). As mentioned previously, phopholipid degradation is mediated by Ca^{2+}-dependent enzymes. Thus, Ca^{2+} overload contributes to free radical generation in ischemia by causing damage to both the electron transport chain and the sarcolemma.

Even in the absence of ischemia, there is continuous production of free radicals. In the final step of aerobic metabolism, oxygen generally accepts electrons from NADH and $FADH_2$ to form water. However, a small proportion of O_2 molecules are instead

reduced to form $O_2^{-\bullet}$ (Beckman and Koppenol, 1996). Under normoxic conditions, these free radicals are eliminated by several mechanisms. Some enzymatic reactions consume reactive oxygen species, such as the conversion of hydrogen peroxide to water in the presence of glutathione peroxidase (Yoshida et al., 1997). As well, a number of substances are present in the cell that can neutralize free radicals. These free-radical "scavengers" include vitamin E, vitamin C, and histidine (Ferrari et al., 1991). During ischemia, neutralization of reactive oxygen species is compromised since free-radical scavengers and the components of eliminatory reactions are less available (Kukreja and Hess, 1992). Therefore, the cell is unable to compensate for the increased production of free radicals in ischemia.

As alluded to in previous sections, accumulation of oxygen-derived free radicals is believed to significantly contribute to cellular damage during ischemia. This cellular damage is wide ranging since free radicals can attack virtually all cellular components. However, of particular interest is the effect of oxygen radicals on ion channels and transporters. Oxidation by free radicals reduces conductance through L-type Ca^{2+} channels (Cerbai et al., 1991) and most types of K^+ channels, with the exception of ATP-sensitive K^+ channels (Jabr and Cole, 1993). In addition, exposure to oxygen radicals causes spontaneous SR Ca^{2+} release, and is thus believed to contribute to the generation of OAPs and triggered arrhythmias in ischemia (Cerbai et al., 1991). Thus, oxidative stress has a number of important consequences for cellular physiology during ischemia. However, as will be discussed in later chapters, the generation of free radicals has even greater implications for reperfusion injury.

D. Summary of EC Coupling in Ischemia:

The conditions of ischemia clearly have complicated effects on the regulators of EC coupling. Several of these ischemic alterations are summarized in Figure 2. $[Na^+]_i$ is increased during ischemia as a result of depressed activity of the Na^+-K^+ ATPase, and by activation of the Na^+-H^+ exchanger following intracellular acidosis. Elevations in $[Na^+]_i$ and acidosis are believed to decrease activity of the $NaCa_{ex}$, thus causing an increase in $[Ca^{2+}]_i$. Ca^{2+} overload during ischemia is also caused by decreased activity of the SR and sarcolemmal Ca^{2+} ATPases.

Ischemic conditions produce a number of cellular alterations which would be expected to reduce the magnitude of Ca^{2+} transients. These include decreased Ca^{2+} entry via L-type Ca^{2+} channels and the $NaCa_{ex}$ (Bersohn et al., 1997), and decreased activity of the SR Ca^{2+} ATPase and SR Ca^{2+} release channel (Griese et al., 1998; Xu et al., 1996). Despite the possibility that the Ca^{2+} transient may be altered, it is not thought that such changes in Ca^{2+} homeostasis are responsible for contractile depression in ischemia (Perez et al., 1999). Instead, it is believed that contractile failure may result from intracellular acidosis and accumulation of inorganic phosphate, which markedly decrease the sensitivity of the myofilaments to Ca^{2+} (Lee and Allen, 1991).

Although it has been suggested that decreased [ATP] / [ADP] could also contribute to inhibition of active myofilament shortening, the time-course of reduction in this ratio does not correlate with changes in contractile function (Koretsune et al., 1991). However, the reduction in [ATP] / [ADP] does correspond to the gradual increase in diastolic pressure that is observed during ischemia (Ventura-Clapier et al., 1994). Indeed, it has been proposed that reduced [ATP] / [ADP] may inhibit myofilament relaxation

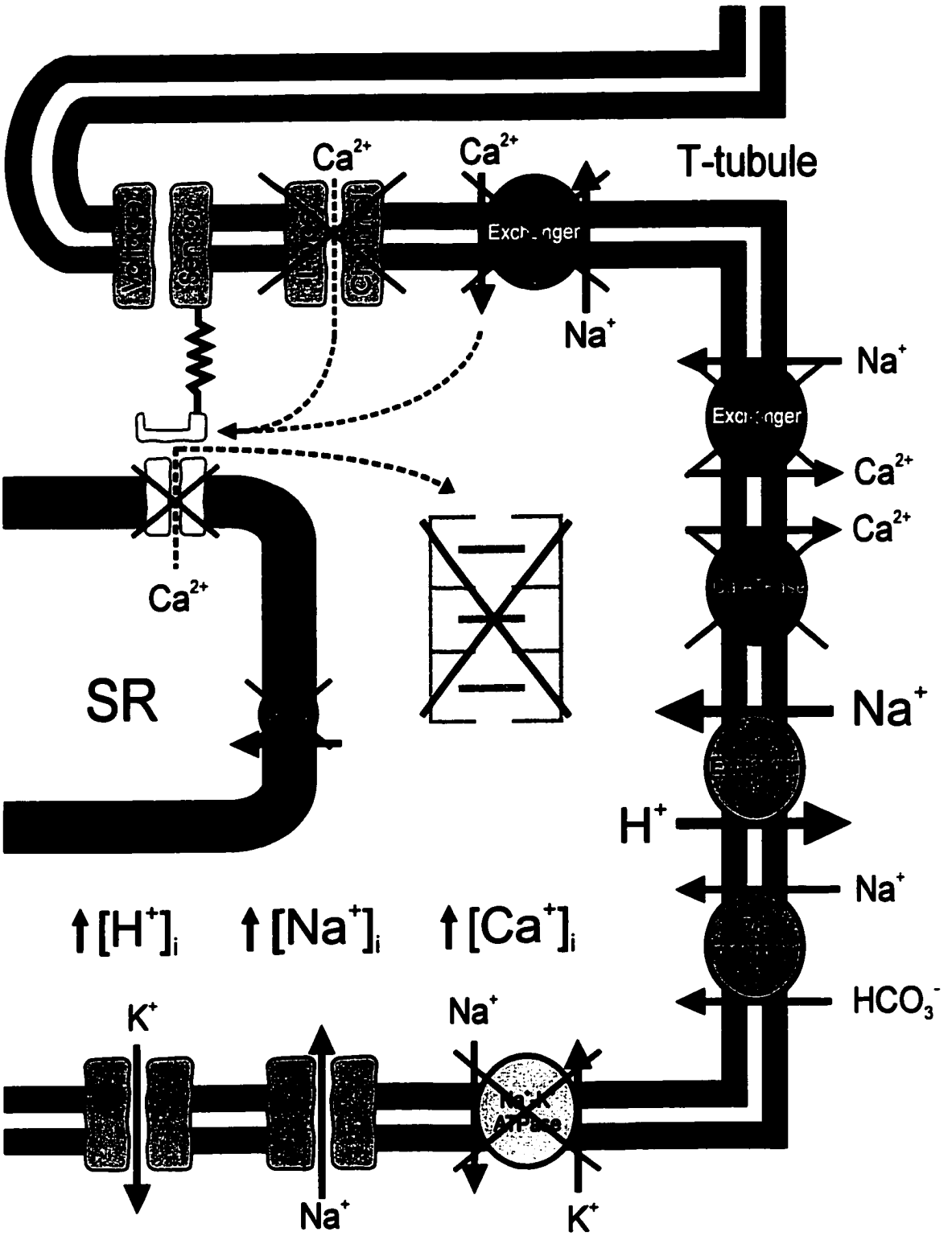

T-tubule
Ca2+
Ca2+
Na+
Na+
Ca2+
Ca2+
Na+
H+
Na+
HCO3-
SR
Ca2+
↑[H+]i
↑[Na+]i
↑[Ca+]i
K+
Na+
Na+
K+

Figure 2

Figure 2. Alterations in EC coupling during ischemia.

Exposure to myocardial ischemia has marked effects on proteins involved in EC coupling. Ischemia causes intracellular acidosis. This rise in $[H^+]_i$ is believed to trigger an increase in $[Na^+]_i$ via the Na^+-H^+ exchanger. Na^+ loading is also caused by decreased activity of the $NaCa_{ex}$. Ischemia is associated with an increase in $[Ca^{2+}]_i$ as a result of decreased Ca^{2+} extrusion by the $NaCa_{ex}$ and decreased activity of the SR and sarcolemmal Ca^{2+} ATPases. Ischemia is also believed to cause reduced Ca^{2+} influx via L-type Ca^{2+} channels and reduced activity of the SR Ca^{2+} release channel. Therefore, Ca^{2+} transients may be decreased. Regardless, contractile depression during ischemia is thought to result from decreased responsiveness of the myofilaments to Ca^{2+}, not alterations in Ca^{2+} homeostasis.

during ischemia by causing actin-myosin crossbridges to lock in the rigor state (Eberli et al., 2000). The resulting diastolic dysfunction can have a number of detrimental consequences in the ischemic myocardium (Kabbani and LeWinter, 2000). As will be discussed in the following chapters of this introduction, myofilament alterations are also of considerable importance in the pathogenesis of reperfusion injury.

4. Myocardial Changes During Reperfusion

While the consequences of myocardial ischemia have been experimentally observed since the 17th century, the consequences of reperfusion were not extensively investigated until the last 25 years. Prior to the late 1970s, little attention was given to experimental observations of reperfusion since it was believed that occluded coronary arteries generally remained blocked (Bolli and Marban, 1999). However, since that time, several studies have demonstrated that many heart disease patients experience spontaneous reperfusion following an ischemic episode (for review see Bolli, 1992). As well, a number of interventional therapies have been developed in recent years which re-establish coronary blood flow, including thrombolytic therapy, angioplasty, and coronary bypass surgery. Therefore, in the modern heart disease patient, reperfusion after myocardial ischemia is a relatively common phenomenon. With this realization, recent research efforts have been directed at examining the nature of myocardial alterations during reperfusion.

Early studies of myocardial reperfusion observed that electrical and contractile abnormalities can result from re-establishment of coronary blood flow (Heyndrickx et al., 1975; Gulker et al., 1977). These observations suggested that although reperfusion is necessary to prevent necrosis of the ischemic tissue, reperfusion can also have harmful

consequences. The extent of the damage caused by reperfusion, however, depends on the severity of the ischemic insult (Bolli, 1992). Reperfusion following relatively brief periods of ischemia (< 30 min), can disrupt cellular function but generally does not cause necrosis or irreversible cell damage (Duncker et al., 1998). However, reperfusion after periods of global ischemia greater than 30 min can cause irreversible contracture and contractile failure, permanent damage to cell structures, and cell death (myocardial infarction) (Langer, 1997). In the remainder of this thesis, however, only myocardial alterations associated with reversible ischemia and reperfusion will be considered.

Reperfusion following reversible myocardial ischemia causes complex myocardial alterations. Early reperfusion is associated with a significant increase in incidence of arrhythmias and a marked increase in diastolic pressure (Vaage and Valen, 1993). Reperfusion also triggers a brief recovery of contractile function, followed by prolonged contractile depression (stunning) (for review see Bolli and Marban, 1999). In this chapter, the biochemical and ionic changes believed to trigger these events will be reviewed. At the end of this chapter, the net effects of these alterations on EC coupling in early reperfusion will be summarized. Changes in EC coupling responsible for stunning will be discussed in chapter 5.

A. Biochemical Changes During Reperfusion

The rapid re-introduction of oxygen upon reperfusion restarts the electron transport chain and halts the progressive decrease in ATP levels that occurs during ischemia (Marban et al., 1990). Despite the resumption of oxidative respiration, ATP levels remain depressed at approximately 50% of normal values with little recovery until

at least 30 min of reperfusion (Marban et al., 1994). However, creatine phosphate levels are restored early in reperfusion, leading to a rapid decrease in [ADP] and thus, an increase in [ATP] / [ADP] (Marban et al., 1990). Therefore, reperfusion following ischemia improves energy availability, but not does not immediately restore energy levels to normal values.

Several mechanisms have been proposed to explain the incomplete recovery of energy levels in early reperfusion. Rates of glycolysis are increased in early reperfusion (Jeremy et al., 1993). However, post-ischemic ATP production is reduced from control levels as a result of abnormalities in mitochondrial function that impact on oxidative respiration. Elevated Ca^{2+} levels in early reperfusion cause opening of the mitochondrial megachannel, which is a non-specific transition pore (Lemasters et al., 1997). Leak of protons into the mitochondrion through the megachannel disrupts the mitochondrial electrical gradient, and compromises production of ATP (Bond et al., 1994). Early reperfusion is also associated with increased ATP demand. Activity of the Na^+-K^+ ATPase may be increased to counteract ischemic elevations in $[Na^+]_i$ (Van Emous et al., 1998). As well, ATP use appears to be relatively inefficient in early reperfusion (Crompton et al., 1993). Indeed, oxygen consumption rapidly recovers to pre-ischemic levels in reperfusion, despite reduced energy availability (Gorge et al., 1991). Therefore, it appears that the reduction in [ATP] / [ADP] during reperfusion results both from decreased ATP production and increased ATP demand. As will be discussed in the following sections, the degree of recovery of [ATP] / [ADP] in early reperfusion has significant implications for re-establishment of ionic homeostasis.

B. Alterations in Ion Concentrations

i) Role of the Na^+-K^+ ATPase

The recovery of Na^+-K^+ ATPase activity is of key importance to post-ischemic changes in $[K^+]_o$, $[Na^+]_i$, and $[Ca^{2+}]_i$. However, the level of pump activity in early reperfusion has been debated. A number of studies have examined Na^+-K^+ ATPase acitivity using *in-vitro* preparations of post-ischemic myocardium (Daly et al., 1984; Kim and Akera, 1987; Avkiran et al., 1996; Ramasamy et al., 1999). These studies have reported that Na^+-K^+ ATPase activity is decreased in reperfusion, which might be expected based on the delayed post-ischemic recovery of [ATP] / [ADP]. Only one study, however, has examined pump activity in the intact post-ischemic myocardium. In this study, Van Emous et al (1998) showed that post-ischemic changes in $[Na^+]_i$ are dependent on rapid recovery of activity of the Na^+-K^+ ATPase upon reperfusion. In fact, they calculated that in the first 40 s of reperfusion, pump activity is dramatically increased above control levels, and is close to maximal values. To explain the dissimilarity of results from their study and *in vitro* studies, Van Emous et al (1998) suggested that ATPase activity may be inaccurately assessed by *in vitro* analysis, due to the absence of pump-regulating factors. Clearly, further *in vivo* studies are needed to verify the finding that Na^+-K^+ ATPase activity is dramatically enhanced upon reperfusion.

If confirmed, the observation that Na^+-K^+ ATPase activity in increased upon reperfusion could partly account for action potential abbreviation during early reperfusion (Van Emous et al., 1998). Activation of the pump creates an outward current (3 Na^+ for 2 K^+) which promotes repolarization of the action potential. However, the exact

contribution of the Na^+-K^+ ATPase to post-ischemic recovery of APD has yet to be determined. As will be discussed below, activity of the Na^+-K^+ ATPase in early reperfusion may also contribute to a number of other myocardial alterations.

ii) Recovery of $[K^+]_o$

As discussed earlier, ischemia produces a marked elevation in $[K^+]_o$. However, re-establishment of blood flow quickly washes away excessive extracellular K^+, returning $[K^+]_o$ to baseline levels (Hill and Gettes, 1980). Thus, resting membrane potential rapidly recovers upon reperfusion (Coronel et al., 1991). Indeed, extracellular K^+ levels have been reported to be temporarily reduced below control levels in early reperfusion, causing hyperpolarization (Aksnes et al., 1989). This excessive reduction in $[K^+]_o$ may result from increased Na^+-K^+ ATPase activity in early reperfusion (Van Emous et al., 1998).

Although ischemic alterations in $[K^+]_o$ are quickly reversed when blood flow is re-established, K^+ channels activated during ischemia may continue to abbreviate the action potential during reperfusion. Shigematsu et al (1995) reported that glibenclamide, an antagonist of ATP-sensitive K^+ channels, attenuated post-ischemic shortening of the action potential. This observation suggests that delayed recovery of [ATP] in reperfusion may cause significant activation of ATP-sensitive K^+ channels. It might also be expected that post-ischemic elevations in $[Na^+]_i$ would cause persistent activation of Na^+-activated K^+ channels. However, the activity of these latter channels has not been investigated in reperfusion.

iii) Recovery From Intracellular and Extracellular Acidosis

Ischemia produces marked intracellular and extracellular acidosis. However, when blood flow is re-established, pH_o recovers rapidly as elevated extracellular levels of H^+, lactate$^-$, and CO_2 are restored (Vandenberg et al., 1993; Hendrikx et al., 1994). With restoration of normal pH_o, a proton gradient develops across the sarcolemma which promotes removal of intracellular H^+ by the Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter (Dennis et al., 1991). Thus, within a few minutes of reperfusion, pH_i and pH_o recover to normal levels (Vandenberg et al., 1993).

Recovery from acidosis has a number of important consequences for cellular physiology in reperfusion. Perhaps most importantly, the removal of intracellular protons by the Na^+-H^+ exchanger and Na^+-HCO_3^- delays recovery of normal $[Na^+]_i$ following Na^+ loading in ischemia (Dennis et al., 1991; Pike et al., 1993). As well, proteins involved in EC coupling which are inhibited by acidosis during ischemia, such as the SR Ca^{2+} release channel and the $NaCa_{ex}$ (Doering and Lederer, 1993, Xu et al., 1996), would be expected to exhibit restored activity upon recovery of pH. In addition, the Ca^{2+} responsiveness of the myofilaments is restored in the initial moments of reperfusion, following the rapid decrease in $[H^+]_i$ and P_i (Mosca et al., 1996; Vandenberg et al., 1993; Hendrikx et al., 1994; Harada et al., 1994). The importance of these alterations to EC coupling in early reperfusion will be discussed in a later section.

iv) Delayed Recovery of $[Na^+]_i$

Although myocytes recover quickly from ischemic elevation of $[K^+]_o$, $[H^+]_o$, and $[H^+]_i$ in early reperfusion, several studies have reported that $[Na^+]_i$ levels remain

significantly elevated for 5-10 min of reperfusion (Pike et al., 1990; Pike et al., 1993, Van Emous et al., 1998). However, intracellular Na^+ levels do not exceed ischemic values in early reperfusion. This observation is surprising since extensive Na^+ influx occurs in reperfusion from activation of the Na^+-H^+ exchanger and the Na^+-HCO_3^- cotransporter (Dennis et al., 1991; Pike et al., 1993). As well, ischemic accumulation of lysophosphatidylcholine continues in reperfusion causing excessive Na^+ entry through Na^+ channels (Sargent et al, 1993). However, increased activity of the Na^+-K^+ ATPase appears to protect against further Na^+ loading in early reperfusion (Van Emous et al., 1998).

Several lines of evidence support the involvement of Na^+ in reperfusion-induced injury in the heart. Measured elevations of $[Na^+]_i$ correspond closely with the time-course of myocardial damage in reperfusion (Pike et al., 1990; Pike et al., 1993; Van Emous et al., 1998). As well, pharmacological inhibitors of the Na^+-H^+ exchanger reduce post-ischemic Na^+ entry and attenuate reperfusion injury (Karmazyn, 1988, Ito et al., 1999). Similar results have been reported when Na^+ influx by the Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter is inhibited by reperfusion with low Na^+ or acidotic solutions (Kitakaze et al., 1988; Tosaki et al., 1989). Thus, recovery of post-ischemic $[Na^+]_i$ has significant implications for reperfusion injury. As will be discussed below, the detrimental consequences of elevated $[Na^+]_i$ appear to be mediated via alterations in Ca^{2+} homeostasis.

v) Exacerbation of Ca^{2+} Overload

It is well established that reperfusion injury is largely the result of post-ischemic alterations in $[Ca^{2+}]_i$ (for reviews see Pierce and Czubryt, 1995; Maxwell and Lip, 1997). Many studies in intact hearts have observed that myocardial reperfusion causes a further elevation of diastolic $[Ca^{2+}]_i$ beyond ischemic levels (Carrozza et al., 1992; Harada et al., 1994; Meissner and Morgan, 1995; Seki et al., 2001; Varadarjan et al., 2001). As well, systolic Ca^{2+} levels are dramatically increased in the first few minutes of reperfusion, leading to an increased magnitude of Ca^{2+} transients (Carrozza et al., 1992; Harada et al., 1994; Meissner and Morgan, 1995; Varadarajan et al., 2001). However, both diastolic and systolic $[Ca^{2+}]_i$ have been observed to return to pre-ischemic levels within 5-10 min of reperfusion (Carrozza et al., 1992; Harada et al., 1994; Varadarajan et al., 2001).

Post-ischemic Ca^{2+} accumulation is prevented when the myocardium is reperfused with solutions containing low $[Ca^{2+}]$ (Shine et al., 1978; Tani and Neely, 1990). Thus, it is believed that Ca^{2+} influx is an important contributor to the increase in cytosolic $[Ca^{2+}]$ triggered by reperfusion. Post-ischemic Ca^{2+} overload may partly result from Ca^{2+} entry via L-type Ca^{2+} channels. Application of Ca^{2+} channel blockers just prior to reperfusion reduces Ca^{2+} accumulation and myocardial damage (Prxyklenk, 1988; Smart et al., 1997). However, the therapeutic benefits of $I_{Ca\text{-}L}$ blockade in reperfusion may be limited, since $I_{Ca\text{-}L}$ is markedly reduced in post-hypoxic cardiomyocytes after only a few seconds of re-oxygenation (Benndorf et al., 1991). These observations suggest that Ca^{2+} influx via $I_{Ca\text{-}L}$ makes only a minor contribution to Ca^{2+} overload in early reperfusion.

Several studies have provided evidence that post-ischemic Ca^{2+} influx may be largely mediated by the $NaCa_{ex}$ acting in reverse mode. Tani and Neely (1989) suggested this role for the $NaCa_{ex}$ after observing that the degree of Ca^{2+} overload in reperfusion is proportional to the magnitude of Na^+ loading. Support for this view comes from the observation that selective inhibition of reverse-mode Na^+-Ca^{2+} exchange by KB-R794 protects against reperfusion injury (Ladilov et al., 1999; Elias et al., 2001). Also, activity of the $NaCa_{ex}$ is believed to be increased in early reperfusion due to oxidative stress and recovery from acidosis (Doering and Lederer, 1993; Goldhaber and Liu, 1994; Goldhaber, 1996; Chesnais, 1999). Therefore, Ca^{2+} entry by reverse-mode exchange is likely an important contributor to post-ischemic elevations in $[Ca^{2+}]_i$ (Elias et al., 2001).

Although Ca^{2+} entry via the $NaCa_{ex}$ appears to be a significant component of the large Ca^{2+} transient observed in early reperfusion, the importance of Ca^{2+} released by the SR is much less clear. A number of *in vitro* studies have reported that Ca^{2+} release may be decreased in reperfusion since protein levels and conductivity of the SR Ca^{2+} release channel are reduced (Holmberg and Williams, 1992; Valdivia et al., 1997; Osada et al., 1998). On the other hand, activity of the SR Ca^{2+} release channel might be expected to increase in early reperfusion as the myocardium recovers from acidosis (Rousseau et al., 1986; Xu et al., 1994). Clearly, additional studies are needed to clarify the contribution of SR Ca^{2+} release to the overshoot of Ca^{2+} transients in early reperfusion.

The elevation in diastolic $[Ca^{2+}]_i$ observed upon reperfusion is believed to result from decreased recycling and extrusion of intracellular Ca^{2+}. The activity of both the sarcolemmal and SR Ca^{2+} ATPases remain reduced in reperfusion (Samouilidou et al., 1998; Temsah et al., 1999; Osada et al., 2000), possibly as a consequence of oxidative

stress and reduced ATP levels (Valdivia et al., 1997; Temsah et al., 1999). As well, the SR becomes leaky in reperfusion as exposure to Ca^{2+} overload and oxygen radicals causes the SR to open spontaneously (Fabiato and Fabiato, 1972; Lappe and Lakatta, 1980; Cerbai et al., 1991). It does not, however, appear that Ca^{2+} influx is a direct cause of the increase in diastolic Ca^{2+} levels. Even under the altered conditions of early reperfusion, the reversal potential of the $NaCa_{ex}$ remains well positive of the membrane potential. Therefore, Ca^{2+} influx by this mechanism probably only directly contributes to the overshoot of $[Ca^{2+}]_i$ during systole.

As previously discussed, myocardial Ca^{2+} overload during ischemia has a number of detrimental consequences. In reperfusion, the repercussions of Ca^{2+} overload are amplified since $[Ca^{2+}]_i$ is elevated beyond ischemic levels. As in ischemia, Ca^{2+} overload in reperfusion is believed to trigger cell injury by activation of Ca^{2+}-dependent proteases and phospholipase A2 (Sargent et al., 1992; Chen et al., 1998). Post-ischemic elevations in $[Ca^{2+}]_i$ are also partly responsible for the high incidence of cardiac arrhythmias in early reperfusion (Bers, 2001). Ca^{2+} overload can facilitate both triggered and re-entrant arrhythmias by mechanisms described earlier. Finally, post-ischemic Ca^{2+} overload enhances inactivation of $I_{Ca\text{-}L}$, which may contribute to action potential abbreviation in early reperfusion. Therefore, Ca^{2+} overload in reperfusion has many important implications for myocardial function in reperfusion.

C. Additional Accumulation of Oxygen-Derived Free Radicals:

As we have previously discussed, free radicals are formed when oxygen accepts high-energy electrons. During ischemia, intracellular Ca^{2+} overload promotes the

formation of oxygen radicals by disrupting the electron transport chain and by triggering degradation of membrane phospholipids (Parr et al., 1975; Prasad and Kalra, 1988; Sargent et al., 1992). However, free radical generation is somewhat limited during ischemia because oxygen availability is reduced (Kukreja and Hess, 1992). In reperfusion, re-introduction of oxygen and exacerbation of Ca^{2+} overload create an environment which is very conducive to radical formation (for review see Lefer and Granger, 2000). In addition, oxygen radicals may be released from neutrophils, which exhibit increased adhesion in reperfusion (Hansen, 1995). It has also been suggested that free radical production could be increased in reperfusion since ischemic conditions cause the breakdown of purine nucleotides to hypoxanthine, and promote the conversion of xanthine dehydrogenase to xanthine oxidase (Xia and Zweier, 1995). Upon re-introduction of oxygen, xanthine oxidase may catalyze the oxidation of hypoxanthine to form urate and $O_2^{-\bullet}$ (Xia and Zweier, 1995). However, in the human heart, xanthine oxidase levels are very low, and thus, this mechanism has been suggested to be only a minor contributor to post-ischemic oxidative stress (Muxfeldt and Schaper, 1987; Coudray et al., 1994). Nevertheless, several studies have reported that total oxygen radical production is markedly increased upon reperfusion (Kramer et al., 1987; Zweier et al., 1987; Garlick et al., 1987).

The consequences of oxidative stress have been discussed previously. Briefly, oxidation by oxygen free radicals during reperfusion has been suggested to cause damage to proteins of the sarcolemma and the SR. In particular, oxygen radicals are believed to reduce $I_{Ca\text{-}L}$ and most K^+ currents, reduce activity of Ca^{2+} ATPases, and to cause spontaneous opening of the SR Ca^{2+} release channel (Cerbai et al., 1991; Jabr and Cole;

1993; Temsah et al., 1999). The importance of these actions on EC coupling in early reperfusion will be discussed in the following section.

D. Changes in EC Coupling During Early Reperfusion:

This chapter has outlined a number of myocardial alterations that occur during reperfusion. These post-ischemic changes are responsible for rather complicated modifications in EC coupling. Indeed, EC coupling is dramatically altered as reperfusion progresses. Therefore, for clarity, only changes in EC coupling that occur in the first few minutes of reperfusion will be summarized here. A review of EC coupling in late reperfusion will be provided in the next chapter which discusses the mechanisms of stunning.

Figure 3 shows that, upon reperfusion, myocytes recover from intracellular acidosis as a result of proton extrusion by the Na^+-H^+ exchanger and Na^+-HCO_3^- cotransporter. This action delays recovery from ischemic elevations in $[Na^+]_i$ for several minutes, although the Na^+-K^+ ATPase may be reactivated. In intact hearts, reperfusion also induces a further increase in both diastolic and systolic $[Ca^{2+}]_i$ beyond ischemic levels. Post-ischemic elevations in diastolic $[Ca^{2+}]_i$ result from reduced activity of the SR and sarcolemmal Ca^{2+} ATPases, which are inhibited by oxidative stress and decreased [ATP] / [ADP]. However, post-ischemic elevation of systolic $[Ca^{2+}]_i$ is believed to largely result from Ca^{2+} influx (Shine et al., 1978; Tani and Neely, 1989). Although $I_{Ca\text{-}L}$ may be reduced in early reperfusion, reverse-mode $NaCa_{ex}$ is thought to be dramatically increased in early reperfusion due to maintained elevations in $[Na^+]_i$ (Tani and Neely, 1990; Elias et al., 2001). Activity of the $NaCa_{ex}$ has also been reported to be increased by

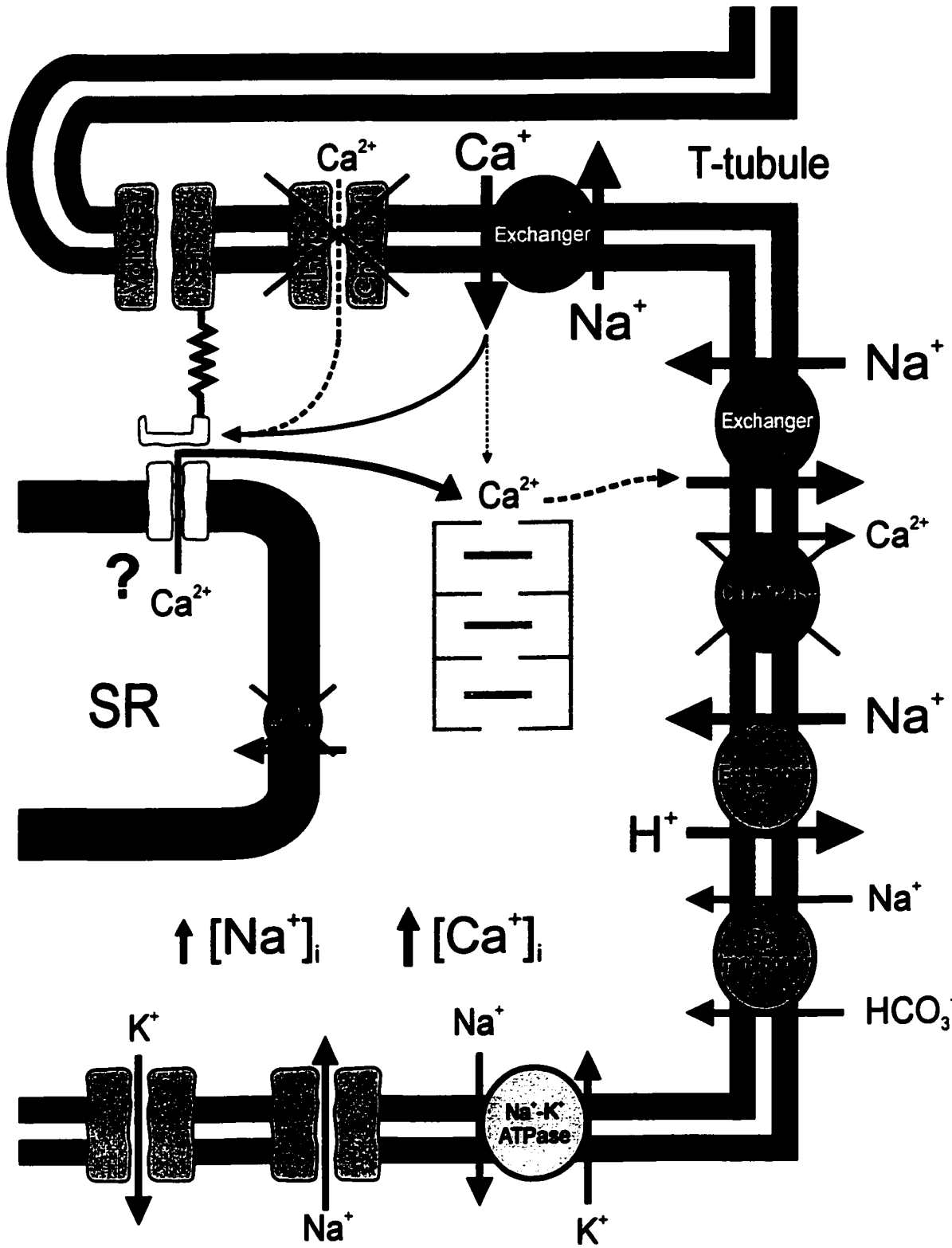

T-tubule
Exchanger
Exchanger
SR
?
Ca^{2+}
Ca^{2+}
Ca^{+}
Na^{+}
Na^{+}
Ca^{2+}
Na^{+}
H^{+}
Na^{+}
HCO_3^-
$\uparrow [Na^+]_i$
$\uparrow [Ca^+]_i$
K^{+}
Na^{+}
Na^{+}-K^{+} ATPase
Na^{+}
K^{+}

Figure 3

Figure 3. Alterations in EC coupling during early reperfusion.

Reperfusion has a number of important effects on proteins involved in EC coupling. Some of these effects rapidly reverse physiological changes induced by ischemia, while other ischemic alterations are exacerbated. The Na^+-K^+ ATPase may be reactivated early in reperfusion. However, the Na^+-H^+ exchanger is also activated to initiate recovery from intracellular acidosis. Thus, recovery from ischemic elevation of $[Na^+]_i$ is somewhat delayed in reperfusion. Diastolic $[Ca^{2+}]_i$ is elevated beyond ischemic levels upon reperfusion as activity of the SR and sarcolemmal Ca^{2+} ATPases remains depressed. Early reperfusion is also associated with a substantial increase in systolic $[Ca^{2+}]_i$, and thus, an increase in Ca^{2+} transients. This brief overshoot of Ca^{2+} transients elicits large contractions since myofilament responsiveness to Ca^{2+} quickly recovers upon reperfusion. The overshoot of Ca^{2+} transients is thought to largely result from Ca^{2+} influx via the $NaCa_{ex}$. Ca^{2+} influx via L-type Ca^{2+} channels may be reduced during early reperfusion. Whether the function of the SR Ca^{2+} release channel is altered in reperfusion is unclear.

post-ischemic oxidative stress and recovery from acidosis (Doering and Lederer, 1993; Goldhaber and Liu, 1994; Goldhaber, 1996; Chesnais, 1999). The contribution of SR Ca^{2+} release to the increase in systolic $[Ca^{2+}]_i$ is unclear, since it is controversial whether activity of the SR Ca^{2+} release channel is altered in early reperfusion.

The increase in systolic $[Ca^{2+}]_i$ that occurs upon reperfusion is larger than the increase in diastolic $[Ca^{2+}]_i$. Thus, an overshoot of Ca^{2+} transients is frequently observed. Importantly, early reperfusion is also associated with rapid recovery of myofilament Ca^{2+} responsiveness, as myocytes recover from ischemic elevations of $[H^+]_i$ and P_i. Thus, the large Ca^{2+} transient triggers a rapid recovery of contractile function in early reperfusion, which sometimes exceeds pre-ischemic levels (Kusuoka and Marban, 1992; du Toit and Opie, 1992).

Early reperfusion is also associated with myocardial contracture and a resulting increase in diastolic pressure (Schaff et al., 1981). Several studies have observed that attenuation of Ca^{2+} overload in reperfusion attenuates this elevation of diastolic pressure (Narita et al., 1983; Applegate et al., 1987; Yamamoto et al., 2000). Therefore, it has been suggested that accumulation of Ca^{2+} during early reperfusion prevents proper myofilament relaxation (Gao et al., 1995). However, diastolic pressure remains elevated in reperfusion long after myocytes recover from diastolic Ca^{2+} overload (Varadarajan et al., 2001; Harada et al., 1994). Thus, other factors must also contribute to post-ischemic contracture. One possibility is that myofilament relaxation could be inhibited in late reperfusion by the same mechanism that causes diastolic pressure to increase during ischemia. Eberli et al (2000) have proposed that a reduction in [ATP] / [ADP] can cause actin-myosin crossbridges to lock in the rigor state, preventing normal relaxation.

Indeed, therapeutic interventions which improve recovery of ATP levels have been shown to attenuate elevations in diastolic pressure (Cargnoni et al., 1999). However, further experiments are necessary to elucidate the exact mechanisms responsible for post-ischemic contracture.

Following the brief recovery of contractions in early reperfusion, the myocardium exhibits a prolonged, but reversible, depression of contractile function. This condition has come to be known as myocardial stunning (Braunwald and Kloner, 1982). The alterations in EC coupling which are responsible for stunning are the subject of the next chapter of this introduction.

5. Stunning

A. History and Definition

Until the mid 1970s, it was generally assumed that myocardial contractile function would recover completely upon reperfusion after a reversible ischemic period (< 30 min). However, in what has proven to be a landmark study, Heyndrickx et al (1975) demonstrated that reperfusion causes significant and prolonged derangements in contractility. In their study of *in vivo* dog hearts, Heyndrickx et al measured regional left ventricular function using ultrasonic segment length transducers. As well, myocardial electrical activity was recorded by ECG. A hydraulic occluder was used to block coronary blood flow and cause ischemia, as illustrated in Figure 4. During ischemia, typical changes in the ECG record were observed and segment length shortening was almost completely abolished (Figure 5). When hearts were reperfused after 5 min of ischemia, electrical activity recovered completely. However, regional contractile

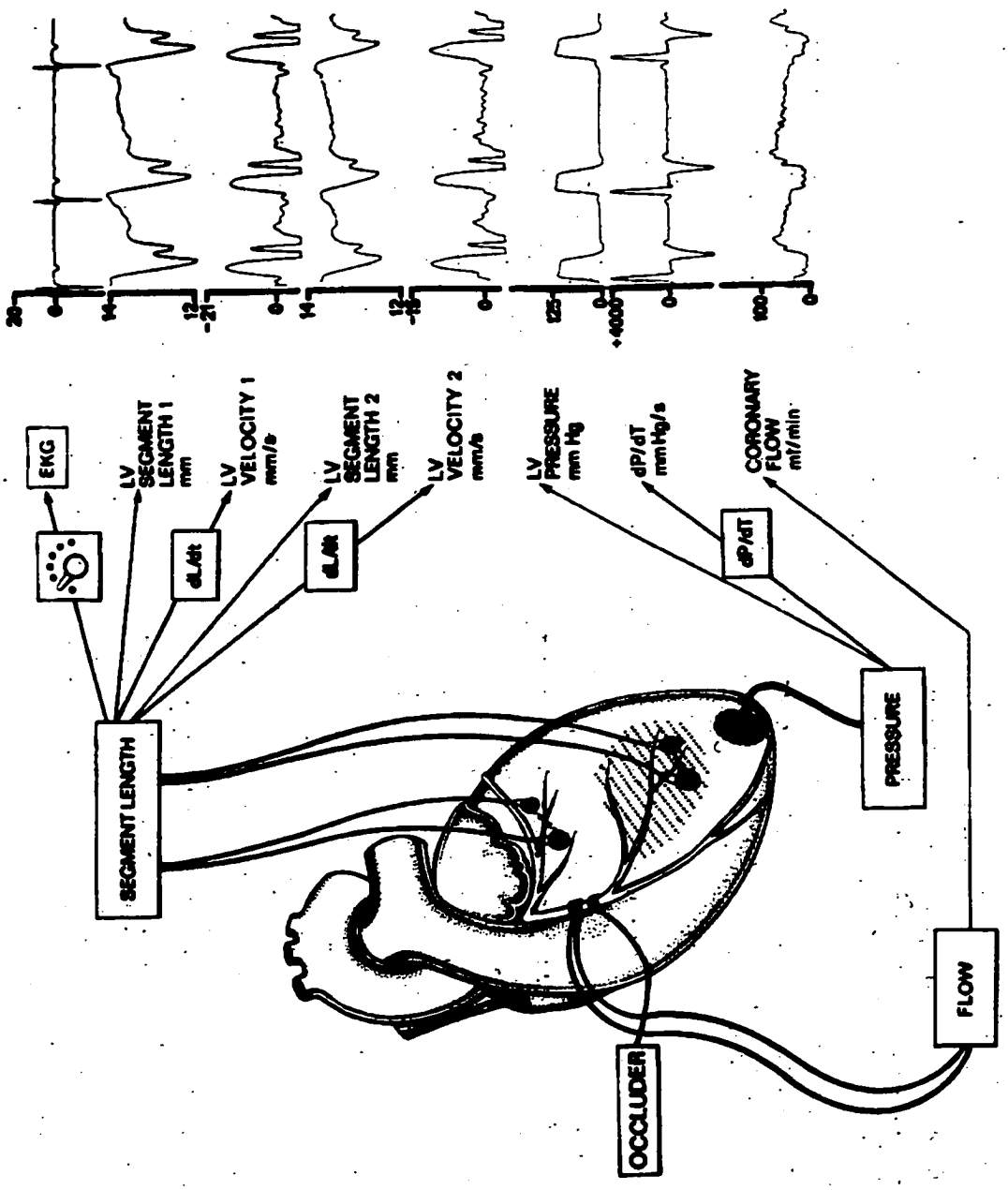

EKG
LV SEGMENT LENGTH 1 mm
LV VELOCITY 1 mm/s
LV SEGMENT LENGTH 2 mm
LV VELOCITY 2 mm/s
LV PRESSURE mm Hg
dP/dT mmHg/s
CORONARY FLOW ml/min
dL/dt
dL/dt
dP/dT
SEGMENT LENGTH
PRESSURE
FLOW
OCCLUDER

Figure 4

Figure 4. Technique for correlating measurements of total and regional LV function with ECG recordings during ischemia and reperfusion.

Heyndrickx et al. (1975) examined the effects of ischemia and reperfusion on the hearts of conscious dogs. A hydraulic occluder was used to block coronary blood flow causing regional myocardial ischemia. Regional segment function was measured in an ischemic and non-ischemic area of the myocardium using ultrasonic segment length transducers. Total left ventricular function was assessed by measuring left ventricular blood pressure (LVP) and dP/dt. ECG was also recorded. Reperfusion of the ischemic region was induced by releasing the occluder. [From Heyndrickx et al. (1975), with permission of the Journal of Clinical Investigation.]

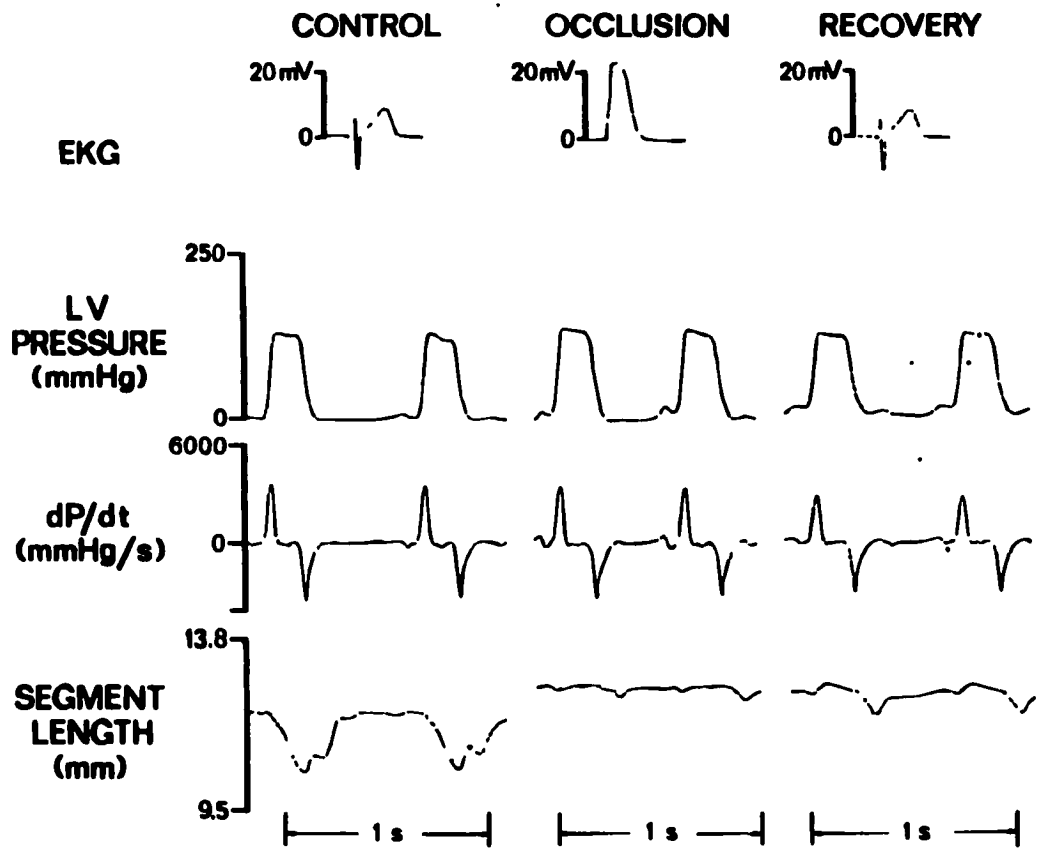

CONTROL
OCCLUSION
RECOVERY
EKG
20mV
0
20mV
0
20mV
0
LV
PRESSURE
(mmHg)
250
0
dP/dt
(mmHg/s)
6000
0
SEGMENT
LENGTH
(mm)
13.8
9.5
1 s
1 s
1 s

Figure 5

Figure 5. Regional contractile function exhibited delayed recovery in reperfusion. During the experiment, Heyndrickx et al. (1975) exposed hearts to 5 min of coronary artery occlusion (ischemia) followed by reperfusion. Shown are recordings of ECG, left ventricular function (LVP, dP/dT), and regional segment length during an experiment. Control recordings are shown in the left panel. After 5 min of ischemia (center panel), ECG recordings exhibited typical alterations. Coronary artery occlusion initially caused a reduction in LVP and dP/dT (not shown), however, left ventricular function quickly recovered and was not altered from control conditions after 5 min of ischemia. Segment length shortening was markedly depressed in the ischemic region of the myocardium. Reperfusion was induced by re-establishing blood flow through the occluded coronary artery. After 5 min of reperfusion (right panel), ECG recordings and total left ventricular function were normal. However, regional segment length shortening remained depressed relative to control levels, and gradually recovered over the next 6 hours of reperfusion. [From Heyndrickx et al. (1975), with permission of the Journal of Clinical Investigation.]

function was markedly depressed in reperfusion, and did not return to control levels for 6 hours. This observation was soon reproduced in a number of laboratories and, in 1982, Braunwald and Kloner coined the term "myocardial stunning" to describe the phenomenon. According to their definition, stunning is characterized by "delayed recovery of regional myocardial contractile function after reperfusion despite the absence of irreversible damage and despite restoration of normal flow" (Braunwald and Kloner, 1982). This clear definition distinguishes stunning from contractile depression caused by myocardial infarction or incomplete reperfusion. In man, stunning has been observed following reperfusion therapy, and during recovery from vasospasm and exercise-induced angina (for review see Bolli, 1992).

B. Pathogenesis of Stunning

In the 25 years that have passed since Heyndrickx et al (1975) made the first observation of myocardial stunning, many studies have been concerned with determining the mechanisms which lead to the development of this condition. It is well established that the severity of contractile depression in reperfusion is related to the severity of the preceeding ischemic period. Therefore, the mechanisms responsible for stunning must either be initiated during ischemia, or triggered by reperfusion in response to ischemic alterations. Experimental observation suggests that both ischemia- and reperfusion-induced mechanisms contribute to the pathogenesis of stunning (Bolli and Marban, 1999). As discussed in the previous chapter, myocardial contractile function briefly recovers in early reperfusion before stunning is observed. This suggests that myocardial changes in the initial moments of reperfusion cause damage to the myocardium (Manche

et al., 1995). In addition, as will be described in the following sections, interventions during early reperfusion can markedly improve contractile recovery. Thus, stunning is at least partly a form of reperfusion injury (Kusuoka and Marban, 1992). However, post-ischemic treatments do not completely prevent the development of stunning (Bolli, 1990), suggesting that ischemic alterations may also directly trigger stunning to some degree. It is currently believed, however, that reperfusion injury is the dominant pathogenetic trigger, and that ischemia mostly contributes to stunning by setting the stage for post-ischemic injury (Bolli and Marban, 1999).

i) Role of Ca^{2+} Overload

Intracellular Ca^{2+} overload is believed to be a key mediator in the development of stunning (for review see Duncker et al., 1998). As in other types of reperfusion injury, increases in $[Ca^{2+}]_i$ during early reperfusion appear to be critically important. Reperfusing the myocardium with low Ca^{2+} solutions decreases Ca^{2+} influx and improves contractile recovery (Kusuoka et al., 1987; Tani and Neely, 1990). Contractile recovery is also improved when post-ischemic Ca^{2+} influx is reduced by pharmacological inhibition of Ca^{2+} channels and the $NaCa_{ex}$ (Prxyklenk, 1988; Smart et al., 1997; Ladilov et al., 1999). In addition, stunning is attenuated when ionic alterations which trigger Ca^{2+} overload are reduced. For example, reperfusion with acidotic or low Na^+ solutions reduces post-ischemic contractile depression (Kitakaze et al., 1988; Tosaki et al., 1989). As well, since ischemic elevations in $[Ca^{2+}]_i$ also contribute to post-ischemic Ca^{2+} overload, stunning is attenuated by interventions that decrease ischemic Ca^{2+} levels, such

as $I_{Ca\text{-}L}$ blockade (Watts, 1986). Thus, several lines of evidence suggest that Ca^{2+} accumulation plays an important role in the development of stunning.

Elevations in $[Ca^{2+}]_i$ can cause cellular injury by different mechanisms. However, recent evidence suggests that stunning could be triggered by the activation of Ca^{2+}-dependent proteases during Ca^{2+} overload (for review see Bolli and Marban, 1999). The mechanism by which Ca^{2+}-dependent proteolysis may contribute to contractile depression in stunning will be discussed in a later section.

ii) Role of Oxygen-Derived Free Radicals

Another key mediator of stunning is oxidative stress (Duncker et al., 1998). As previously described, production of oxygen radicals is increased during ischemia and greatly amplified in early reperfusion (Kramer et al., 1987; Kukreja and Hess, 1992). A number of studies have observed that reducing oxidative stress during ischemia and/or early reperfusion attenuates stunning (for review see Duncker et al., 1998). Enzymes such as superoxide dismutase and catalase protect against stunning by catalyzing the conversion of oxygen-derived radicals to less reactive species (Myers et al., 1985; Gross et al., 1986). Similarly, free radical scavengers also improve contractile recovery when applied either during ischemia or immediately prior to reperfusion (Bolli et al., 1987; Bolli et al., 1989). However, Bolli et al (1989) observed that, when antioxidants are applied one minute after reperfusion, the myocardium is not protected from contractile depression. Therefore, as with Ca^{2+} overload, it is believed that exacerbation of oxidative stress in the initial moments of reperfusion is critically important to the pathogenesis of stunning.

The exact mechanism by which oxygen-derived free radicals cause stunning is unclear. Since the cellular damage caused by oxidative stress is wide ranging, a number of cellular alterations could theoretically contribute to post-ischemic contractile abnormalities. Free radicals are known to impair Ca^{2+} homeostasis by altering activity of SR and sarcolemmal proteins (for review see Carmeliet, 1999). Therefore, a popular hypothesis is that oxidative stress indirectly contributes to the pathogenesis of stunning by exacerbating Ca^{2+} overload (Kusuoka and Marban, 1992). However, it has also been suggested that oxidative stress may cause prolonged damage to myofilament proteins, which could directly contribute to contractile depression (Bolli and Marban, 1999). These putative mechanisms will be fully described in the following discussion of the pathophysiology of this phenomenon.

C. Pathophysiology of Stunning

As outlined in this introduction, exposure of the myocardium to ischemia and reperfusion causes significant changes in cardiac function. Of these changes, Ca^{2+} overload and oxidative stress appear to be of key importance in initiating stunning. However, while $[Ca^{2+}]_i$ and free radical levels recover to normal with 20 min of reperfusion (Sekili et al., 1993; Varadarajan et al., 2001), stunning is a prolonged condition which can be observed for many hours following an ischemic event. This suggests that the mechanisms responsible for contractile depression in stunning may be the maintained result of Ca^{2+}- and/or free radical-mediated cellular damage. Since protein damage caused by these conditions would be expected to be long lasting, it has been suggested that protein degradation may contribute to the pathophysiology of

stunning. Indeed, the time course of recovery of post-ischemic contractile function is consistent with the expected rates of protein resynthesis after reperfusion (Thomas et al., 1999). However, identifying proteins which may be involved in this process has been difficult since nearly any cellular protein could theoretically be damaged during ischemia and reperfusion. The ongoing search for the lesion responsible for stunning has been aided, however, by experimental observation of the stunned myocardium.

It is well established that electrical activity of the stunned myocardium is normal (Hanich et al., 1993). Therefore, the pathophysiology of this condition must involve deficiencies which are downstream from sarcolemmal excitation. In other words, contractile depression in stunning is believed to result from defective EC coupling (Bolli, 1990). Two different types of lesions in EC coupling could theoretically cause stunning (for review see Bolli and Marban, 1999). The first possibility is that stunning could result from decreased myofilament responsiveness to Ca^{2+}. Alternatively, Ca^{2+} cycling could be altered, causing reduced activation of the myofilaments. There is much debate as to whether one or both of these mechanisms are involved in stunning. There is also debate concerning the specific protein derangements responsible for these alterations in EC coupling. In the following sections, the evidence that supports each of these arguments will be evaluated.

i) Reduced Myofilament Responsiveness in Stunning

The ability of the contractile proteins to generate force is described by both the Ca^{2+} sensitivity and the maximal force-generating capacity of the myofilaments (Gao et al., 1995). These parameters can be assessed by experiments in either the intact heart or

isolated tissue preparations. The sensitivity of the myofilaments to Ca^{2+} is determined by examining the range of $[Ca^{2+}]_i$ which activates the contractile proteins, while the maximal force-generating capacity of the myofilaments can be determined by measuring force production at saturating levels of $[Ca^{2+}]_i$ (Bolli and Marban, 1999). Using these procedures, a number of studies have examined post-ischemic myofilament responsiveness to determine if reductions in either Ca^{2+} sensitivity or maximal force-generating potential are responsible for contractile depression in stunning. The findings of these studies are controversial. While several studies have observed that both aspects of myofilament function are depressed in the stunned myocardium (Kusuoka et al., 1990; Kusuoka et al., 1987; Gao et al., 1995), others have observed a decrease in only maximal Ca^{2+}-activated force (Carrozza et al., 1992) or Ca^{2+} sensitivity (Hofmann et al., 1993). Therefore, it is generally accepted that myofilament responsiveness to Ca^{2+} is decreased in stunning, although the exact nature of this deficit is unclear (Bolli and Marban, 1999).

Several mechanisms could theoretically cause a reduction in myofilament responsiveness in the post-ischemic heart. One possibility is that contractile depression results from decreased binding of Ca^{2+} to troponin C as a result of defects in one or more of the troponin subunits (Solaro, 1986; McDonald et al., 1998). Alternatively, defects in contractile proteins or regulatory proteins other than the troponin molecules could also reduce myofilament responsiveness (Solaro, 1986; McDonald et al., 1998). To examine these possibilities, several studies have conducted structural analysis of proteins within the contractile apparatus of the stunned myocardium. Gao et al (1997) used immunoblots of tissue from stunned hearts to examine the structure of actin, tropomyosin, myosin light chain-1, myosin light chain-2, and troponin C, T and I. They observed that troponin I

was partially degraded in the stunned myocardium while all other proteins examined remained intact. This observation is of potential interest because when troponin C binds Ca^{2+}, troponin I triggers a conformational change in the troponin-tropomyosin complex that initiates contraction. Thus, degradation of this protein could contribute to contractile depression in stunning. Support for this view comes from a recent transgenic study by Murphy et al. (2000). They observed that transgenic mice which express the major degradation product of troponin I exhibit cardiac abnormalities that resemble stunning (Murphy et al., 2000). However, the role of troponin I in stunning remains highly controversial. Although a recent study by Van Eyk et al (1998) has corroborated the observation that troponin I is degraded in stunning, other studies have not confirmed these findings (Luss et al., 2000; Thomas et al., 1999). Thus, further studies are needed to clarify whether troponin I degradation is important in the pathophysiology of stunning.

Protein degradation is mediated in reperfusion by Ca^{2+}-dependent proteolysis and oxidation following Ca^{2+} overload and oxidative stress (Bolli and Marban, 1999). Thus it is possible that proteolytic and/or oxidative damage to the myofilaments may be an important trigger for stunning (Bolli, 1990). In particular, several studies have suggested an involvement of the Ca^{2+}-dependent protease calpain I. Exposure of the myocardium to this enzyme produces contractile depression which resembles stunning (Gao et al., 1996). Further support for an involvement of this enzyme in stunning comes from the observation that calpain inhibitors have been observed to improve post-ischemic contractile recovery (Matsumura et al., 1993). Interestingly, *in vitro* analysis has shown that this enzyme effectively digests troponin I (Di Lisa et al., 1995). Thus, it has been suggested that calpain I activation may trigger stunning via selective proteolysis of

troponin I (Gao et al., 1996). However, this hypothesis is controversial since it remains unclear whether troponin I degradation is a pre-requisite for stunning (Luss et al., 2000). Clearly, further studies are needed to clarify the mechanisms which lead to altered myofilament responsiveness in the stunned myocardium.

ii) Calcium Cycling in Stunning

Although it is well established that myofilament responsiveness is reduced in stunning, it is possible that alterations in Ca^{2+} homeostasis might also contribute to the pathophysiology of this condition. To examine this possibility, a number of studies have examined the Ca^{2+} transient in the intact stunned myocardium to determine whether Ca^{2+} availability is reduced. With few exceptions, these studies have reported that following a brief overshoot in early reperfusion, the magnitude of the Ca^{2+} transient returns to control levels for the remainder of reperfusion (for review see Bolli and Marban, 1999). However, in apparent contradiction to these observations, studies which have examined SR vesicles or homogenates from stunned myocardium have reported alterations in SR proteins involved in Ca^{2+} homeostasis. Both activity and protein levels of the SR Ca^{2+} ATPase have been reported to be reduced in stunning, suggesting that SR Ca^{2+} uptake is compromised (Zucchi et al., 1996; Smart et al., 1997; Osada et al., 1998; Temsah et al., 1999; Osada et al., 2000; c.f. Luss et al., 1998). Such an effect would be expected to contribute to the physiology of stunning by reducing SR Ca^{2+} stores, thus decreasing the magnitude of the Ca^{2+} transient (Valdivia et al., 1997). Other studies have observed that protein levels of the SR Ca^{2+} release channel are also reduced in stunned myocardium (Holmberg et al., 1992; Zucchi et al., 1994; Valdivia et al., 1997), and that the

conductivity of this channel may be decreased (Osada et al., 1998). In addition, phosphorylation of the SR Ca^{2+} ATPase and Ca^{2+} release channel by Ca^{2+} calmodulin-dependent protein kinase (CaMK) may be decreased in stunning (Osada et al., 1998). Based on these findings, it would be expected that SR Ca^{2+} uptake and release may be reduced in stunning.

It is unclear why most intact heart studies have observed normal transients in stunning while many *in vitro* studies have reported abnormalities in SR Ca^{2+} handling. One possible explanation is that the SR performs much differently in isolated vesicles than *in situ*, perhaps because the isolation procedure produces an unrepresentative sample of the whole-tissue SR (Rapundalo et al., 1986). Therefore, the observations of these studies may not be representative of what occurs in the intact stunned heart. On the other hand, it is not unreasonable to think that SR proteins could be damaged by oxidative stress and Ca^{2+}-dependent proteolysis during ischemia and reperfusion. Clearly, further experiments are needed to clarify the role of SR function in stunning.

In addition to alterations in SR Ca^{2+} handling, it is also possible that alterations in $I_{Ca\text{-}L}$ could contribute to stunning. Several studies have reported that $I_{Ca\text{-}L}$ is reduced under conditions of Ca^{2+} overload and oxidative stress (Gill et al., 1995; Hammerschmidt et al., 1998; McDonald et al., 1994). A reduction in $I_{Ca\text{-}L}$ during stunning might be expected to lead to reduced SR Ca^{2+} release by decreasing CICR and/or SR Ca^{2+} stores. However, because it is not possible to measure transmembrane currents in intact heart models, no previous studies have examined $I_{Ca\text{-}L}$ in stunning. Therefore, the contribution of $I_{Ca\text{-}L}$ and CICR to the Ca^{2+} transient during stunning is unknown. The role of the VSRM in stunning has also not been investigated. However, it is quite possible that

stunning is associated with either potentiation or inhibition of SR Ca^{2+} release by this mechanism.

In summary, several questions remain concerning the pathophysiology of stunning. These are illustrated in Figure 6. There is general agreement that myofilament responsiveness is reduced in stunning. However, whether other alterations in EC coupling may also contribute to contractile depression is controversial. In particular, it is unclear whether SR Ca^{2+} cycling and $I_{Ca\text{-}L}$ are altered in the intact stunned heart, and whether these changes affect the Ca^{2+} transient through effects on CICR and/or the VSRM. If Ca^{2+} transients are, in fact, normal in stunned myocardium, it is possible that SR function and/or $I_{Ca\text{-}L}$ alterations could be compensated for by other alterations in Ca^{2+} homeostasis.

D. Hypothesis

The hypothesis investigated in Part I of this thesis is that Ca^{2+} homeostasis is altered in stunning.

E. The Need For a Cellular Model

The majority of studies that have investigated the mechanisms of stunning have examined whole animal or isolated heart models of ischemia and reperfusion. However, it is difficult to assess dysfunction at the level of the cardiomyocyte using these models (Gao et al., 1995). Indeed, as described above, studies in intact post-ischemic hearts have been unable to answer several important questions concerning the pathophysiology of stunning. To address these questions, it would be useful to develop a cellular model of

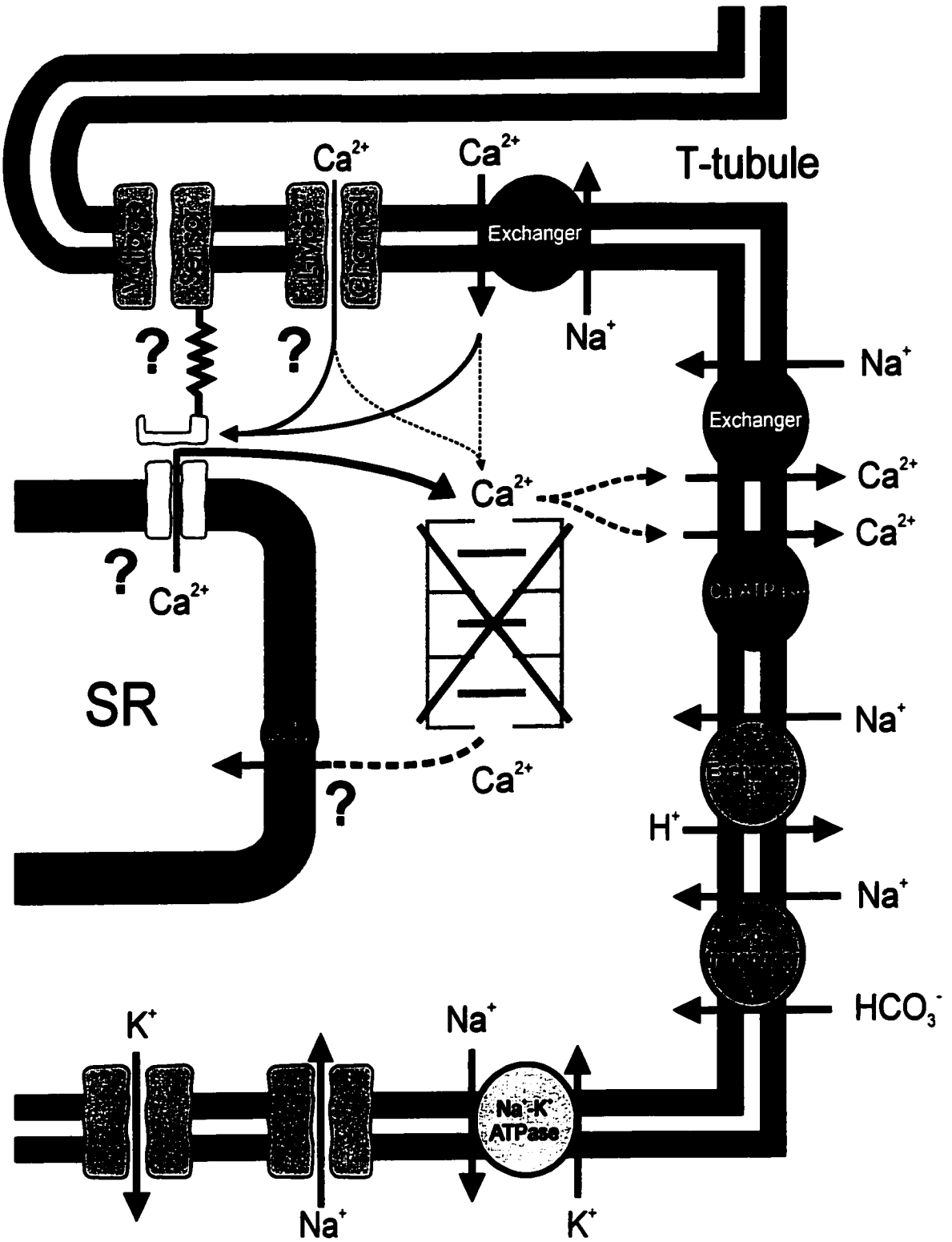

T-tubule
Exchanger
Exchanger
Na+
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
Na+
Na+
Na+
H+
Na+
HCO3-
K+
Na+
Na+
K+
Na+-K+ ATPase
SR
?
?
?
?

Figure 6

Figure 6. Alterations in EC coupling during stunning.

The precise mechanisms responsible for stunning are unclear. It is well established that contractile depression in late reperfusion at least partly results from decreased responsiveness of the myofilaments to Ca^{2+}. However, it is unclear whether alterations in Ca^{2+} transients could also contribute to defective EC coupling in stunning. Changes in Ca^{2+} transients could be mediated by alterations in SR Ca^{2+} uptake and release, $I_{Ca\text{-}L}$, CICR, and/or the VSRM.

stunning. A cellular model would allow the use of voltage-clamp techniques to examine post-ischemic changes in transmembrane currents and various mechanisms of EC coupling in a well-controlled environment. An isolated myocyte model would also enable post-ischemic SR function to be examined in the intact cardiomyocyte, whereas most previous studies have examined *in vitro* SR preparations. Therefore, a cellular model of myocardial stunning used in conjunction with existing models could aid in revealing the cellular mechanisms involved in this phenomenon.

Several single cell models of stunning have been previously described. Some of these have examined isolated myocytes following exposure to anoxia (Boekstegers et al., 1992; Koyama et al., 1996; Gandhi et al., 1999). Other models have been described in which myocytes are isolated from stunned myocardium (Hofmann et al., 1993; Lew et al., 1994). However, the validity of these cellular models has been questioned since other studies have not observed stunning in myocytes under these conditions (Stern et al., 1985; Silverman et al., 1991; Chandrashekhar et al., 1999; Straznicka et al., 2000). In addition, none of these single cell studies have examined the electrical activity of "stunned" myocytes. Since electrical activation of stunned myocardium is normal (Heyndrickx et al., 1975; Hanich et al., 1993), a useful cellular model of stunning should generate cells which exhibit both normal electrical activity and depressed contractile function.

A major limitation of single cell models of ischemia and reperfusion is that assumptions must be made on how to mimic these conditions. In the past, nearly all such models have simulated ischemia using hypoxia or anoxia, with or without metabolic inhibition. However, these conditions are rather poor approximations of ischemia, as

they generate responses in cardiac tissue which are quite different from those seen in response to true ischemia (Lee, 1995). This may explain why several studies of simulated ischemia have not observed characteristic ischemic events, or post-ischemic stunning (Stern et al., 1985; Silverman et al., 1991; Boekstegers et al., 1992). However, a single cell model of ischemia and reperfusion that more closely mimics myocardial ischemia has been recently developed (Cordeiro et al., 1994). Specifically, this model employs hypoxia, hypercapnia, hyperkalemia, acidosis, lactate accumulation, and substrate deprivation. Previous studies have shown that myocytes exposed to these conditions show contractile and electrophysiological changes that resemble those observed in true ischemic myocardium (Cordeiro et al., 1994, 1995). In the present investigation, this model was adapted to develop a model of myocardial stunning.

F. Objectives (Part I)

1) To develop a cellular model of stunning in which myocytes exposed to simulated ischemia and reperfusion exhibit contractile depression associated with normal electrical activity.

2) To examine EC coupling in this model to determine whether stunning is related to changes in:

a) transmembrane currents,

b) CICR or the VSRM,

c) SR Ca^{2+} stores,

d) the magnitude of Ca^{2+} transients.

6. Therapeutic Interventions That Attenuate Stunning

A. Overview

It is well established that the severity of post-ischemic contractile depression is related to the severity of the preceeding ischemic period (for review see Bolli, 1990). Therefore, it is no surprise that interventions which decrease the length of time the myocardium remains ischemic generally improve contractile recovery in reperfusion (Bolli and Marban, 1999). However, reperfusion injury can also be reduced by therapeutic strategies that attenuate or compensate for myocardial alterations during ischemia and/or early reperfusion (Duncker et al., 1998). As will be discussed below, a number of drugs and other interventions have been shown to attenuate stunning by reducing oxidative stress and Ca^{2+} overload. Some of these therapeutic strategies have been mentioned earlier in this introduction but are reviewed here to provide a general overview.

Interventions that reduce oxidative stress during ischemia and early reperfusion protect against stunning (for review see Duncker et al., 1998). Post-ischemic contractile recovery is improved when production of free radicals is reduced by xanthine oxidase inhibitors (Headrick et al., 1990) or by gradual re-introduction of oxygen during reperfusion (Yamazaki et al., 1986). Similarly, agents that remove oxygen radicals also can attenuate stunning. These substances include enzymes such as superoxide dismutase and catalase that convert free radicals to less active species (Myers et al., 1985; Gross et al., 1986). As well, a number of compounds that contain thiol groups such as

dimethylthiourea, or sulphydryl groups such as mercaptopropionyl glycine, are believed to protect against myocardial stunning by acting as free radical scavengers (for review see Bolli and Marban, 1999).

An alternative therapeutic strategy which can be used to inhibit the development of stunning is to attenuate elevations in $[Ca^{2+}]_i$ during ischemia and early reperfusion. Agents that prevent Ca^{2+} influx such as Ca^{2+} channel blockers and inhibitors of the $NaCa_{ex}$ protect against stunning (Przyklenk, 1988; Smart et al., 1997; Ladilov et al., 1999). Increasing ATP levels by direct infusion of adenosine also improves post-ischemic contractile function by increasing activity of the Na^+-K^+ ATPase, and thus, reducing $[Na^+]_i$ and $[Ca^{2+}]_i$ (McFalls et al., 1991; Sekili et al., 1995). Similarly, Ca^{2+} influx and stunning can be attenuated by reperfusing the myocardium with acidotic, low Na^+, or low Ca^{2+} solutions (Kusuoka et al., 1987; Kitakaze et al., 1988; Tosaki et al., 1989). Reducing SR Ca^{2+} release, especially in the initial moments of reperfusion, may also improve contractile recovery. Elevating intracellular Mg^{2+} levels has been observed to protect against stunning (Atar et al., 1994), perhaps because Mg^{2+} competes with Ca^{2+} for the SR release channel (Terada et al., 1996). However, the protective actions of Mg^{2+} could also be explained by competition with Ca^{2+} for binding sites on Ca^{2+}-dependent proteases. Inhibition of protease activity may improve post-ischemic contractile recovery by attenuating degradation of the contractile machinery following Ca^{2+} overload (Matsumura et al., 1993; Gao et al., 1996).

Several other interventions have been observed to attenuate stunning, although the mechanisms underlying these protective actions remain poorly understood. Contractile function in reperfusion is improved if the myocardium is subjected to ischemic

preconditioning, a technique whereby exposure of the heart to brief ischemic episodes increases tolerance to subsequent prolonged ischemia (for review see Cohen and Downey, 1996). The precise mechanisms responsible for preconditioning are unclear but may involve events triggered by free radical production or alterations in Ca^{2+} homeostasis (Das et al., 1999; Przyklenk et al., 1999). Anti-inflammatory drugs have also been reported to attenuate stunning (Ravingerova et al., 1991). It has been hypothesized that these drugs may reduce reperfusion injury by stabilizing cell membranes, thus attenuating accumulation of Na^{+} during ischemia and reperfusion (Maxwell and Lip, 1997; Rossoni et al., 2001). Finally, post-ischemic contractile recovery is improved following treatment with inhibitors of the renin-angiotensin system (Mehta et al., 1990; Morales et al., 1998). The mechanisms responsible for the protective actions of selective inhibitors of angiotensin receptors will be examined in the second part of this thesis. First, however, the renin-angiotensin system will be reviewed.

B. The Renin-Angiotensin System

i) The Circulating Renin-Angiotensin System

The renin-angiotensin system (RAS) is known to perform a variety of functions in the body. These roles include regulation of vascular smooth muscle tone, cell growth, and control of fluid and electrolyte balance. The primary end product of the RAS is angiotensin II (AII). Its synthetic pathway involves several stages as shown in Figure 7.

The first step in the synthesis of AII is secretion of renin from the kidney. Renin is an aspartyl protease enzyme that is produced and stored in renal juxtaglomerular cells (Inagami, 1993). The release of renin into plasma can be triggered by a number of

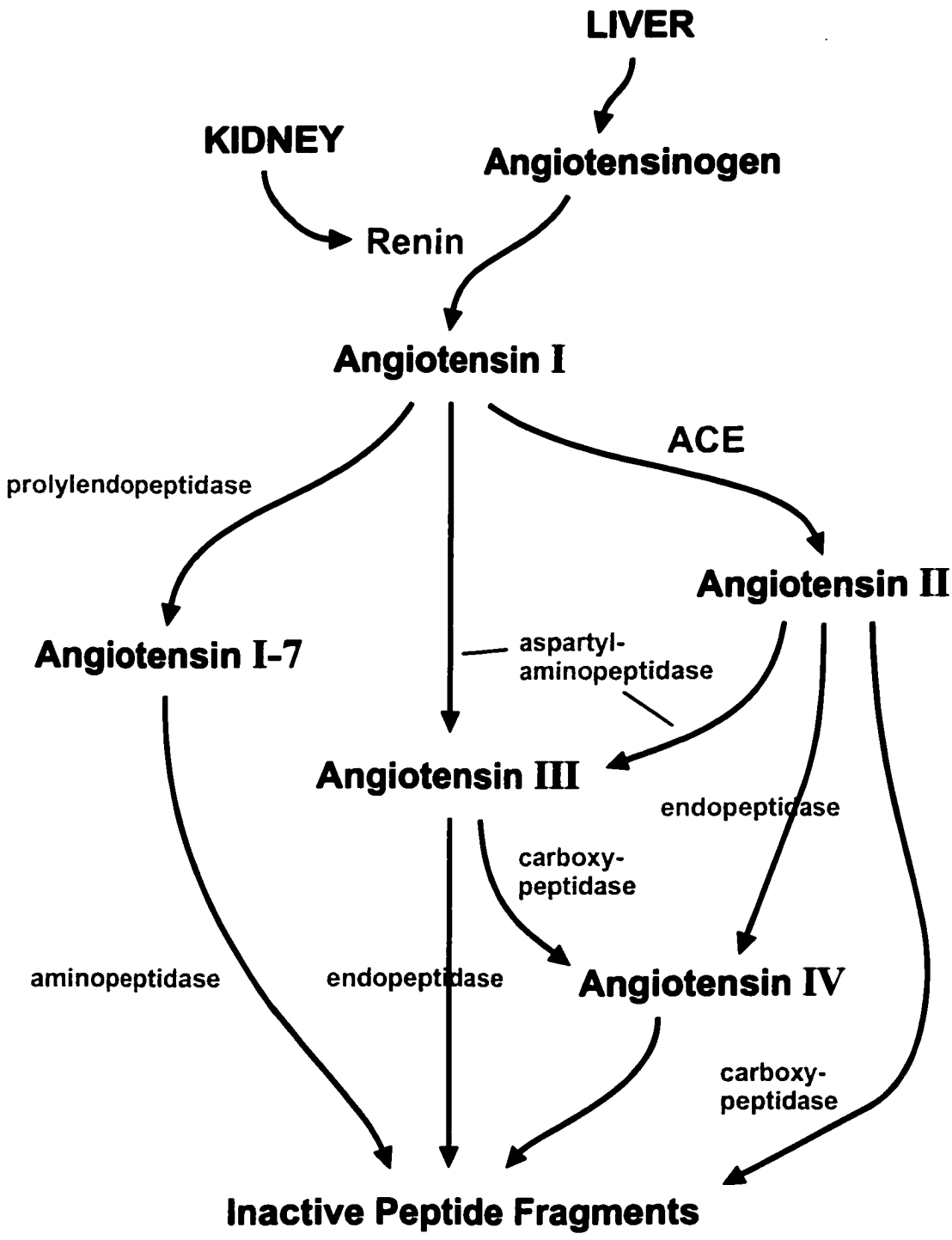

LIVER
KIDNEY
Angiotensinogen
Renin
Angiotensin I
ACE
prolylendopeptidase
Angiotensin II
Angiotensin I-7
aspartyl-
aminopeptidase
Angiotensin III
endopeptidase
carboxy-
peptidase
aminopeptidase
endopeptidase
Angiotensin IV
carboxy-
peptidase
Inactive Peptide Fragments

Figure 7

Figure 7. The Renin-Angiotensin System (RAS).

Synthesis of angiotensin II (AII), the primary effector of the RAS, begins with secretion of renin from the kidney. Once in the plasma, renin catalyzes the conversion of the liver-derived glycoprotein, angiotensinogen, to angiotensin I (AI). In the presence of angiotensin converting enzyme (ACE), AI is then converted to AII. AI is also converted to angiotensin III (AIII) by aspartylaminopeptidase and to angiotensin I-7 by prolylendopeptidase. Once formed, AII may be degraded directly to inactive peptide fragments by carboxypeptidase, or converted to AIII or angiotensin IV (AIV) by aspartylaminopeptidase and endopeptidase, respectively. AIII is converted to AIV by carboxypeptidase or to inactive fragments by endopeptidase. Angiotensin I-7 is degraded to peptide fragments by aminopeptidase. (Adapted from Robertson and Nicholls, 1993)

stimuli including decreased blood pressure in the kidney, circulating catecholamines, or the sympathetic nervous system (Robertson and Nicholls, 1993). It is believed that renin secretion is the rate-determining step in the eventual production of AII (Mulrow, 1999). Once in circulation, renin catalyzes the hydrolysis of angiotensinogen to form angiotensin I (AI) and a slightly smaller protein of unknown function (Riordan, 1995). Angiotensinogen is a glycoprotein that is synthesized predominantly in the liver, but also in other organs such as the brain and kidney. Gene transcription and plasma concentrations of angiotensinogen are known to be increased by estrogens, glucocorticoids, thyroid hormones, and AII (Roberston and Nicholls, 1993). Angiotensinogen is the only known substrate for renin (Inagami, 1994).

Following its synthesis from angiotensinogen, AI is further metabolized by a variety of enzymes, yielding several different peptide products. As AII is the most biologically active of these peptides, the enzymes involved in its synthesis are of significant interest. Included in this category is angiotensin converting enzyme (ACE) which is the major producer of circulating AII. ACE is located on the surface of vascular endothelial cells where it is in contact with plasma and, therefore, a constant supply of AI (Ehlers and Riordan, 1989). In addition, soluble ACE has been shown to circulate with plasma, although this form of the enzyme is believed to generate only minor amounts of AII (Robertson and Nicholls, 1993).

ACE is not the only enzyme capable of generating AII. In cardiac tissue, serine proteinase (a chymase) also converts AI to AII (Urata et al., 1990). As well, other enzymes such as tonin, elastase, and tissue plasminogen have been shown to produce AII

directly from angiotensinogen (Dzau et al., 1993). However, the significance of these enzymatic pathways is not well understood.

Once synthesized, AII performs a number of functions, several of which counteract the stimuli that initially lead to activation of the RAS. AII acts at AII receptors to cause contraction of vascular smooth muscle, and thus, an increase in blood pressure. AII also stimulates secretion of a steroid hormone, called aldosterone, from the adrenal cortex (Inagami, 1993). Aldosterone causes an increase in blood pressure by inducing sodium reabsorption in the kidney. Importantly, AII also acts on juxtaglomerular cells to inhibit renin release (Robertson and Nicholls, 1993). Therefore, activity of the RAS is self-limiting. However, it is still necessary to inactivate circulating AII once it is synthesized.

AII degradation is known to procede by several different enzymatic pathways (Figure 7). Depending on which enzymes are involved, AII may be converted to either angiotensin III (AIII) or angiotensin IV (AIV) (Inagami, 1993). However, regardless of the route taken, AII is ultimately metabolized to inactive fragments. Aspartylamino-peptidase catalyzes the conversion of AII to AIII, a peptide that is known to have considerable biological activity at AII receptors (Goodfriend et al., 1975). AIV, which is much less active than AII or AIII, is synthesized either from AIII by carboxypeptidase, or from AII by endopeptidase (Stroth and Unger, 1999).

Figure 7 also shows that there are several metabolic pathways in the RAS that do not involve the formation of AII. AI may be converted directly to AIII or to angiotensin I-7 (Inagami, 1993). It is currently unclear whether angiotensin I-7 is biologically active (Stroth and Unger, 1999).

ii) Evidence for the Existence of a Local Renin-Angiotensin System in the Heart

Traditionally, the RAS has been understood to be an endocrine system that regulates circulating levels of AII. However, in the past decade a number of studies have provided evidence suggesting that local renin-angiotensin systems also exist in many tissues. These systems are believed to influence local tissue functions with only minimal contribution to the activity of the circulating RAS (Dzau, 1988).

According to Danser (1996), one of two circumstances must exist to define the presence of a local RAS in a tissue. In the first situation, a local RAS exists if AII synthesis in a tissue is independent of circulating RAS activity. For this to be true, the components of the RAS (renin, angiotensinogen, and ACE) must be synthesized within the tissue. In the second situation, RAS components are not generated *in situ*, but must be taken up from plasma. Therefore, although AII is synthesized within the tissue, this local production is dependent on plasma levels of RAS components. A local RAS can also be defined by any combination of the above situations.

A substantial body of evidence has indicated that a local RAS exists in the heart. Linz et al. (1986) observed that addition of AI to isolated, perfused rat heart resulted in rapid production of AII. This action was prevented with the inclusion of ACE inhibitors in the perfusate. Since these experiments were performed in the absence of circulating plasma, it was concluded that ACE must be functionally present in the heart or blood vessels. More recent studies have detected significant levels of ACE mRNA and ACE activity in isolated cardiac tissue, suggesting that cardiac ACE may be produced predominantly in the heart (Yamada et al., 1991; Pieruzzi et al., 1995). In addition to ACE, chymases present in cardiac tissue can also catalyze AII synthesis (Urata et al.,

1990). These observations suggest that conversion of AI to AII may occur in the heart by mechanisms largely independent of the circulating RAS (Dostal and Baker, 1999).

Other studies have observed that renin and angiotensinogen mRNA are also present in cardiac tissue (Boer et al., 1994; Passier et al., 1996). However, Passier et al (1996) detected only tiny amounts of renin mRNA in cardiac tissue which contrasts with the observation that normal hearts contain relatively high concentrations of renin. It has, therefore, been suggested that under physiological conditions, cardiac renin is predominantly of kidney origin and taken up from the plasma (Ruzicka and Leenan, 1997). Similarly, Passier et al (1996) also detected only moderate levels of angiotensinogen mRNA in cardiac tissue, suggesting that the local RAS of the heart may also rely on angiotensinogen from plasma to meet physiological demands.

In summary, it has been shown that AII can be produced in the heart, possibly by locally synthesized ACE and chymases found in cardiac cells and/or blood vessels. However, it appears unlikely that the local cardiac RAS is capable of producing sufficient levels of renin and angiotensinogen to meet normal tissue demands. Therefore, the cardiac RAS is not believed to be completely independent of the circulating plasma (Ruzicka and Leenan, 1997). Thus, it appears that a local RAS exists in the heart according to Danser's definition (1996). As will be discussed in later sections, the discovery of a local cardiac RAS has drawn considerable attention as it may have significant implications for the pathogenesis and treatment of cardiovascular disease.

iii) AII Signal Transduction

Whether synthesized by the circulating or cardiac RAS, AII exerts its effects by acting at cell surface receptors. There are at least two types of AII receptors (AT_1 and AT_2) that are known to exist in humans (Robertson and Nicholls, 1993). These receptor types are defined by their differing affinities for various AII antagonists (Timmermans et al., 1993). Two further subtypes of the AT_1 receptor, AT_{1A} and AT_{1B}, have also been identified (Sandberg et al., 1992; Elton et al., 1992; Sasamura et al., 1992).

Most physiological functions of the circulating RAS have been shown to be mediated by AT_1 receptors (Horiuchi et al., 1999). For example, AT_1 receptor activation induces vasoconstriction, promotes cell growth, and inhibits apoptosis (for review see Dostal, 2000). Although the functions of the AT_2 receptor are generally less understood, recent studies have suggested that activation of the AT_2 receptor opposes the actions of the AT_1 receptor. Indeed, AT_2 receptor activation can trigger vasodilation, inhibit cell growth, and promote apoptosis (Hayashida et al., 1996; van Kesteren et al., 1997; Dimmeler et al., 1999). Thus, it appears that the functions of AT_1 and AT_2 receptors are mutually antagonistic.

Following activation of AT_1 receptors, signal transduction is mediated via G-proteins. Two major signaling pathways exist for AT_1 receptors, and these can be initiated by activation of either of the receptor isoforms (AT_{1A} or AT_{1B}). Some AT_1 receptors inhibit adenylate cyclase through G_i (Riordan, 1995). Activation of these receptors inhibits cAMP formation, and cellular mechanisms that require the presence of cAMP. For example, decreased activity of cAMP-dependent PKA would be expected to decrease phosphorylation of ion channels. The second type of AT_1 receptor is coupled by

G_q to the phosphoinositide pathway (Riordan, 1995). When AII binds to this receptor, PLC is activated which increases the hydrolysis of phosphatidylinositol bisphosphate to diacyl glycerol and inositol triphosphate (Griendling et al., 1987). Diacylglycerol activates PKC, which performs a variety of functions including activation of the Na^+/H^+ exchanger. Inositol trisphosphate releases Ca^{2+} from internal stores, an action that is believed to mediate most cardiovascular actions of AII (Vallotton et al., 1990). The AT_1 receptor has also been reported to be functionally coupled to tyrosine kinase-dependent signaling pathways (Sadoshima et al., 1995; Touyz and Schiffrin, 1997). These pathways may be involved in mediating the regulatory effects of AII on gene transcription and cell growth (Dostal et al., 1997).

The AT_2 receptor is also believed to be coupled to G-proteins (Horiuchi et al., 1999). However, unlike the AT_1 receptor, the mechanisms of AT_2 receptor-mediated signal transduction are not well understood. The growth-inhibitory effects of the AT_2 receptor have been reported to be mediated by the activation of tyrosine phostphatase, which may result in inactivation of the AT_1 receptor (Tsuzuki et al., 1996). As well, proapoptotic signaling linked to AT_2 receptor activation is believed to be mediated, at least in part, by the Bcl-2 family of cytoplasmic proteins (Horiuchi et al., 1997). Finally, vasodilatory effects of AT_2 receptor activation have been shown to involve increased production of nitric oxide (Seyedi et al., 1995; Siragy et al., 1996). However, the precise mechanism responsible for this action is unknown (Horiuchi et al., 1999). Clearly, further study is needed to more firmly establish the mechanisms responsible for AT_2 receptor-mediated signal transduction.

C. Inhibition of the Renin-Angiotensin System During Ischemia and Reperfusion

Drugs that inhibit the RAS are commonly used to treat clinical conditions such as hypertension and congestive heart failure. However, recent evidence has suggested that blockade of the RAS could also be beneficial in the treatment of ischemic heart disease. Sigurdsson et al (1993) observed that plasma levels of AII are elevated in patients with ischemic heart disease. This observation has drawn widespread interest since AII has been reported to have deleterious effects on the ischemic myocardium. Exogenous AII applied before or during ischemia has been observed to exacerbate myocardial stunning (Linz and Scholkens, 1987; Scholkens and Linz, 1988) and to increase the duration and incidence of reperfusion arrhythmias (Linz et al., 1986; Scholkens and Linz, 1988). Further support for an involvement of AII in reperfusion injury comes from the observation that AT_{1a} receptor knockout mice are less susceptible to reperfusion arrhythmias than wild-type mice (Harada et al., 1998). These findings support the hypothesis that activation of the RAS may contribute to myocardial injury during ischemia and reperfusion.

Since coronary perfusion is reduced or abolished during myocardial ischemia, the circulating RAS may play only a minor role in the pathogenesis of ischemia / reperfusion injury (Dzau et al., 1988). Instead, the increase in AII levels observed during ischemia may result from activation of the cardiac RAS (Werrman and Cohen, 1996; So et al., 1998; Barsotti et al., 2001). In support of this hypothesis is the observation that conversion of AI to AII is enhanced during ischemia, suggesting that the activity of ACE in the heart and/or vessels is increased under these conditions (Tian et al., 1991).

Several studies have suggested that AII synthesized by the cardiac RAS could contribute to ischemia / reperfusion injury by causing coronary vasoconstriction, thus worsening the ischemic insult. Administration of either AI or AII has been observed to cause dose-dependent reductions in coronary blood flow during ischemia (Tian et al., 1991; Yoshiyama et al., 1994). As well, application of ACE inhibitors, which decrease AII production, has been reported to increase coronary perfusion during ischemia (Ertl et al., 1983; Yoshiyama et al., 1994). Interestingly, ACE inhibitors have also been observed to attenuate stunning and post-ischemic arrhythmogenesis when applied during ischemia and/or early reperfusion (Przyklenk and Kloner, 1987; Westlin and Mullane, 1988; Ehring et al., 1994; Nakai et al., 1999; Zhu et al., 2000). However, it is unclear whether these protective actions of ACE inhibitors are mediated by reductions in AII production. It is well established that many ACE inhibitors act as free radical scavengers since they contain sulphydryl groups (Duncker et al., 1998). Thus, it is possible that these ACE inhibitors improve tolerance to ischemia and reperfusion simply by reducing oxidative stress. As well, the protective actions of ACE inhibitors could also result from elevated levels of bradykinin (Ehring et al., 1994; Matoba et al., 1999), since ACE catalyzes the breakdown of bradykinin in addition to its actions in the RAS. Bradykinin is believed to improve coronary blood flow during ischemia and reperfusion by stimulating NO synthesis and improving endothelial function (Remme, 1997). Therefore, the protective actions of ACE inhibitors during ischemia and reperfusion may result, at least in part, from the ability of these compounds to scavenge free radicals and/or increase levels of bradykinin.

i) AII Receptor Antagonists

As described above, studies using ACE inhibitors have not clearly established a role of the RAS in ischemia / reperfusion injury. However, the recent development of AII receptor antagonists has provided an additional means to examine the RAS in ischemic heart disease. Antagonists of both AT_1 and AT_2 receptors have been described, although AT_1 receptor antagonists have been more extensively examined in ischemia / reperfusion experiments. The majority of these studies have investigated the actions of either losartan or candesartan, two specific non-peptide antagonists of the AT_1 receptor. Both antagonists have been observed to protect against stunning (Paz et al., 1998; Yang et al., 1998; Dorge et al., 1999; Shimizu et al., 1999; Wang and Sjoquist, 1999; Zhu et al., 1999; c.f. Ford et al., 1996; Ford et al., 1998; So et al., 1998) and reperfusion arrhythmogenesis (Lee et al., 1997; Harada et al., 1998; Zhu et al., 2000).

As in the case of ACE inhibitors, it is unclear whether the protective actions of losartan and candesartan result from AII blockade. It might be expected that the cardioprotective actions of AT_1 antagonists would be associated with increased coronary perfusion. While some studies have observed such a correlation (Shimizu et al., 1999; Paz et al., 1998) others have observed no change in coronary flow following AT_1 blockade, despite improved contractile function in reperfusion (Shimizu et al., 1998; Dorge et al., 1999; Wang and Sjoquist, 1999). One possible explanation for these findings is that AT_1 antagonists could have protective actions that are not related to the actions of AII. A recent study by Thomas et al (1996) supports this hypothesis. In their study, losartan was observed to have anti-arrhythmic actions in an isolated tissue model of ischemia and reperfusion. This protective effect was attributed to attenuation of

depressed cardiac impulse conduction during ischemia and early reperfusion. Interestingly, Thomas et al (1996) observed that losartan improved impulse conduction both in the presence and absence of exogenous AII, although AII alone did not promote conduction defects. Based on these observations, it was hypothesized that losartan may have an intrinsic anti-arrhythmic action that is independent of AT_1 receptor blockade. In addition, because elevation of $[Ca^{2+}]_i$ slows impulse conduction (Weingart, 1977; Jalife et al., 1989), Thomas et al (1996) suggested that the anti-arrhythmic actions of losartan might result from reduced Ca^{2+} overload during ischemia and reperfusion. Such an action could also explain the protective actions of losartan on post-ischemic contractile function, since Ca^{2+} overload is believed to be a key mediator of stunning (Maxwell and Lip, 1997).

To investigate the hypothesis proposed by Thomas et al. (1996), the actions of losartan and AII on Ca^{2+} homeostasis, contractile function, and arrhythmogenesis could be examined during ischemia and reperfusion. An isolated myocyte model of stunning would be appropriate for such a study since $I_{Ca\text{-}L}$, $[Ca^{2+}]_i$, SR Ca^{2+} stores, and post-ischemic contractile function can be examined. Isolated myocytes exposed to these conditions of simulated ischemia and reperfusion exhibit I_{Ti} in early reperfusion (Cordeiro et al., 1994). Therefore, the mechanisms responsible for the anti-arrhythmic actions of losartan could also be examined in this model. Finally, a cellular model of stunning has the advantage that any actions of losartan observed in this model must result from direct actions on cardiomycotyes and not alterations in coronary perfusion.

D. Hypothesis

The hypothesis investigated in Part II of this thesis is that losartan protects against stunning and post-ischemic arrhythmogenesis by attenuating cellular Ca^{2+} overload during ischemia and early reperfusion, through an action independent of AT_1 receptor blockade.

E. Objectives (Part II)

1) To determine whether losartan protects against stunning by a direct action on cardiomyocytes.

2) To determine whether this action is dependent on AT_1 blockade by examining the effects of losartan, AII, and losartan plus AII treatment during ischemia.

3) To determine whether the protective actions of losartan are mediated by effects on Ca^{2+} homeostasis by examining I_{Ti}, $I_{Ca\text{-}L}$, $[Ca^{2+}]_i$, and SR Ca^{2+} stores during ischemia and reperfusion, in the presence and absence of losartan.

METHODS

1. Animals

All experiments were conducted on freshly isolated guinea pig ventricular myocytes. Albino guinea pigs (250-400 g; Charles River, St. Constant, Quebec), approximately 90% of which were male and 10% female, were used in this investigation. Studies were performed in accordance with guidelines published by the Canadian Council on Animal Care (2 Volumes, Ottawa, Ontario: CCAC, 1980-1984), and approval for this research was obtained from the Dalhousie University Committe on Animal Care. Animals were housed in the Dalhousie Animal Care facility with a 12 hour day/night cycle. Food and water were freely available.

2. Myocyte Isolation and Superfusion

A. Isolation Procedures

One guinea pig was sacrificed on each experiment day, and isolated ventricular myocytes were shared between experimenters in two laboratories. Myocyte isolation duties were alternated between the laboratories, although each laboratory employed a different isolation procedure. These two procedures were employed with approximately equal frequency, and are described below.

Procedure 1:

Guinea pigs were intraperitoneally injected with heparin (3.3 IU/g) and anesthetized with sodium pentobarbital (160 mg/kg) 10 minutes prior to opening the

chest cavity by parasternal incision. Once the chest was opened, the aorta was retrogradely cannulated *in situ*, and the heart was perfused with oxygenated (100% O_2; Praxair Canada Inc.) nominally Ca^{2+}-free solution (Table 1). The perfusion rate was maintained at 10-12 ml/min using a peristaltic pump (Cole-Parmer Instruments Co., Chicago, IL) and the purfusate temperature was maintained at 37°C with a heated water bath (Model Haake D1-L, Fisher Scientific, Berlin, Germany). Following 7-8 min of Ca^{2+}-free perfusion, the heart was subjected to enzymatic dissociation by including collagenase A (25 mg/50 ml buffer, Boehringer Mannheim) and protease (4.8 mg/50 ml, Sigma type XIV) in the perfusate for 5 min. The perfusion pressure was monitored by a pressure gauge. A drop in pressure indicated adequate tissue dissociation. The ventricles were then excised from the heart and minced in a high-potassium substrate enriched solution (Table 2, You et al., 1994).

Procedure 2:

In the second isolation procedure, guinea pigs were injected with heparin and sodium pentobarbital as described above. Following 10 min of anaesthetization, the chest cavity was opened and the heart was rapidly excised and placed in nominally Ca^{2+}-free solution (Table 3). The heart was quickly trimmed of excess tissue and fat to expose the aorta, and then mounted via the aorta on a Langendorff column. Using the same nominally Ca^{2+}-free solution, the heart was retrogradely prefused through the aorta. The purfusate was bubbled with 95% O_2 / 5% CO_2 (Praxair Canada Inc.) and maintained at 37°C using a circulating water bath. Since the perfusion pressure is dependent on gravity

Table 1: Composition of Nominally Ca^{2+}-Free Solution for Myocyte Isolation (Procedure 1)

Compound	Concentration (mM)
NaCl	120
KCl	4
$MgSO_4$	1.2
KH_2PO_4	1.2
HEPES	10
Glucose	11

pH 7.4 with NaOH

Table 2: Composition of High-Potassium Solution

Compound	Concentration (mM)
KCl	30
KOH	85.5
KH_2PO_4	30
$MgSO_4$	3
Glutamic Acid	50
Taurine	20
HEPES	10
EGTA	0.5
Glucose	10

pH 7.4 with KOH

- You et al. (1994)

Table 3: Composition of Nominally Ca^{2+}-Free Solution For Myocyte Isolation (Procedure 2)

Compound	Concentration (mM)
NaCl	120
KCl	3.8
$MgSO_4$	1
NaH_2PO_4	4
$NaHCO_3$	22
Glucose	5.5

pH 7.4 with NaOH

in a Langendorff column, the fluid level of the column was kept constant to maintain pressure. Following 7 min of perfusion with the Ca^{2+}-free solution, the heart was subjected to 3-5 min of enzymatic digestion by including collagenase (Worthington I, 50 mg/50 ml) and protease (Sigma type XIV, 5 mg/ 50 ml) in the perfusate. The ventricles were then excised from the heart and minced in high-potassium substrate enriched solution (Table 2).

Following isolation by either of the above procedures, myocytes were rinsed in the high-potassium solution to remove debris and residual enzyme. Myocytes were then stored in this solution for the duration of the experiment day. Fresh cells were decanted from the chunks of ventricular tissue for use in experiments. Both procedures of myocyte isolation produced rod-shaped myocytes with clear striations. Myocyte viability was approximately 90% with each method.

B. Experimental Setup

Isolated myocytes were placed in an experimental chamber (approximate volume = 0.75 mL), on the stage of an inverted microscope (Model INT-2, Olympus Optical Co., Ltd., Tokyo, Japan). The perfusion chamber, illustrated in Figure 8, was custom made from a piece of plexiglass and had a plastic or glass coverslip as its base. Myocytes were left undisturbed in the chamber for 5-10 min to allow them to adhere to the coverslip. The cells were then superfused with Tyrode's solution (Table 4, bubbled with 95% O_2 / 5% CO_2), which was delivered to the experimental chamber from a reservoir by a perisaltic pump (Model P2OT, Dungey Inc., Agincourt, Ontario) at a rate of 3 ml/min. An inlet and outlet at opposite ends of the chamber allowed constant

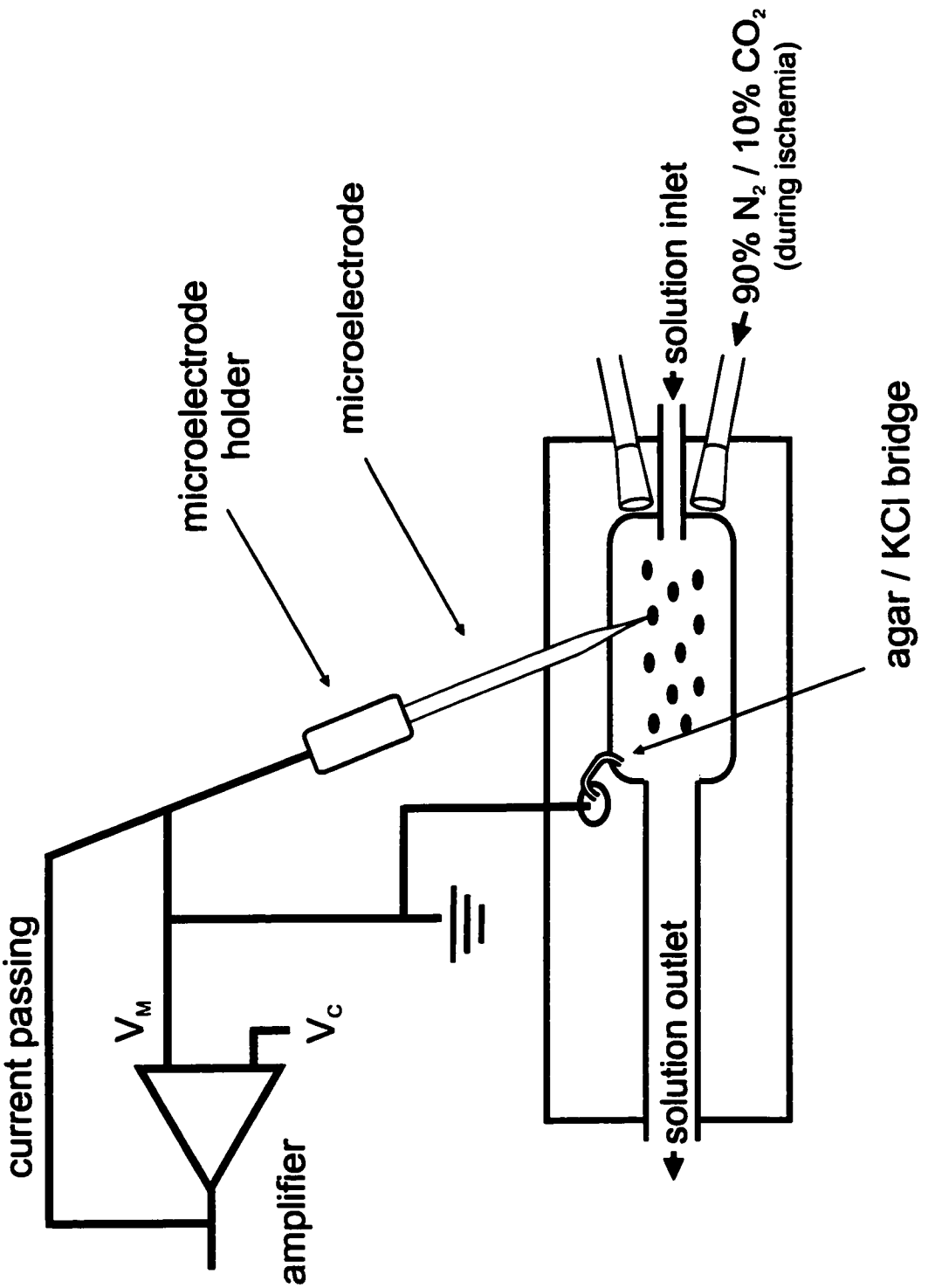

current passing
V_M
V_C
amplifier
microelectrode holder
microelectrode
solution inlet
90% N_2 / 10% CO_2 (during ischemia)
agar / KCl bridge
solution outlet

Figure 8

Figure 8. Experimental Setup.

Isolated myocytes were placed in an experimental chamber as shown and continuously superfused with extracellular solution. Under control conditions, myocytes were superfused with Tyrode's solution. During simulated ischemia, the extracellular solution was altered to an "ischemic" Tyrode's solution, and 90% N_2 / 10% CO_2 gas was distributed over the experimental chamber through two lengths of hosing (approximate diameter = 1 cm). In some experiments, myocytes were impaled with high-resistance microelectrodes. The electrode was mounted in an electrode holder and connected to a ground. To complete the circuit, a wire connected to the ground was placed in a small well of KCl, which was electrically connected to the experimental chamber via an agar / KCl bridge. In single-electrode voltage-clamp experiments, the membrane voltage (V_m) is measured and compared with a designated command voltage (V_c) using an amplifier. If V_m is found to differ from V_c, then current is passed through the microelectrode to return V_m to V_c. In our experiments, the voltage and current signals measured by the amplifier were sent to a computer where they were displayed and recorded.

Table 4: Composition of Tyrode's Solution

Compound	Concentration (mM)
NaOH	129
KCl	4
NaH_2PO_4	0.9
$NaHCO_3$	20
Glucose	5.5
$MgSO_4$	0.5
$CaCl_2$	2.5
Bubbled with	95% O_2 / 5% CO_2
pH	7.4
PO_2 (mmHg)	811.7 ± 25.9

- Cordeiro et al., 1994

superfusion of the myocytes. The solution change time was measured as approximately 1 min. Tyrode's solution and all other extracellular solutions were maintained at 37°C with a circulating heated water system driven by an immersion ciculating heater (Haake E2 model, Fisher Scientific, Berlin, Germany).

C. General Methods

Experiments were performed only on cells that were free of membrane blebs and were not spontaneously contracting. Some myocytes were randomly selected to serve as time controls and were superfused with Tyrode's solution for the duration of the experiment. All other myocytes were superfused for 10 min with Tyrode's solution, and then for 30 min with a solution mimicking specific conditions of myocardial ischemia, including: hypoxia, hypercapnia, hyperkalemia, acidosis, lactate accumulation and substrate deprivation (Ferrier et al., 1985; Ferrier and Guyette, 1991). This "ischemic solution" (Table 5) was gassed with 90% N_2 / 10% CO_2 (Praxair Canada Inc.) which reduces PO_2 to 26.9 ± 6.0 mm Hg (Cordeiro et al., 1995). As well, atmospheric oxygen was excluded by a 90% N_2 / 10% CO_2 gas phase which was layered over the experimental chamber via two lengths of hosing at the end of the chamber (see Figure 8). Reperfusion was simulated by return to Tyrode's solution, and removal of the gas phase. Cells were exposed to only one cycle of ischemia and reperfusion.

In Part II of this thesis, the effects of losartan were examined in some experiments by including 10 μM losartan in the "ischemic solution". In other experiments, cells were exposed to either 0.1 μM AII or AII plus losartan during simulated ischemia. It has previously been reported that at these concentrations, losartan

Table 5: Composition of "Ischemic" Tyrode's Solution

Compound	Concentration (mM)
NaOH	123
KCl	8
NaH_2PO_4	0.9
$NaHCO_3$	6
Lactate	20
$MgSO_4$	0.5
$CaCl_2$	2.5
Bubbled with	90% N_2 / 10% CO_2
pH	6.8
PO_2 (mmHg)	26.9 ± 6.0

- Cordeiro et al., 1995

has anti-arrhythmic actions during ischemia and reperfusion, and can block the effects of AII (Thomas et al., 1996). Drugs were not applied during reperfusion.

The actions of losartan in the absence of ischemia and reperfusion were also examined. In these experiments, cells were exposed to Tyrode's solution containing 10 μM losartan for 30 min followed by 40 min of washout. Therefore, the treatment period was identical to that in ischemia / reperfusion experiments.

3. Recording Techniques

A. Experiments in Impaled Myocytes

In some experiments, cells were impaled with high-resistance microelectrodes (18-25 MΩ) to minimize dialysis and avoid buffering intracellular Ca^{2+} levels. The electrodes were made of borosilicate capillary tubes with a filament, and outer and inner diameters of 1.2 mm and 0.69 mm, respectively (Sutter Instruments Co., Novato, CA). These high-resistance electrodes were made each experiment day with a Flaming/Brown micopipette puller (Model P-87, Sutter Instruments Co., Novato, CA). Electrodes were filled with 2.7 M KCl by placing a drop of KCl in the middle of the electrode and allowing the tip of the electrode to fill by capillary action. The remainder of the electrode was then back-filled with KCl, and the electrode was inserted into a microelectrode holder (World Precision Instruments, Inc., Sarasota, FL) containing 2.7 M KCl and a Ag/AgCl pellet. The microelectrode holder was inserted into the pin jack of an amplifer headstage (Axoclamp 2B, Axon Instruments) which, in turn, was connected via a cable to the main amplifier. The headstage itself was mounted on a micromanipulator (Leitz Inc.,

Wetzlar, Germany) which allowed the electrode to be precisely positioned in the experimental chamber.

The headstage was grounded by means of a Ag/AgCl wire which was chlorided in household bleach prior to each experiment. One end of the wire was connected to the headstage, while the other was immersed in a well filled with 2.7 M KCl. The well was electrically connected to the experimental chamber by a small U-shaped piece of glass tubing filled with 1% agar in 2.7 M KCl. This agar / KCl bridge design limits the movement of ions through the bridge, and thus, minimizes junction potentials when the extracellular solution is changed.

In preparation for impalement, the tip of the microelectrode was placed slightly above a selected cardiomyocyte. Liquid junction potentials were then cancelled by zeroing the input voltage offset dial on the amplifier. Next, the resistance of the electrode was measured by passing a 1 nA current. Since resistance = voltage / current, the voltage displayed on the amplifier in mV in response to a 1 nA current is equal to the electrode resistance in MΩ. If the resistance was observed to be between 18 and 25 MΩ, the electrode was deemed suitable for use, and the electrode resistance was then cancelled. When using the Axoclamp 2B amplifier, this can be accomplished by adjusting the bridge dial so that a voltage proportional to the voltage drop across the electrode is subtracted from all recordings. Therefore, in our experiments voltage records only reflected the membrane voltage. Finally, electrode capacitance was neutralized so that, in voltage-clamp experiments, the amplifier could accurately control membrane voltage. This conpensation was attained by placing the amplifier in discontinuous current clamp (DCC) mode and passing 1 nA current through the electrode. The switching rate for current

passing and membrane potential recording was set to approximately 8-10 kHz, and the associated voltage waveform was observed on an oscilloscope (Kikusui Model COR5541U, Kikusui Electronic Corp., Japan). Using the capacitance adjustment dial on the amplifier, this waveform was optimized so that the voltage response triggered by one cycle of current application returned to baseline before the next current application.

Once liquid junction potentials, electrode resistance, and electrode capacitance were cancelled, the electrode was ready for impalement and the amplifier was returned to bridge mode. Using the micromanipulator, the electrode was slowly lowered until it gently pressed against the cell. At this point, impalement was facilitated by briefly overcompensating for the electrode capacitance. This technique, commonly referred to as "buzzing", helps the cell membrane seal around the tip of the electrode, and increases the likelihood of a successful impalement. In some cells, stable resting membrane potentials were not immediately observed, so small negative currents were applied to maintain membrane potential at values more negative than -80 mV. Usually, after a few minutes, membrane potential stabilized and this negative current was removed. Cells in which resting membrane potential did not stabilize were not used in experiments.

Once a myocyte was successfully impaled, it was stimulated by a series of action potentials and voltage clamp protocols. Both were generated with pCLAMP software (Axon Instruments, Inc., Foster City, CA). To record the experimental changes in current and voltage measured by the amplifier, these analog signals were digitized by a Labmaster analog-to-digital (A/D) interface (TL1-125, Axon Instruments Inc.) and sent to a computer for display and storage. pCLAMP software was used to analyze data.

i) Measurement of Action Potentials and Associated Contractions

In impaled myocytes, action potentials were initiated by 3.5 ms current pulses delivered through the recording electrodes, and were recorded with conventional microelectrode techniques at 5 min intervals (averages of 5 initiated at 2.8 Hz). Resting membrane potential (RMP) was measured as the membrane potential immediately prior to the upstroke of the action potential. Action potential duration (APD) was measured at 80% repolarization with respect to the action potential amplitude.

Myocyte contractions elicited by action potentials were also recorded and measured. A video camera (Pulnix America, Model TM-640) connected to a monitor (Model OVM-12E12, Oriental Precision Co Ltd., Sung Nam City, Korea) was used to visualize cells. Unloaded cell shortening was sampled at 120 Hz using a video edge detector (Model VED 103, Crescent Electronics, Sandy, UT). In these experiments, one edge of the myocyte was tracked with the edge detector, although in other experiments both edges were monitored (see below). As with voltage and current data, the analog contraction signal was sent via a Labmaster A/D interface to a computer for display and storage. pCLAMP software was used to acquire and analyze contraction data. Contraction amplitudes were measured with respect to rest length immediately before the onset of cell shortening.

ii) Measurement of Transmembrane Currents and Contractions Elicited by Voltage-Clamp Steps

During the 5 min intervals between sets of action potentials recordings, cells were exposed to discontinous single-electrode voltage clamp (dSEVC) protocols (Figure 8). With dSEVC, the same electrode is used to both measure the cell voltage and to apply current. The amplifier controls the membrane voltage by rapidly (8-10 kHz) switching between these two modes. When the measured membrane voltage differs from the command voltage designated by the experimenter, current is applied which is proportional to this voltage error. Thus, the membrane voltage is returned to the command potential. After application of current, the amplifier switches back to voltage recording mode. During the experiment, the voltage waveform produced by the current passing-voltage measuring cycle was continuously monitored on the oscilloscope. This was done to ensure that capacitance compensation was such that elicited voltage recovered to baseline before another current pulse was applied.

In all voltage-clamp experiments, a holding potential of -80 mV was used. Test steps were preceded by trains of ten 200-ms conditioning pulses to 0 mV to provide a consistent history of activation. Three voltage clamp protocols were used. In one protocol, conditioning pulses were followed by a 500 ms step to -40 mV to inactivate sodium current, followed by a 200 ms test step to 0 mV to elicit $I_{Ca\text{-}L}$ and contraction. Peak inward $I_{Ca\text{-}L}$ was measured with respect to steady-state current (I_{SS}) at the end of the 200 ms step. The absolute magnitude of I_{SS} was measured with respect to 0 nA.

A second voltage-clamp protocol was used to separate the VSRM and CICR components of EC coupling (Ferrier and Howlett, 2001). In this protocol, conditioning

pulses were followed by a 500 ms repolarization to -52 mV to inactivate sodium current but allow activation of the VSRM, and two sequential 250 ms test steps to -40 and 0 mV, respectively.

In the third voltage-clamp protocol, conditioning pulses were followed by a 500 ms step to –52 mV, and a 200 ms test step to 0 mV to elicit both VSRM and CICR components of contraction. Contractions elicited by both voltage-clamp protocols were measured as described above for action potential contractions. Voltage-clamp protocols were run once every 5 min during the experiment, immediately following action potential recordings.

iii) Fluorescence Measurements

In other experiments, $[Ca^{2+}]_i$ was measured by whole cell photometry using the fluorescent dye fura-2. This molecule has several characteristics which make it very useful for fluorescence experiments. Myocytes are loaded with the membrane-permeant form of the dye, fura-2 AM ester. When fura-2 AM crosses the sarcolemma, cytosolic esterases catalyze the conversion of this molecule to the cell impermeant Ca^{2+} chelator, fura-2 potassium salt. Therefore, after being loaded with dye, cells maintain a high concentration of fura-2. Importantly, de-esterified fura-2 is also not able to pass into the SR (Sipido and Callewaert, 1995). Therefore, fura-2 can be used to measure the Ca^{2+} transient since it reflects the Ca^{2+} concentration in the cytosol. Another important feature of this dye is that the fura-2 molecule absorbs light differently when it binds Ca^{2+}. In its Ca^{2+}-unbound form, fura-2 absorbs light maximally near 380 nm, while fura-2-Ca^{2+} absorbs maximally near 340 nm (Grynkiewicz et al., 1985). However, in both states, the

dye emits light at 510 nm. Therefore, fura-2 is described as a dual-excitation, single emission dye. These fluorescent properties are useful because the ratio of emission at each excitation wavelength (E_{340} / E_{380}) can be used to calculate $[Ca^{2+}]_i$. This ratiometric method for estimating $[Ca^{2+}]_i$ minimizes error resulting from uneven dye distribution and photobleaching, since these alterations should equally affect measurements at both wavelengths.

Before fluorescence experiments were conducted, myocytes were first loaded with fura-2 AM in Tyrode's solution. To isolate cells from the high-potassium storage solution, approximately 2 ml of this solution were placed in a test tube and briefly centrifuged, producing a small pellet of cells. The storage solution was then decanted from the tube and replaced with 2 ml of Tyrode's solution, and the cells were resuspended. Myocytes were loaded in the dark with the cell permeant fura-2 AM ester (4 μM) for 20 min.

After loading with fura-2, cells were placed in the experimental chamber (described above) mounted on the stage of a Nikon Eclipse fluorescence microscope (Model TEG220, Mississauga, ON). Other equipment for fluorescence experiments was purchased from Photon Technology International (PTI, Brunswick, NJ). UV light for fluorescence excitation was provided by a PTI DeltaRam high speed multiwavelength illuminator with LPS-220 lamp power supply and SC-500 shutter controller. Light was transmitted from the illuminator to the microscope via a fiber optic cable. Myocytes were alternately excited at 340 nm and 380 nm, and emission at 510 nm was measured only from the myocyte using an adjustable rectangular window. Light emitted within this window was collected by the microscope optics, directed to the D-104 microscope

photometer (PTI), and detected by an Analog Photomultiplier Detection System (Model 814, PTI). This analog fluroescence signal was then sent via a computer interface (PTI) to a computer for display and storage.

Myocytes were impaled with high resistance electrodes as described previously, with the exception that a Bertrand lens was used to position the electrode tip. Following impalement, the microscope light was turned off and and the photometer was switched to measurement mode. The experimental chamber was then darkened to prevent stray light from interfering with fluorescence measurements. During the experiment, fluorescence emission, membrane voltage, and currents were recorded using Felix software (version 1.4, PTI). Following each experiment, background fluorescence was measured by moving the rectangular window away from the cell. This background level was subtracted from E_{340} and E_{380} before the emission ratio was calculated.

In order to calculate $[Ca^{2+}]_i$, a calibration curve was prepared by measuring the E_{340} / E_{380} ratio for fura-2 at known concentrations of Ca^{2+}. These standard solutions were prepared using K-MOPS / EGTA buffers containing 1 μM cell-impermeant Fura-2 (Grynkiewicz et al., 1985). Two calibration curves at pH 7.2 were performed and were virtually identical. The average of these two curves was used to calculate $[Ca^{2+}]_i$ and is shown in Figure 9. $[Ca^{2+}]$ measured with this calibration curve is expected to slightly underestimate the actual $[Ca^{2+}]_i$ during ischemia, since intracellular acidosis increases the dissociation constant of fura-2 (Martinez-Zaguilan et al., 1991). However, in the present study, calibration curves determined at pH 6.8, 7.0, and 7.2, indicated that differences were negligible at $[Ca^{2+}]$ from 100 nM to 1μM (not shown). Estimations of $[Ca^{2+}]_i$ during reperfusion were expected to be accurate since intracellular pH has been reported

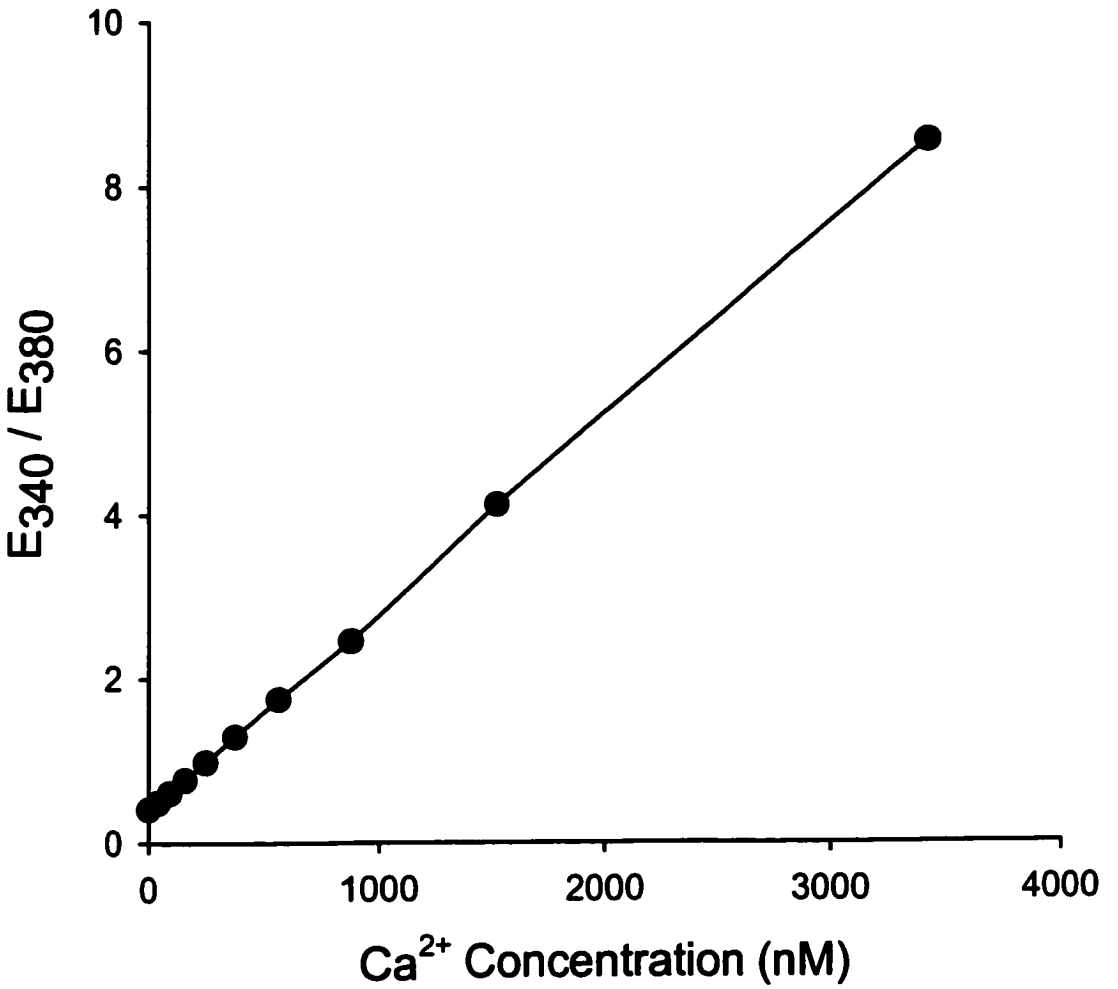

10
8
6
4
2
0
E_{340} / E_{380}
0
1000
2000
3000
4000
Ca^{2+} Concentration (nM)

Figure 9

Figure 9: Calibration curve for calculating $[Ca^{2+}]_i$ from E_{340} / E_{380}.

A calibration curve was produced by measuring E_{340} / E_{380} over a range of Ca^{2+} concentrations at pH 7.2. Standard Ca^{2+} solutions containing cell-impermeant fura-2 were prepared using K-MOPS / EGTA buffers. The displayed curve is the average of two calibration experiments. This curve was used to convert measured values of E_{340} / E_{380} to $[Ca^{2+}]_i$.

to recover to pre-ischemic levels rapidly upon reperfusion (Kusuoka et al, 1987; Kitakaze et al., 1988).

iv) Estimation of SR Ca^{2+} Stores

In impaled myocytes, fluorescence experiments were used to examine SR Ca^{2+} stores during ischemia / reperfusion and parallel time controls. Since caffeine causes release of Ca^{2+} from stores by activating the SR Ca^{2+} release channel (O'Neill and Eisner, 1990), caffeine-elicited Ca^{2+} transients were used to estimate SR Ca^{2+} content. Myocytes were stimulated with current pulses (3.6 ms, 2 Hz) delivered through the microelectrode using Pulsar 6i stimulators (Model 04011, Frederick Haer & Co, Brunswick, ME). At 10, 40, 55, 70, and 80 min during the experiment, stimulation was briefly interrupted and 10 mM caffeine was applied to cells for 1 sec with a rapid solution changer, triggered by one of the Pulsar 6i stimulators. The rapid solution changer allowed the extracellular solution to be changed within 300 ms, while the temperature of the applied solution was maintained at 37°C by a heated circulating water system. Although the rapid solution changer applies solution to myocytes at high velocity, cells were effectively anchored in place by impaled electrodes, and were not observed to be displaced from the measurement window during caffeine application. Caffeine-elicited Ca^{2+} transients were measured relative to diastolic $[Ca^{2+}]_i$ immediately prior to activation of the rapid solution changer. Felix software (version 1.4, PTI) was used to acquire and analyze $[Ca^{2+}]_i$ data.

B. Experiments in field-stimulated myocytes

In some experiments, it was not necessary to impale myocytes with electrodes since neither the rapid solution solution changer nor voltage-clamp protocols were used. Instead, myocytes were continuously field-stimulated (100 V, 2 Hz, 3 ms pulse duration) through a pair of platinum electrodes placed on opposite sides of the experimental chamber. Electrodes were connected to a Grass stimulator (Model 5D9B, Grass Medical Instruments, Quincy, MA). Cell length and $[Ca^{2+}]_i$ were separately examined in field-stimulation experiments during ischemia / reperfusion and parallel time controls. Cell length was recorded by tracking both edges of the myocyte using equipment described previously. In another set of experiments, $[Ca^{2+}]_i$ was monitored by methods discussed above. Diastolic cell length and $[Ca^{2+}]_i$ were measured at a point immediately before responses. Systolic cell length and $[Ca^{2+}]_i$ were measured at peak values during responses. Cell shortening and Ca^{2+} transients were calculated as the difference between diastolic and systolic measurements. Cell length and $[Ca^{2+}]_i$ were recorded at 5 min intervals throughout the experiment, except for the first 5 min of reperfusion when recordings were made every minute. Three responses were averaged for each recording period. Cell length data and $[Ca^{2+}]_i$ data were acquired and analyzed using Axoscope (version 8.0, Axon Instruments, Inc.) and Felix software (version 1.4, PTI), respectively.

4. Drugs and Chemicals

Caffeine was purchased from the Sigma Chemical Company (St. Louis, MO, USA) and was dissolved in de-ionized water. The chemicals for buffer solutions were

purchased from BDH Inc. (Toronto, Ontario, Canada), Fisher Scientific (Nepean, Ontario, Canada), or the Sigma Chemical Company (Oakville, Ontario, Canada).

Fura-2 AM and cell-impermeant Fura-2 were purchased from Molecular Probes (Eugene, OR, USA). Fura-2 AM was dissolved in anhydrous DMSO (Sigma Chemical Co.) to form a 2.5 mM stock solution. Cell-impermeant Fura-2 was dissolved in de-ionized water to form a 1.2 mM stock solution which was used in standard solutions for calibration of $[Ca^{2+}]_i$.

Losartan was a gift from Merck Frosst Canada Inc. (Kirkland, Quebec) and was dissolved in de-ionized water to form a 10 mM stock solution. AII was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and was dissolved in de-ionized water to form a 1 mM stock.

5. Statistical Analyses

Data are presented as means ± SEM. Differences in incidence of I_{TI} between cell populations were determined with a chi square test. All differences between experimental groups and time controls were tested for statistical significance with a two-way repeated measures analysis of variance. All other data were analyzed relative to pre-ischemic values using a one-way repeated measures analysis of variance. Post-hoc comparisons were made with a Bonferroni test. Differences were considered significant when $P < 0.05$. Statistical analyses were performed with Sigma Stat (Jandel, version 2.0). The value of n represents the number of myocytes sampled. No more than 3 myocytes from a single heart were used for any one experiment.

RESULTS – PART I

1. Development of a Cellular Model of Stunning

In previous studies from this laboratory, isolated myocytes exposed to simulated ischemic conditions exhibited contractile and electrophysiological changes that are characteristic of the ischemic myocardium (Cordeiro et al., 1994, 1995). However, reperfusion following 20 min of simulated ischemia was not observed to cause contractile depression (stunning) in these studies (Cordeiro et al., 1994, 1995). Therefore, in the present study, the duration of the ischemic period was increased to 30 min with the hope that a more severe ischemic insult would increase the degree of reperfusion injury. Since the objective of this study was to develop a model in which post-ischemic ventricular myocytes exhibited both normal electrical activity and stunning, contractions and action potential configuration were examined during 30 min of ischemia and throughout reperfusion.

Figure 10 shows recordings of action potentials (Panel B) and associated contractions (Panel A) elicited by a 3.5 ms current stimulus (illustrated at the bottom) delivered through the microelectrode. Representative traces are shown at several selected times during an experiment. Under pre-ischemic conditions, contractions consisted of an initial phasic component, and often a smaller sustained component (Fig. 10A). During ischemia, myocytes exhibited a marked reduction in the amplitude of contractions. Ischemia also caused abbreviation of action potentials and depolarization of cell membranes (Fig. 10B). Amplitudes of contractions increased in early reperfusion, but

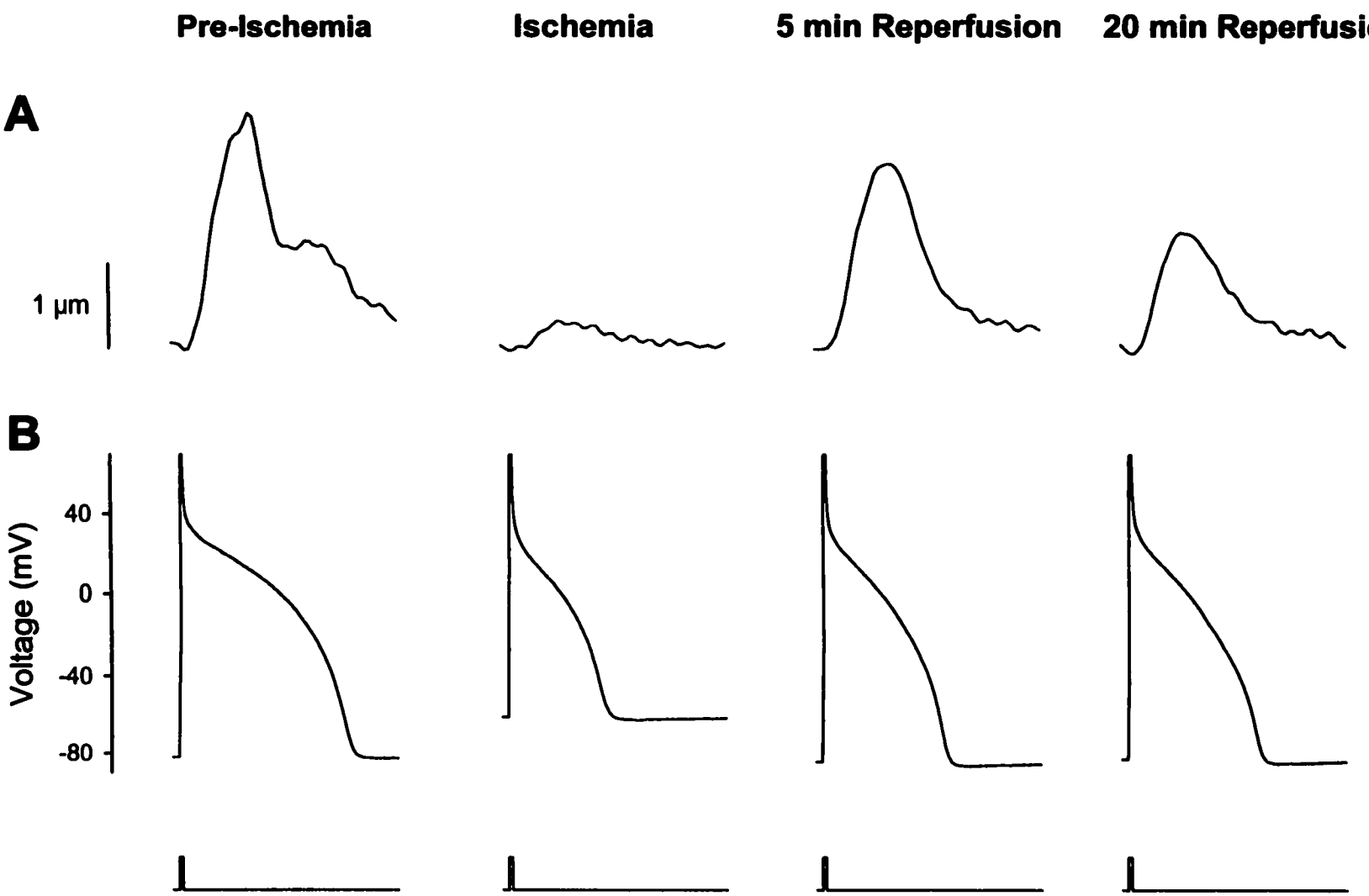

Pre-Ischemia
Ischemia
5 min Reperfusion
20 min Reperfusion
A
1 µm
B
Voltage (mV)
40
0
-40
-80
50 ms

Figure 10

Figure 10. Changes in membrane potential and cell shortening in cells exposed to simulated ischemia and reperfusion.

Representative recordings are shown for a myocyte during pre-ischemia, ischemia, early (5 min) reperfusion and late (20 min) reperfusion. Action potentials (**Panel B**) and associated contractions (**Panel A**) were elicited by passing depolarizing current (**Panel B**, bottom). During ischemia, action potentials shortened, cell membranes depolarized, and contractions were reduced in magnitude. During reperfusion, action potentials gradually recovered to pre-ischemic levels. However, contractions recovered in early reperfusion, but decreased again in late reperfusion.

decreased with continued reperfusion (Fig. 10A). Action potential configuration recovered with reperfusion (Fig. 10B).

Figure 11 shows mean data describing changes in contraction amplitudes, APD, and RMP, during the series of experiments. During ischemia, contractions decreased significantly from pre-ischemic levels (Fig. 11A). Ischemia also resulted in a gradual reduction in APD (Fig. 11B) and a marked decrease in RMP (Fig. 11C). Upon reperfusion, contractions initially recovered to pre-ischemic values, but then were significantly reduced in late reperfusion (Fig. 11A). Membrane potential recovered rapidly in reperfusion (Fig. 11C), and APD gradually returned to control levels (Fig. 11B). Therefore, in late reperfusion myocytes exhibited contractile depression associated with normal electrical activity as assessed by measurements of RMP and APD. This observation suggests that isolated ventricular myocytes exposed to 30 min of simulated ischemia followed by reperfusion may be used to model myocardial stunning.

2. EC Coupling in a Cellular Model of Stunning

A. Effects of ischemia and reperfusion on contraction and transmembrane currents elicited by voltage-clamp protocols. The cellular model of stunning was used to examine changes in EC coupling during ischemia and reperfusion. $I_{Ca\text{-}L}$ and associated contractions were examined using the voltage-clamp protocol shown at the top of Figure 12 (described in methods). Representative currents and contractions, recorded at selected times during an experiment, are shown in Figures 12A and 12B, respectively. Under pre-ischemic conditions, the 200 ms test step from -40 mV to 0 mV elicited an inward current (Fig. 12A) and contraction (Fig. 12B) that were blocked with the Ca^{2+} channel antagonist,

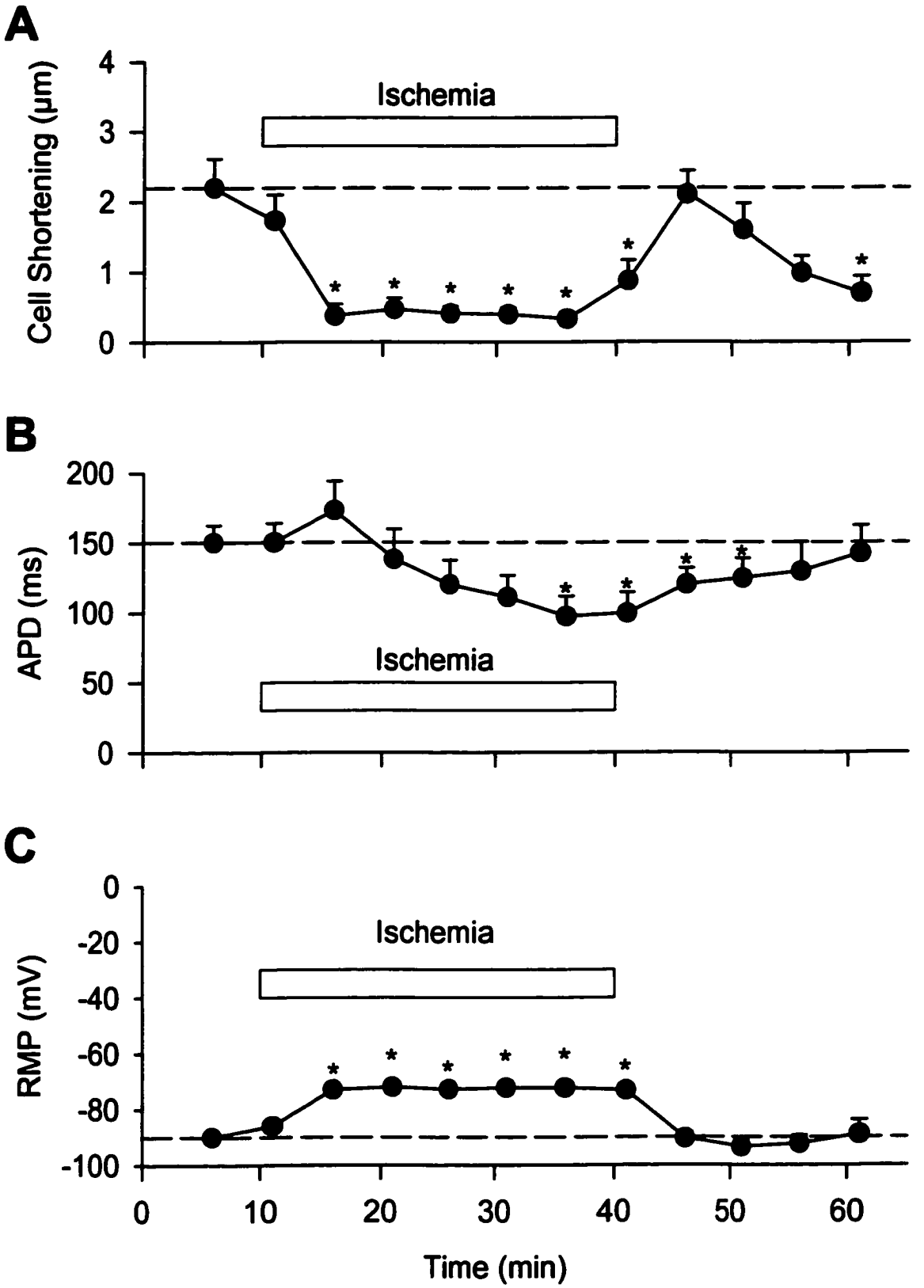

A
Cell Shortening (µm)
4
3
2
1
0
Ischemia
B
APD (ms)
200
150
100
50
0
Ischemia
C
RMP (mV)
0
-20
-40
-60
-80
-100
Ischemia
0
10
20
30
40
50
60
Time (min)

Figure 11

Figure 11. Action potential configuration was normal but myocytes exhibited contractile depression (stunning) in late reperfusion.

Ischemia was associated with a gradual decrease in APD (**Panel B**) and membrane depolarization (**Panel C**). Mean contraction amplitude also was decreased during ischemia (**Panel A**). RMP and APD both recovered to pre-ischemic levels by late reperfusion, when significant contractile depression (stunning) was observed (* denotes significant difference from pre-ischemic values, $P < 0.05$, n=21).

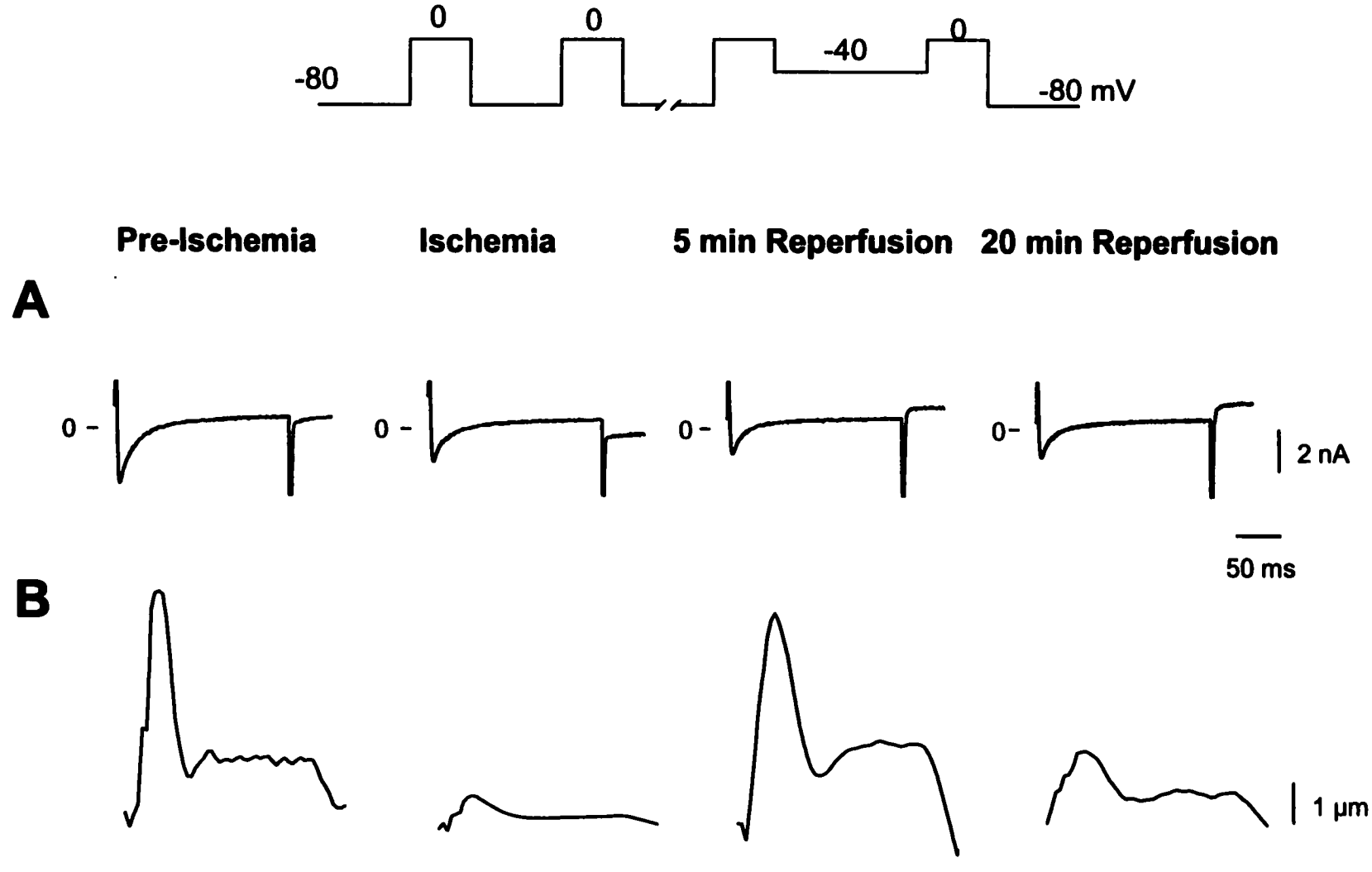

0
0
-40
0
-80
-80 mV
Pre-Ischemia
Ischemia
5 min Reperfusion
20 min Reperfusion
A
0 -
0 -
0 -
0 -
2 nA
50 ms
B
1 µm

Figure 12

Figure 12. Changes in membrane currents and contractions in voltage-clamped myocytes exposed to simulated ischemia and reperfusion.

The voltage-clamp protocol shown at the top was used to elicit $I_{Ca\text{-}L}$ **(Panel A)** and associated contractions **(Panel B)**. Representative recordings show that peak $I_{Ca\text{-}L}$ was reduced during ischemia and did not recover in reperfusion. Contractions were decreased in ischemia, recovered in early reperfusion, and were reduced in late reperfusion.

nifedipine (data not shown). Peak inward current declined in ischemia and showed little recovery in reperfusion. Contractions also were depressed by ischemic conditions, but, in contrast to current, exhibited rapid recovery early in reperfusion, followed by sustained depression later in reperfusion.

Figure 13 shows mean measurements of currents and contractions. $I_{Ca\text{-}L}$ was measured as peak inward current relative to the steady-state current (I_{SS}) at the end of the test step. Myocytes exhibited a gradual decrease in $I_{Ca\text{-}L}$ during ischemia that continued in early reperfusion (Fig. 13A). In late reperfusion, $I_{Ca\text{-}L}$ magnitude remained significantly depressed relative to pre-ischemic values. Panel B shows that I_{SS} became significantly more outward during ischemia, but recovered early in reperfusion. Contractions were significantly depressed during ischemia, but showed a rapid return to control levels in early reperfusion (Fig. 13C). Recovery of contractions in early reperfusion was transient, and contraction amplitudes became significantly decreased in later reperfusion. These observations suggest that post-ischemic contractile depression is associated with reduced $I_{Ca\text{-}L}$ in our model of stunning.

B. A Comparison of CICR and VSRM Contractions During Ischemia and Reperfusion

Under the voltage-clamp protocol described above, contractions were elicited by CICR in response to $I_{Ca\text{-}L}$. In the next set of ischemia / reperfusion experiments, CICR contractions were compared with VSRM contractions to examine the contributions of these mechanisms of EC coupling to stunning. These components of contraction were

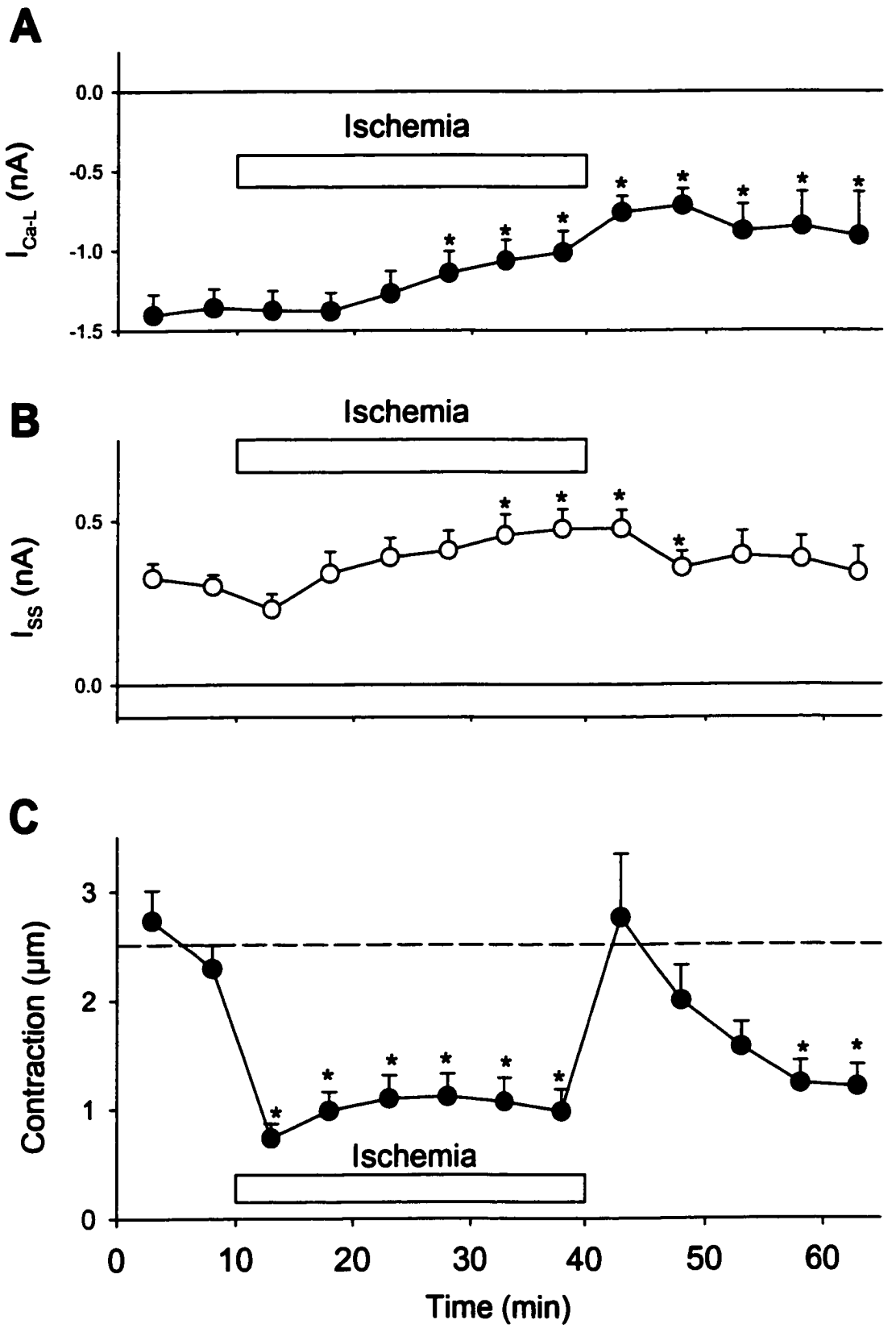

A
0.0
-0.5
-1.0
-1.5
I_{Ca-L} (nA)
Ischemia
B
Ischemia
0.5
0.0
I_{ss} (nA)
C
3
2
1
0
Contraction (μm)
Ischemia
0
10
20
30
40
50
60
Time (min)

Figure 13

Figure 13. Stunned myocytes exhibited reduced $I_{Ca\text{-}L}$ and recovery of I_{SS}.

Mean $I_{Ca\text{-}L}$ amplitude was gradually reduced during ischemia, and did not recover in reperfusion (**Panel A**, top). In contrast, outward I_{SS} increased during ischemia and recovered to control levels in late reperfusion (**Panel B**, bottom). As was observed for contractions elicited by action potentials, contractions elicited by $I_{Ca\text{-}L}$ (**Panel C**) were reduced during ischemia and recovered rapidly in early reperfusion. In late reperfusion, contractions were significantly reduced (* denotes significant difference from pre-ischemic values, $P < 0.05$, n=21).

separately elicited by the two-step voltage-clamp protocol shown at the top of Figure 14 (described in methods). The 250 ms voltage step from -52.5 mV to -40 mV elicited a VSRM contraction that was associated with a small inward current (Fig. 14A). The voltage step to 0 mV elicited a CICR contraction accompanied by $I_{Ca\text{-}L}$ (Fig. 14A). Both VSRM and CICR contractions consisted of a large phasic component and a smaller sustained component. The magnitudes of VSRM and CICR contractions were affected similarly by ischemia and reperfusion (Fig. 14B and 14C). Contractions initiated by both mechanisms decreased in amplitude during ischemia, recovered in early reperfusion, and exhibited significant depression in late reperfusion. Therefore, contractions initiated by either the VSRM or CICR are similarly depressed in stunned myocytes. This suggests that the lesion underlying stunning may be common to both mechanisms of EC coupling.

C. $[Ca^{2+}]_i$ and Cell Length During Ischemia and Reperfusion.

Alterations in Ca^{2+} homeostasis could conceivably contribute to post-ischemic depression of both VSRM and CICR contractions. Therefore, to determine whether Ca^{2+} transients are altered in stunned myocytes, Ca^{2+} transients and cell shortening were examined in the next set of experiments. Time control experiments were included in this data set to determine whether time-dependent alterations in fura-2 fluorescence occur in experiments of this duration. Figure 15 shows original recordings from field-stimulated myocytes that served as time controls. The magnitudes of both Ca^{2+} transients (Fig 15A) and contractions (Fig 15B) were only slightly decreased during 80 min of recording. Mean data show that the slight run-down in magnitude of Ca^{2+} transients (shaded region, Fig. 16A) was associated with a gradual increase in diastolic $[Ca^{2+}]_i$. By 60 min of

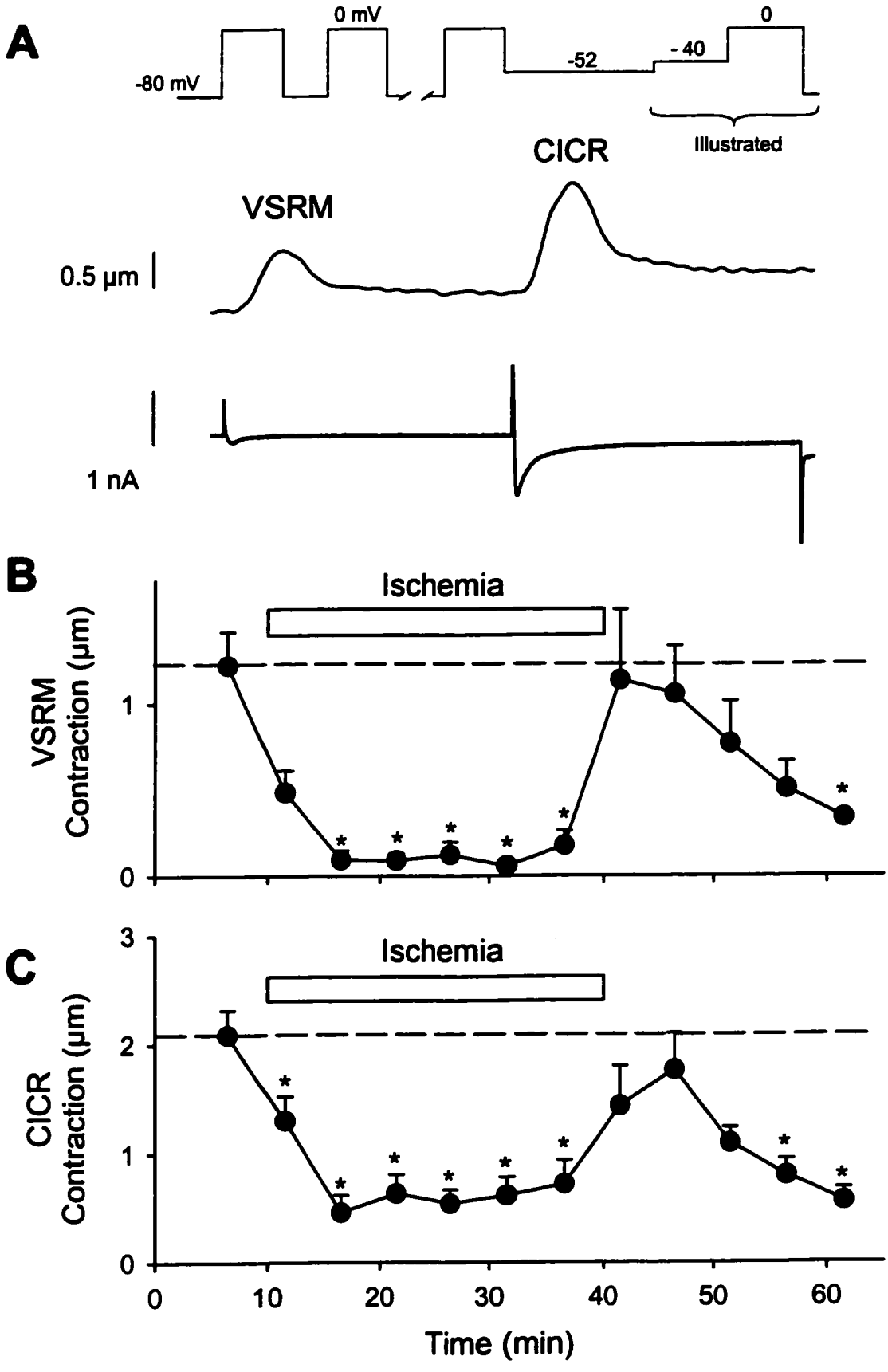

A
0 mV
-80 mV
-52
- 40
0
Illustrated
CICR
VSRM
0.5 µm
1 nA
B
Ischemia
VSRM Contraction (µm)
1
0
C
Ischemia
CICR Contraction (µm)
3
2
1
0
0
10
20
30
40
50
60
Time (min)

Figure 14

Figure 14. Contractions initiated by the VSRM and CICR were similarly depressed in late reperfusion.

The two-step voltage protocol shown in **Panel A** was used to separately elicit VSRM and CICR contractions. VSRM contractions were elicited by a voltage step from –52 mV to –40 mV, and were associated with a small inward current. $I_{Ca\text{-}L}$ and CICR contractions were observed in response to the voltage-step to 0 mV. VSRM and $I_{Ca\text{-}L}$ contraction amplitudes were similarly affected by ischemia and reperfusion (**Panels B** and **C**, respectively). Contractions initiated by both mechanisms were depressed during ischemia and late reperfusion when compared to pre-ischemic levels (* denotes significant difference from pre-ischemic values, $P < 0.05$, n=21).

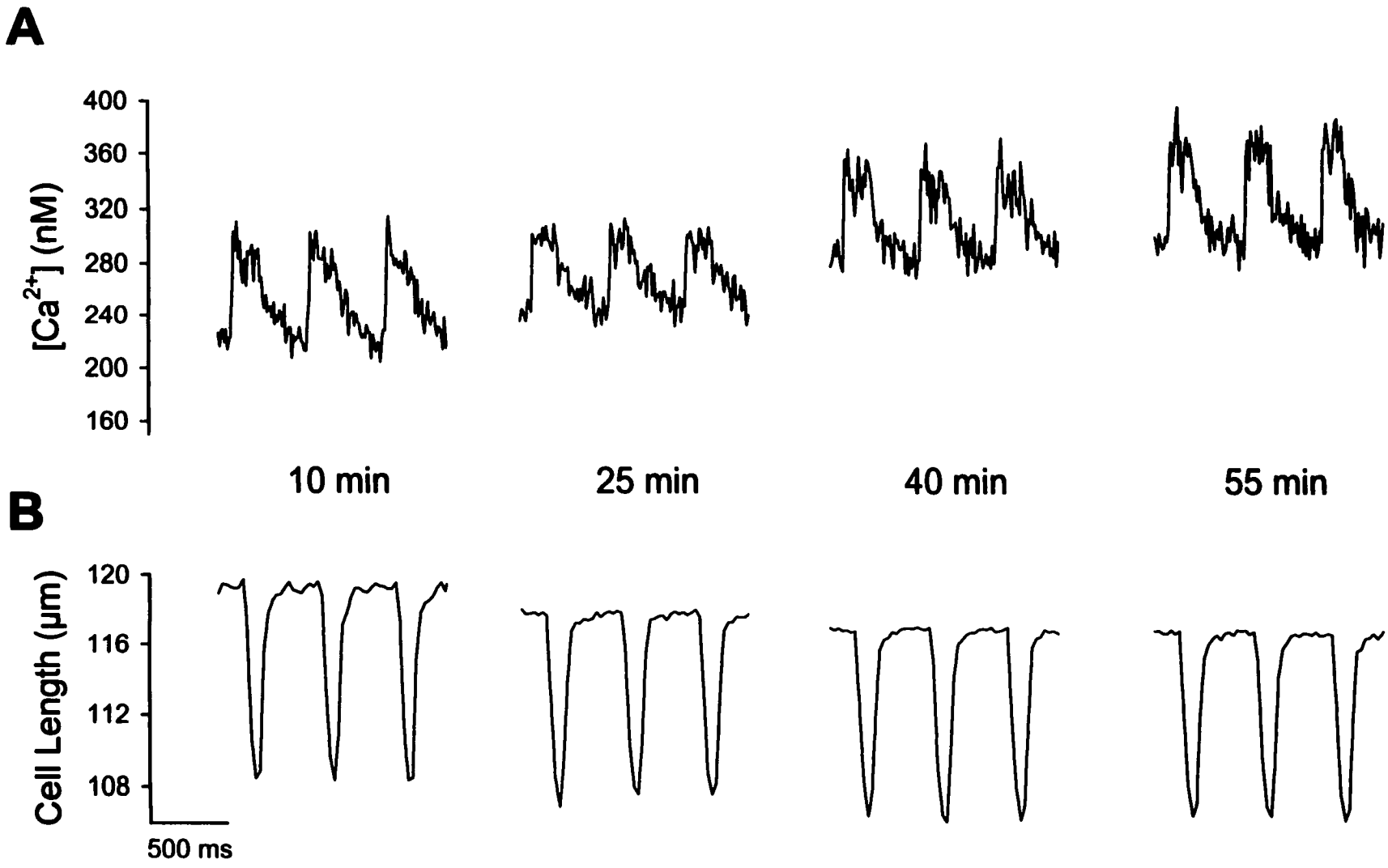

A
400
360
320
280
240
200
160
[Ca2+] (nM)
10 min
25 min
40 min
55 min
B
120
116
112
108
Cell Length (µm)
500 ms

Figure 15

Figure 15. Ca^{2+} transients and contractions in cells that served as time controls. Field-stimulated time controls exhibited only slight reductions in Ca^{2+} transients (**Panel A**) and contractions (**Panel B**) with time. Diastolic $[Ca^{2+}]_i$ appeared to increase slightly during these experiments.

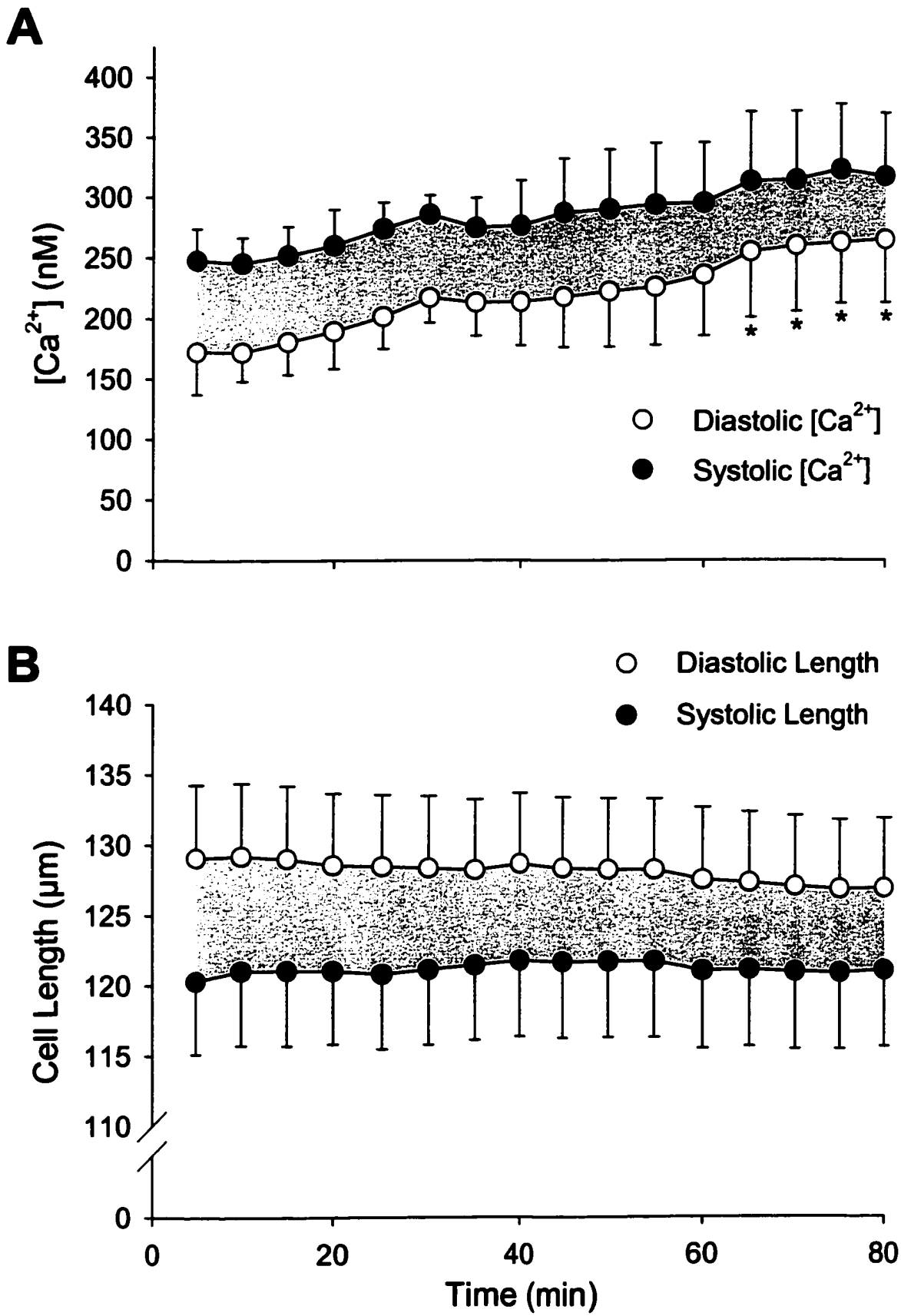

A
400
350
300
250
200
150
100
50
0
[Ca2+] (nM)
Diastolic [Ca2+]
Systolic [Ca2+]
*
*
*
*
B
Diastolic Length
Systolic Length
140
135
130
125
120
115
110
0
Cell Length (µm)
0
20
40
60
80
Time (min)

Figure 16

Figure 16. Ca^{2+} transients and contractions were relatively unchanged during time control experiments.

Panel A: Mean measurements of $[Ca^{2+}]_i$ show that, during 80 min of recording, myocytes exhibited a small decrease in the magnitude of Ca^{2+} transients (shaded region), and a gradual increase in diastolic $[Ca^{2+}]_i$. **Panel B** shows that the magnitude of contractions (shaded region) and mean diastolic length were also slightly decreased with time (* denotes significant difference from pre-ischemic values, $P < 0.05$; $[Ca^{2+}]_i$ measurements, $n = 5$; length measurements, $n = 12$).

recording, this increase in diastolic $[Ca^{2+}]_i$ was significantly different from values at 10 min. Figure 16B shows that there was only a slight decrease in diastolic cell length and contraction magnitude (shaded region).

Figure 17 shows representative recordings of $[Ca^{2+}]_i$ and cell length from field-stimulated myocytes exposed to ischemia and reperfusion. During ischemia, although diastolic $[Ca^{2+}]_i$ increased, the magnitude of Ca^{2+} transients was unchanged (Fig. 17A). Contractions were markedly depressed during ischemia (Fig. 17B). Early reperfusion was associated with an increase in magnitudes of both contractions and Ca^{2+} transients that exceeded pre-ischemic levels. Myocytes also exhibited a partial recovery of diastolic $[Ca^{2+}]_i$ in early reperfusion although diastolic cell length was markedly depressed. Some myocytes exhibited aftercontractions (arrow, Fig. 17B). With continued reperfusion contractions were markedly decreased, but Ca^{2+} transients were only slightly depressed relative to pre-ischemic levels.

Figure 18 shows mean measurements of $[Ca^{2+}]_i$ and cell length (A and B, respectively) during ischemia and reperfusion. During ischemia, myocytes exhibited a significant increase in diastolic $[Ca^{2+}]_i$ relative to control levels (Fig. 18A). However, myocytes also exhibited an increase in systolic $[Ca^{2+}]_i$ during ischemia, and thus, there was no marked change in the magnitude of Ca^{2+} transients (shaded region). Diastolic $[Ca^{2+}]_i$ partially recovered in early reperfusion, but then gradually increased with continued reperfusion, and was significantly elevated by 80 min of recording. Reperfusion was also associated with a gradual reduction in the magnitude of Ca^{2+} transients (shaded region).

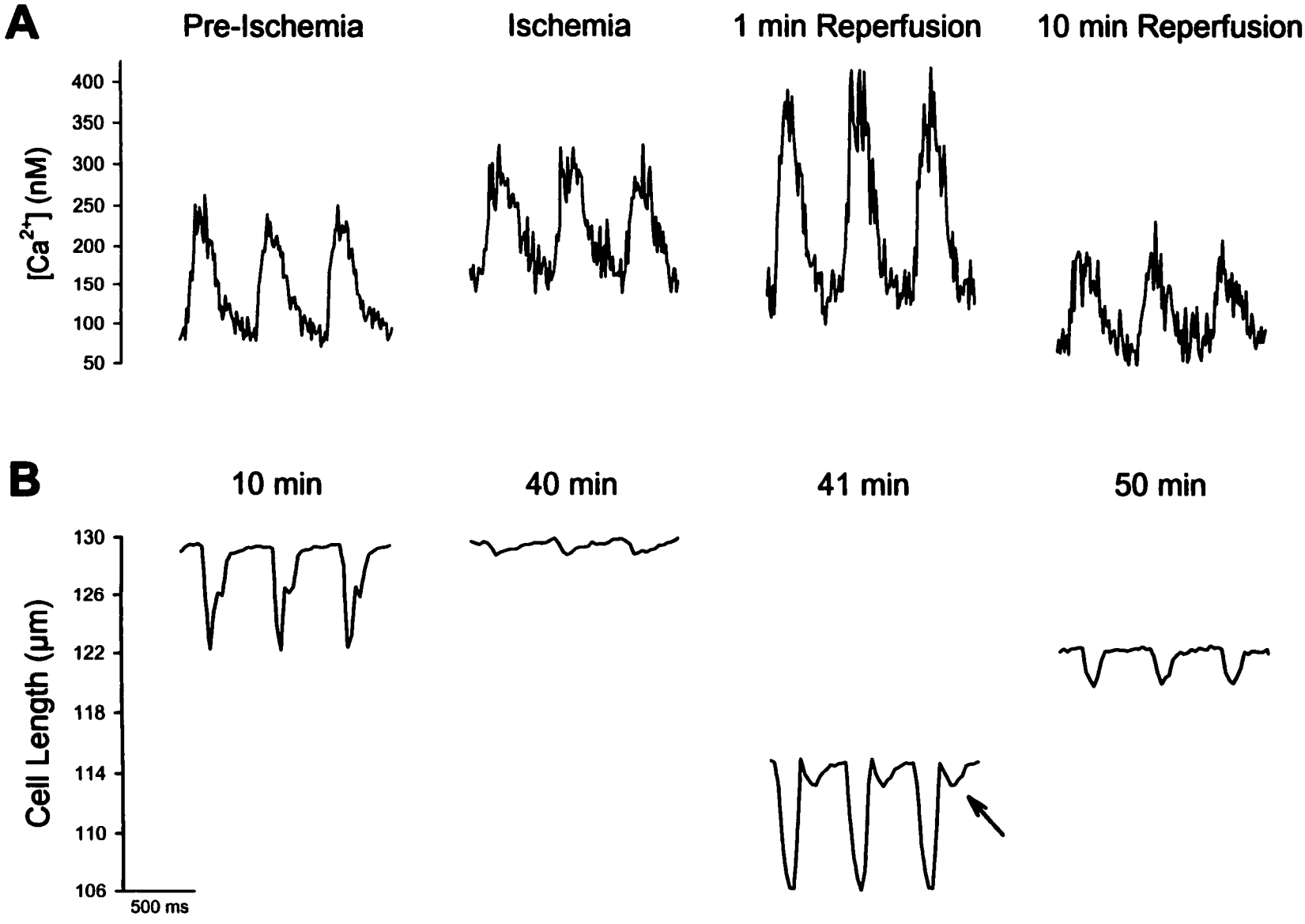

A
Pre-Ischemia
Ischemia
1 min Reperfusion
10 min Reperfusion
[Ca2+] (nM)
400
350
300
250
200
150
100
50
B
10 min
40 min
41 min
50 min
Cell Length (µm)
130
126
122
118
114
110
106
500 ms

Figure 17

Figure 17. Ca^{2+} transients and contractions in field-stimulated myocytes exposed to simulated ischemia and reperfusion.

Representative recordings of Ca^{2+} transients and contractions are shown in **Panels A** and **B**, respectively. During ischemia, diastolic $[Ca^{2+}]_i$ increased although the magnitude of Ca^{2+} transients was unchanged from control levels (**Panel A**). Myocytes exhibited a partial recovery of diastolic $[Ca^{2+}]_i$ upon reperfusion, and an overshoot of Ca^{2+} transients. With continued reperfusion, the magnitude of Ca^{2+} transients decreased slightly. **Panel B** shows that contractions were markedly decreased during ischemia. In early reperfusion, myocytes exhibited a decrease in diastolic length and recovery of contractions. Aftercontractions were occasionally observed in early reperfusion (**arrow**). With further reperfusion, diastolic length partially recovered and contraction magnitude was markedly reduced.

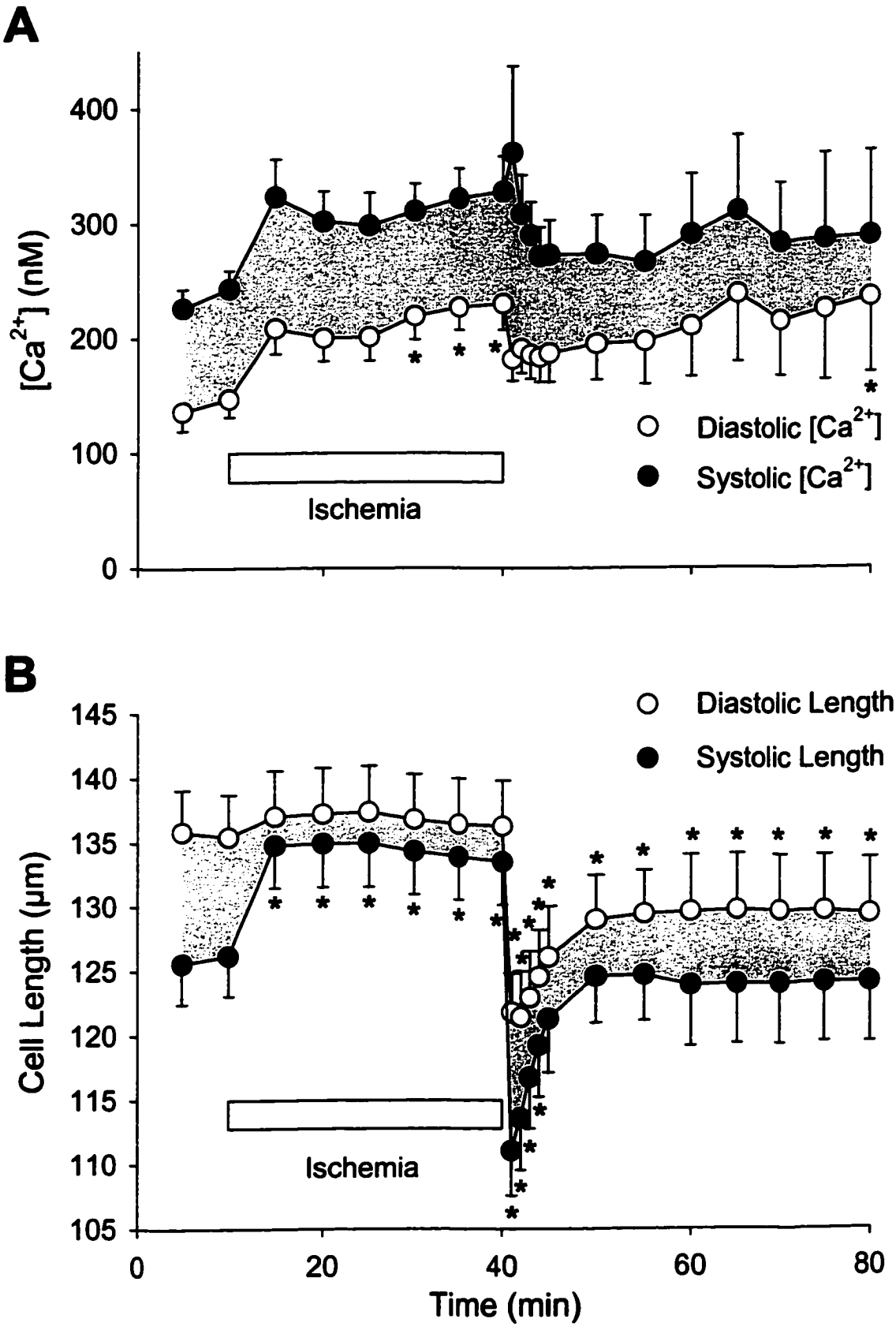

A
400
300
200
100
0
[Ca2+] (nM)
Diastolic [Ca2+]
Systolic [Ca2+]
Ischemia
B
145
140
135
130
125
120
115
110
105
Cell Length (µm)
Diastolic Length
Systolic Length
Ischemia
0
20
40
60
80
Time (min)

Figure 18

Figure 18. Contractile depression in reperfusion was not associated with changes in Ca^{2+} transients.

Mean measurements of $[Ca^{2+}]_i$ are shown in **Panel A.** Ischemia was associated with increased diastolic $[Ca^{2+}]_i$ and no obvious change in the magnitude of Ca^{2+} transients (shaded region). In early reperfusion, mean diastolic $[Ca^{2+}]_i$ partially recovered but increased gradually with continued reperfusion. Ca^{2+} transients were slightly reduced in late reperfusion. **Panel B** shows mean measurements of cell length. During ischemia, myocytes exhibited a marked reduction in the magnitude of cell shortening (shaded region) that was associated with a significant decrease in systolic length. With reperfusion, both diastolic and systolic cell length were decreased and the magnitude of contractions was briefly increased. Contractions were reduced in late reperfusion and diastolic cell length remained below control levels (* denotes significant difference from mean pre-ischemic values, $P < 0.05$, $n = 15$).

Diastolic cell length was relatively unchanged during ischemia (Fig. 18B). However, ischemia was associated with a marked decrease in cell shortening (shaded region) as systolic length measurements were significantly reduced from pre-ischemic values. In early reperfusion, myocytes exhibited a significant decrease in both diastolic and systolic length. With continued reperfusion, diastolic length partially recovered, however contractions (shaded region) were reduced from control levels.

Figure 19 presents a comparison of post-ischemic myocytes and time controls during diastole. As was described in Figure 18A, diastolic $[Ca^{2+}]_i$ appeared to be elevated during ischemia, exhibited a partial recovery in early reperfusion, and gradually increased with continued reperfusion (Fig. 19A). However, diastolic $[Ca^{2+}]_i$ was also gradually increased in time control experiments. When the two groups of myocytes are compared directly, Figure 19A shows that diastolic $[Ca^{2+}]_i$ was elevated relative to time controls during ischemia, but was unchanged from time controls throughout reperfusion.

Normalized diastolic cell length measurements are shown in Figure 19B. Time controls exhibited a slight decrease in diastolic length during 80 min of recording. During ischemia, cells lengthened slightly relative to time controls. Upon reperfusion, diastolic length was markedly reduced, but partially recovered during the first 10 min of reperfusion. In late reperfusion, diastolic cell length remained below the level of time controls, however this difference was not statistically significant.

Figure 20 shows a direct comparison of post-ischemic myocytes and time controls during systole. In untreated myocytes, Ca^{2+} transients were slightly reduced in magnitude during 80 min of recording (Fig. 20A). Ca^{2+} transients remained at the level of time controls during ischemia, but exhibited a brief overshoot in early reperfusion.

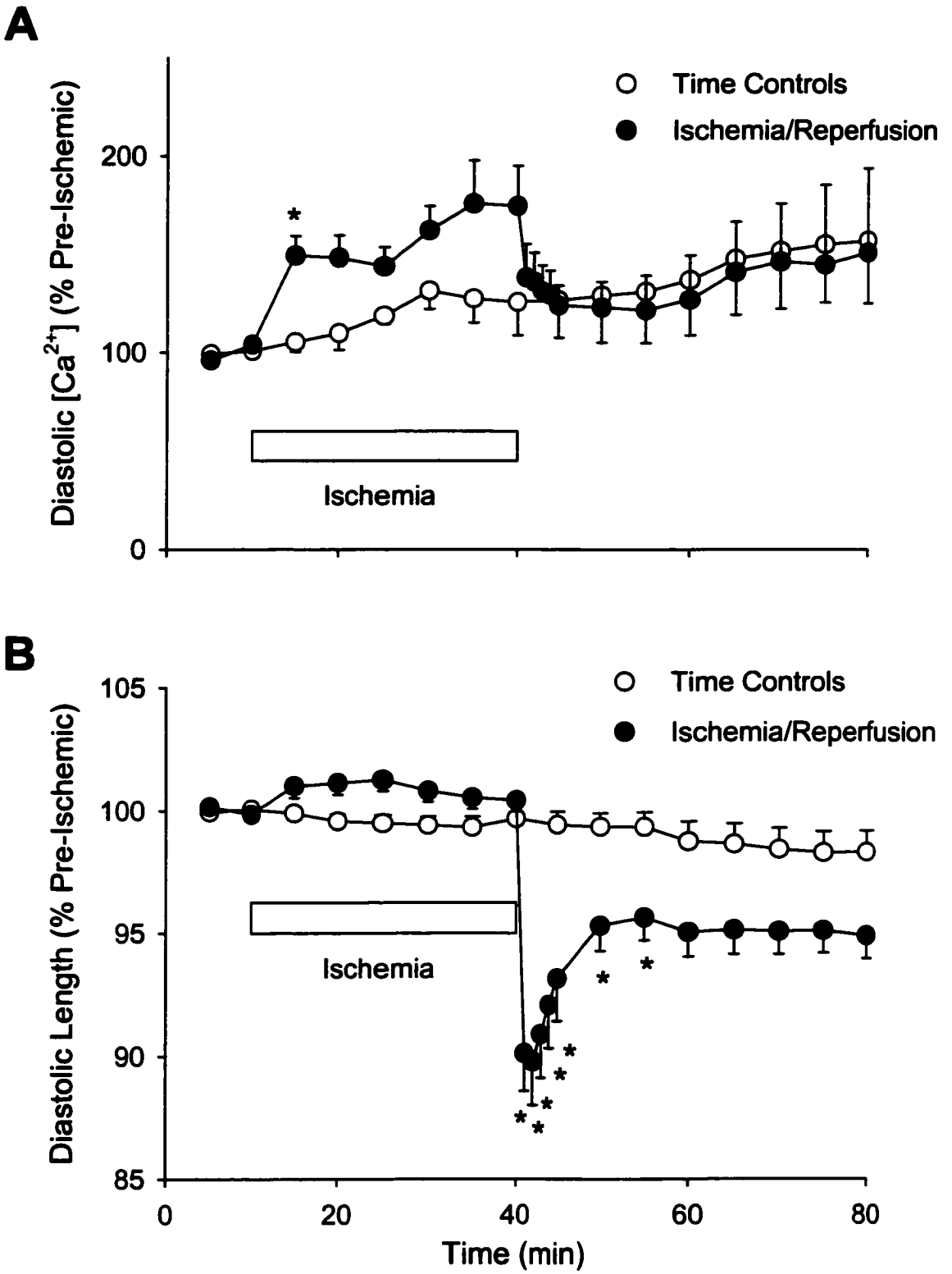

A
Time Controls
Ischemia/Reperfusion
Diastolic [Ca2+] (% Pre-Ischemic)
200
100
0
*
Ischemia
B
Time Controls
Ischemia/Reperfusion
Diastolic Length (% Pre-Ischemic)
105
100
95
90
85
Ischemia
*
0
20
40
60
80
Time (min)

Figure 19

Figure 19. Diastolic $[Ca^{2+}]_i$ was normal in late reperfusion.

Panel A shows mean measurements of diastolic $[Ca^{2+}]_i$ for time controls and myocytes exposed to simulated ischemia and reperfusion. Data are normalized to mean pre-ischemic values. Diastolic $[Ca^{2+}]_i$ was significantly elevated from time controls during the first 5 min of ischemia and remained markedly elevated for the duration of the ischemic period. In early reperfusion, diastolic $[Ca^{2+}]_i$ rapidly recovered to the level of time controls. With continued reperfusion, the two treatment groups exhibited a similar gradual increase in diastolic $[Ca^{2+}]_i$. Measurements of diastolic length normalized to pre-ischemic values are shown in **Panel B**. Diastolic length was slightly increased from time controls during ischemia, but significantly reduced upon reperfusion. With further reperfusion, diastolic length partially recovered and was not significantly different from time controls after 60 min (* denotes significant difference from time controls, $P < 0.05$).

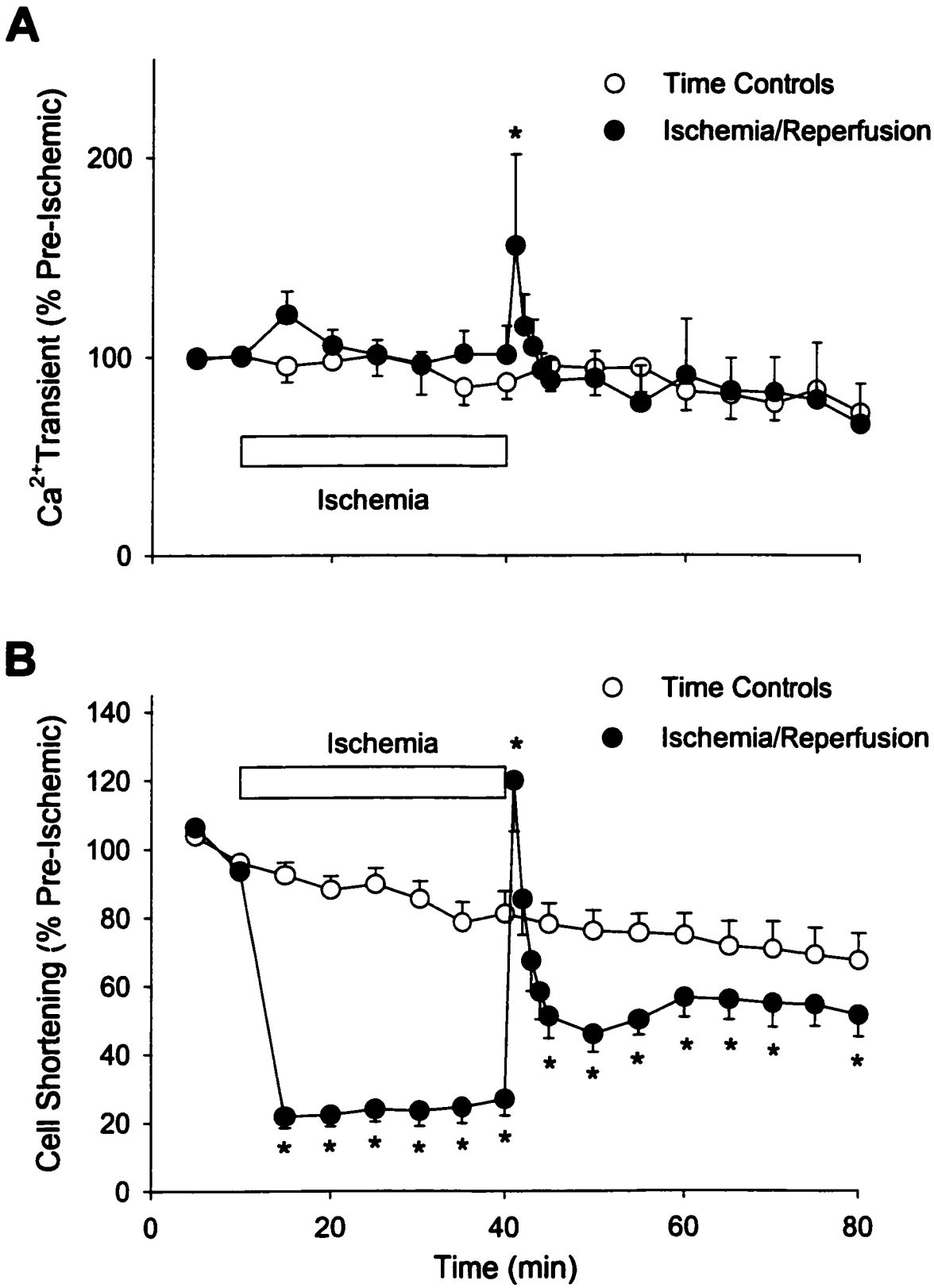

A
Time Controls
Ischemia/Reperfusion
Ca2+Transient (% Pre-Ischemic)
200
100
0
*
Ischemia
B
Time Controls
Ischemia/Reperfusion
Cell Shortening (% Pre-Ischemic)
140
120
100
80
60
40
20
0
Ischemia
*
0
20
40
60
80
Time (min)

Figure 20

Figure 20. Ca^{2+} transients were normal in stunning.

Measurements of Ca^{2+} transients are presented in **Panel A** as a percent of pre-ischemic values, and compared in time controls and in cells exposed to simulated ischemia and reperfusion. **Panel B** shows contraction measurements normalized to pre-ischemic values. During ischemia, Ca^{2+} transients were unchanged from time controls but contractions were significantly depressed. In early reperfusion, myocytes exhibited a significant overshoot in the magnitudes of both Ca^{2+} transients and contractions. In late reperfusion, myocytes exhibited stunning as contractions were reduced to 60% of time control values. This reduction in contraction amplitude occurred with no change in Ca^{2+} transient magnitude (* denotes significant difference from time controls, $P < 0.05$).

However, following this overshoot, Ca^{2+} transients were unchanged relative to time controls for the remainder of reperfusion.

Time control myocytes also exhibited a gradual decrease in cell shortening during the experiment (Fig. 20B). During ischemia, cell shortening was significantly depressed relative to time controls. An overshoot of contractions was observed in early reperfusion that paralleled the overshoot observed in Ca^{2+} transients (Fig. 20A). After the first few minutes of reperfusion, contractions were depressed relative to time controls for the remainder of the experiment.

In summary, the results of field-stimulation experiments showed that in late reperfusion, isolated myocytes exhibited stunning that was associated with normal diastolic $[Ca^{2+}]_i$ and normal magnitude of Ca^{2+} transients.

D. Effects of ischemia and reperfusion on the magnitude of SR Ca^{2+} stores.

The objective of the next set of experiments was to determine whether alterations in SR stores could contribute to the pathophysiology of stunning. SR Ca^{2+} content was assessed by measuring caffeine-elicited Ca^{2+} transients at several points during the 80 min ischemia / reperfusion protocol. Figure 21A shows a representative recording of $[Ca^{2+}]_i$ from an impaled myocyte stimulated at 2 Hz under control conditions. Each 3.5 ms stimulus elicited a rapid transient rise in $[Ca^{2+}]_i$. Stimulation was briefly interrupted and the superfusate was switched for 1 sec to one containing 10 mM caffeine using a computer controlled rapid-solution changer. Caffeine application elicited a Ca^{2+} transient which decayed gradually during the 1 sec of application. Following caffeine application, Ca^{2+} transients elicited by stimulation were temporarily reduced in magnitude, but

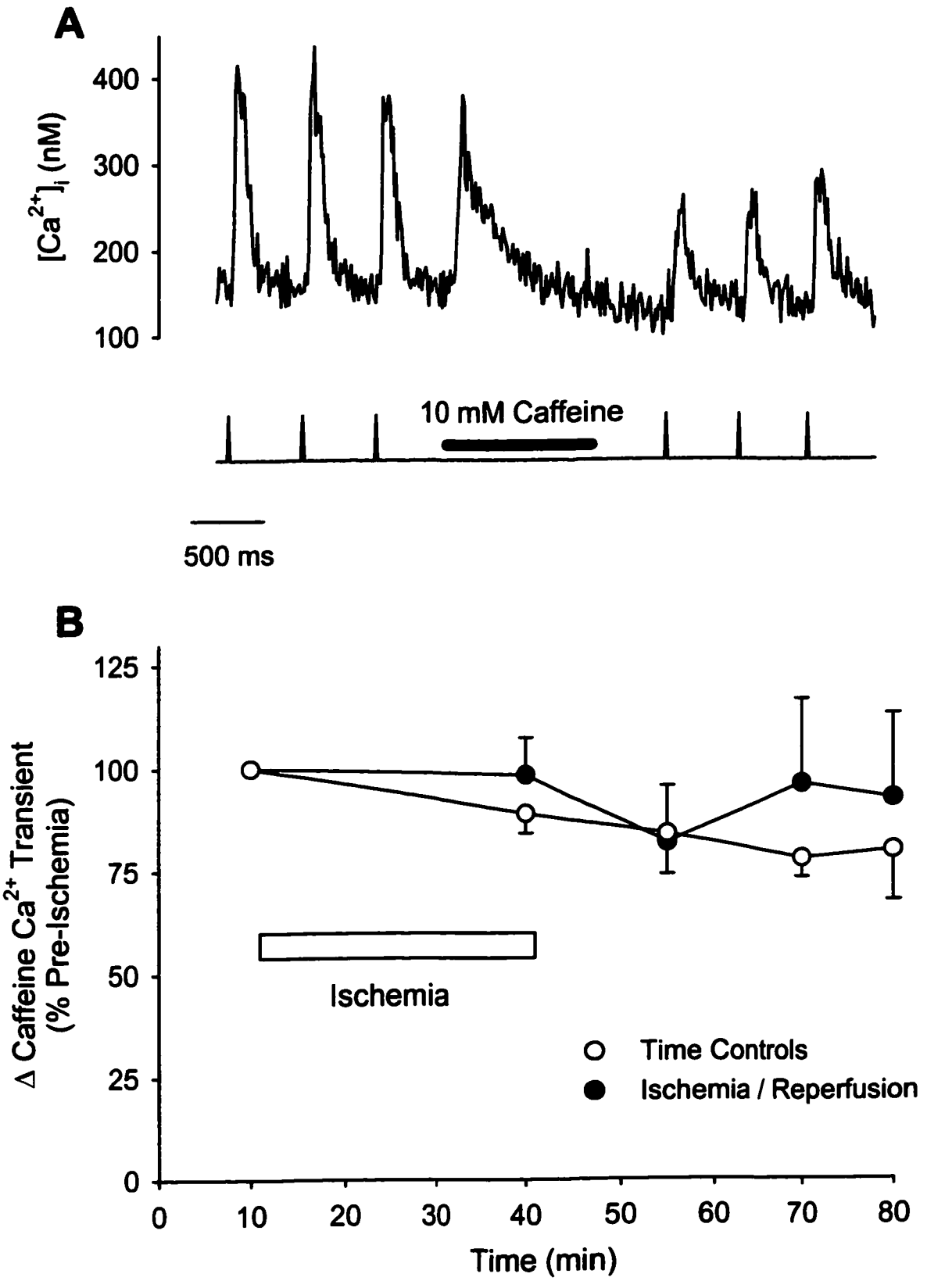

A
400
300
200
100
[Ca2+]i (nM)
10 mM Caffeine
500 ms
B
125
100
75
50
25
0
Δ Caffeine Ca2+ Transient
(% Pre-Ischemia)
Ischemia
Time Controls
Ischemia / Reperfusion
0
10
20
30
40
50
60
70
80
Time (min)

Figure 21

Figure 21. Caffeine-elicited Ca^{2+} transients were normal in stunned myocytes. **Panel A** shows a representative recording of $[Ca^{2+}]_i$ in a myocyte exposed to 10 mM caffeine. A 1 s caffeine application was interpolated in a 2 Hz stimulus train as indicated. **Panel B** shows that, in time control experiments, the magnitude of caffeine–elicited Ca^{2+} transients was not altered from values at 10 min. In myocytes exposed to ischemia and reperfusion, normalized caffeine transients were also not altered from pre-ischemic levels during the experiment, and were not significantly different from time controls. Data are presented as a percent of pre-ischemic values (ischemia / reperfusion, n = 13; time controls, n = 6).

recovered after approximately 10 s. Caffeine-elicited transients were measured at 10, 40, 55, 70, and 80 min during both ischemia / reperfusion and time control experiments (Fig 21B). In the absence of ischemia, the magnitude of caffeine transients declined slightly, but not significantly during 80 min of recording. Interestingly, caffeine transients also did not change significantly in myocytes during exposure to ischemia and reperfusion. These data indicate that the decrease in contraction observed late in reperfusion (Figures 11,13,14) cannot be attributed to a decline in SR stores of Ca^{2+}.

RESULTS - PART II

3. Does Losartan Protect Against Stunning in Isolated Cardiomyocytes?

In the second part of this thesis, the putative protective actions of the AT_1 receptor antagonist, losartan, were investigated using the cellular model of stunning described above. In these studies, observations of losartan-treated cells were compared with observations in time control experiments and/or myocytes exposed to control ischemia and reperfusion. In most cases, the control ischemia / reperfusion and time control data have been presented previously in this thesis, but are repeated here for clarity.

In the first set of experiments, contractions were examined in field-stimulated myocytes. Representative recordings of cell length during ischemia and reperfusion are shown in Figure 22 for a control and losartan-treated myocyte (A and B, respectively). Contractions were markedly reduced from pre-ischemic levels during ischemia in both the presence and absence of losartan, and diastolic length was slightly increased. After 1 min of reperfusion, contractions in the two treatment groups recovered to and exceeded pre-ischemic levels. Early reperfusion was also associated with a marked reduction in diastolic length in both drug-treated cells and in cells exposed to control ischemia. With continued reperfusion, diastolic cell length recovered slightly in the two treatment groups. However, contractions were much less depressed in the losartan-treated group in late reperfusion. Representative traces of cell length in time controls have been described previously in Figure 15. These myocytes exhibited only a slight reduction in cell shortening with time.

Figure 23 shows mean measurements of cell shortening during ischemia and

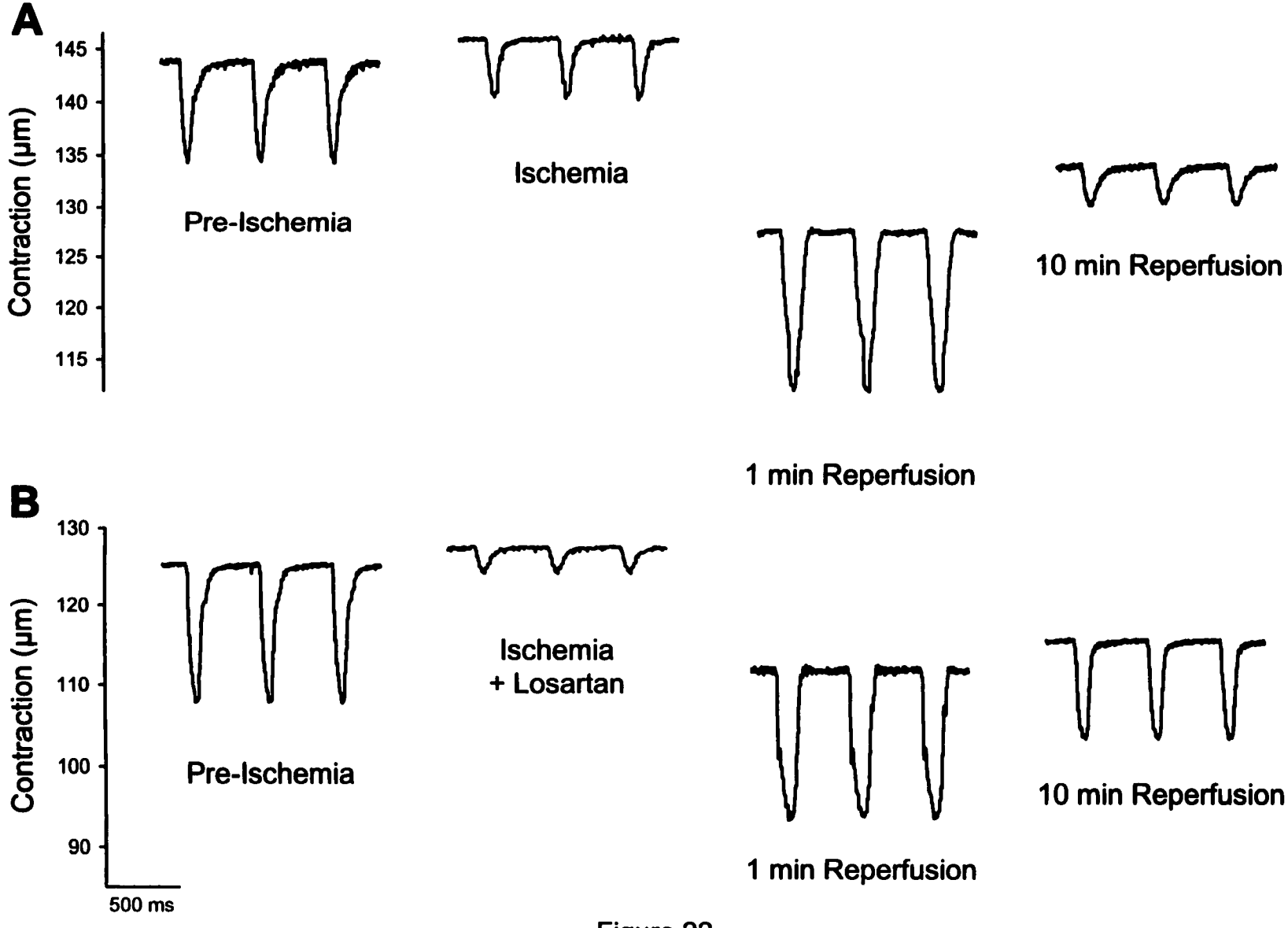

A
Contraction (µm)
145
140
135
130
125
120
115
Pre-Ischemia
Ischemia
1 min Reperfusion
10 min Reperfusion
B
Contraction (µm)
130
120
110
100
90
500 ms
Pre-Ischemia
Ischemia
+ Losartan
1 min Reperfusion
10 min Reperfusion

Figure 22

Figure 22. Losartan treatment during ischemia attenuated contractile depression in late reperfusion.

Panel A shows representative recordings of cell length from a myocyte exposed to control ischemia and reperfusion. Contractions were markedly reduced during ischemia. Upon reperfusion, myocytes exhibited a decrease in diastolic length and an increase in cell shortening. With continued reperfusion, diastolic length partially recovered but contractions became depressed. In losartan-treated myocytes (**Panel B**), changes in diastolic length during ischemia and early reperfusion were similar to those observed in control cells. Contractions were also reduced during ischemia and increased in early reperfusion following drug treatment. However, in later reperfusion, contractile depression was attenuated in losartan-treated myocytes.

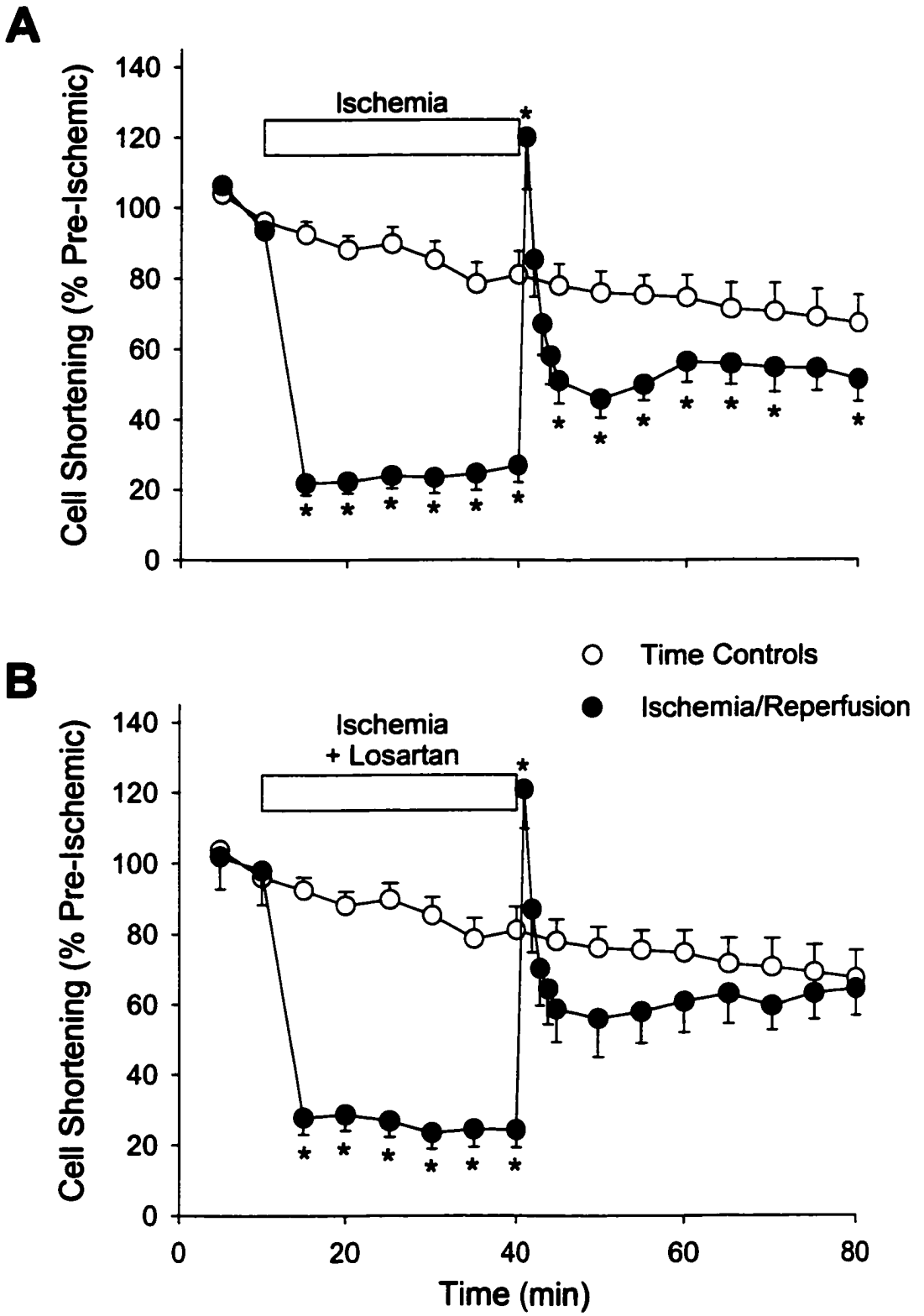

A
Ischemia
Cell Shortening (% Pre-Ischemic)
140
120
100
80
60
40
20
0
B
Ischemia
+ Losartan
Time Controls
Ischemia/Reperfusion
Cell Shortening (% Pre-Ischemic)
0
20
40
60
80
Time (min)

Figure 23

Figure 23. Losartan protects against stunning in isolated cardiomyocytes.

In control cells (**Panel A**), ischemia caused a significant reduction in cell shortening relative to time controls. Upon reperfusion, contractions were initially increased, however significant contractile depression (stunning) was observed with continued reperfusion. Losartan-treated myocytes (**Panel B**) also exhibited significantly reduced contractions during ischemia and an overshoot of contraction magnitude in early reperfusion. However, stunning was attenuated following losartan treatment as contractions were not significantly reduced from time control values in late reperfusion. Cell shortening is presented as a percent of pre-ischemic values (* denotes significant difference from time controls, $P < 0.05$; control group, $n = 15$; losartan group, $n = 18$).

reperfusion. During ischemia, myocytes exhibited a significant decrease in contractions relative to time controls in both the absence and presence of losartan (A and B, respectively). In early reperfusion, contractions in the two treatment groups were briefly but significantly increased from time control values. After the first few minutes of reperfusion, myocytes exposed to control ischemia and reperfusion exhibited a significant reduction in cell shortening for the remainder of the protocol (Fig. 23A). In losartan-treated cells, post-ischemic contraction magnitude was not significantly altered from the level of myocytes exposed to control ischemia and reperfusion. However, when compared with time controls (Fig. 23B), it was observed that post-ischemic contractile depression was attenuated following losartan treatment. Indeed, cell shortening in this group of myocytes was not significantly reduced from time control values at any point in reperfusion. Therefore, losartan treatment during ischemia appears to attenuate stunning in isolated cardiomyocytes.

4. What is the Mechanism for the Protective Actions of Losartan?

A. Does Losartan Improve Post-ischemic Contractile Function by Altering Action Potential Configuration?

Action potentials were elicited by a 3.5 ms current stimulus delivered through the electrode. Figure 24 shows representative recordings of action potentials in control and losartan-treated cells (A and B, respectively) during ischemia and reperfusion. In both treatment groups, action potentials were abbreviated and cell membranes depolarized during ischemia. During reperfusion, action potential configuration recovered in control and drug-treated myocytes.

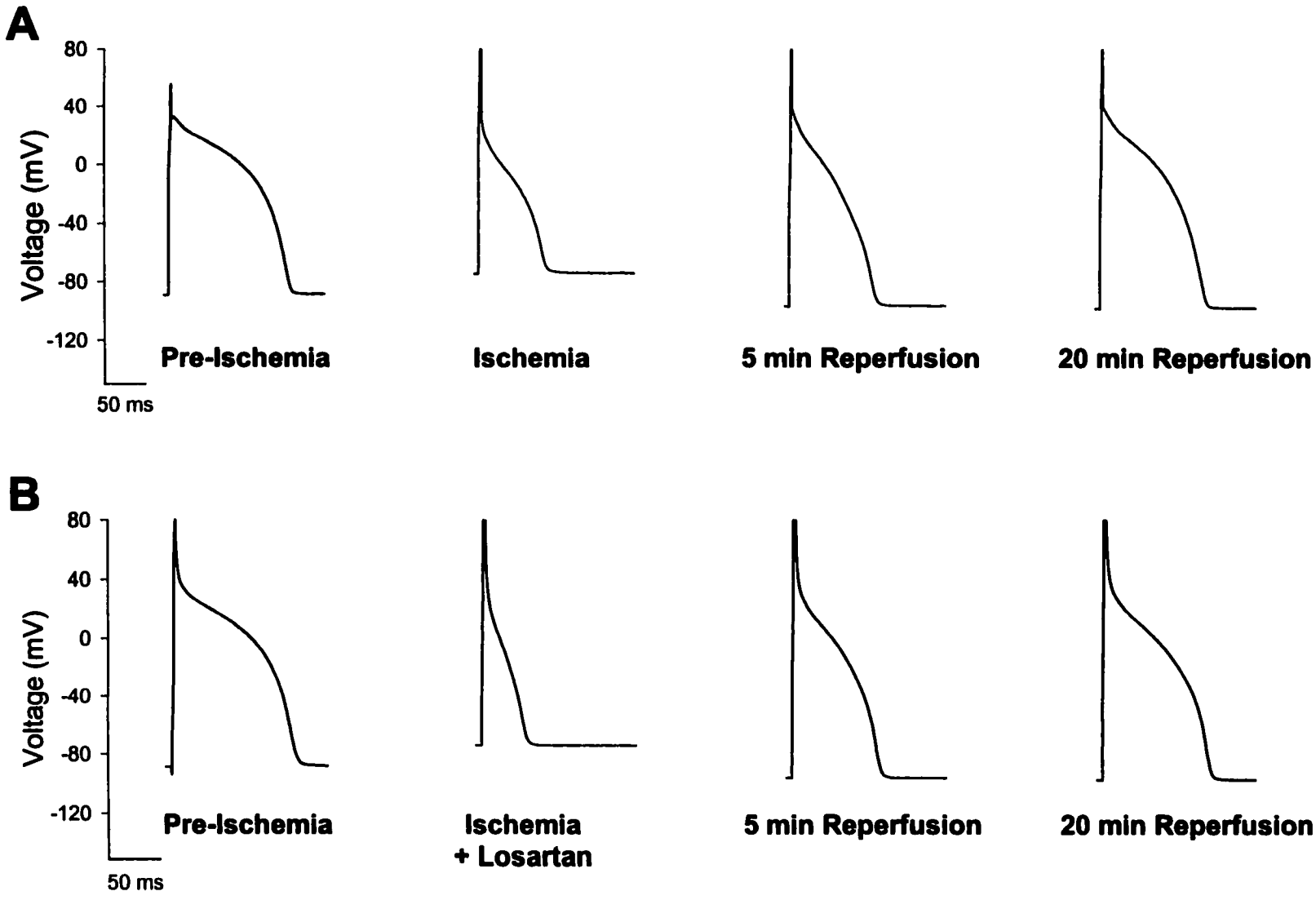

A
80
40
0
-40
-80
-120
Voltage (mV)
50 ms
Pre-Ischemia
Ischemia
5 min Reperfusion
20 min Reperfusion
B
80
40
0
-40
-80
-120
Voltage (mV)
50 ms
Pre-Ischemia
Ischemia
+ Losartan
5 min Reperfusion
20 min Reperfusion

Figure 24

Figure 24. Action potentials were similar in control and losartan-treated cells. Representative recordings show that action potentials were abbreviated during control ischemia (**Panel A**) and cell membranes depolarized. However, action potential configuration recovered in reperfusion. **Panel B** shows that action potentials were similarly altered during the protocol when cells were treated with losartan in ischemia. Although it appears from this example that losartan treatment may have exacerbated shortening of the action potential during ischemia, this effect was not consistently observed.

Figure 25 shows mean measurements of RMP (Panel A) and APD (Panel B) during the experiment. During ischemia, control and losartan-treated cells exhibited significant membrane depolarization from pre-ischemic values. Both treatment groups also showed a gradual reduction in APD that reached statistical significance by late ischemia. In reperfusion, control and drug-treated myocytes exhibited a rapid recovery of RMP to pre-ischemic levels and a gradual recovery of APD. Thus, it does not appear that losartan treatment significantly alters RMP and APD during ischemia and reperfusion.

Although RMP and ADP may be normal following losartan treatment, it is possible that other important alterations in action potential configuration may occur which are not accounted for by these measurements. To examine this possibility, contractions were examined using a voltage-clamp protocol to control the membrane voltage. This protocol was similar to that illustrated in Figure 12, but with a test step from –52 to 0 mV (shown at the top of Figure 26) to inactivate sodium current, but allow activation of both the VSRM and CICR (Howlett and Ferrier, 1997; Wier and Balke, 1999). The test step elicited phasic contractions as shown in Figure 26. In both control and losartan-treated cells (A and B, respectively), contractions were markedly decreased during ischemia but recovered in early reperfusion. However, in late reperfusion, marked contractile depression was only observed in control myocytes.

Figure 27 shows mean measurements of voltage-clamp elicited contractions during ischemia and reperfusion. In control cells (Fig. 27A), contractions were markedly reduced during ischemia and then recovered to near pre-ischemic levels in early reperfusion. With continued reperfusion, however, contractions gradually decreased again, and maximal depression occurred at 20 min of reperfusion. Losartan-treated cells

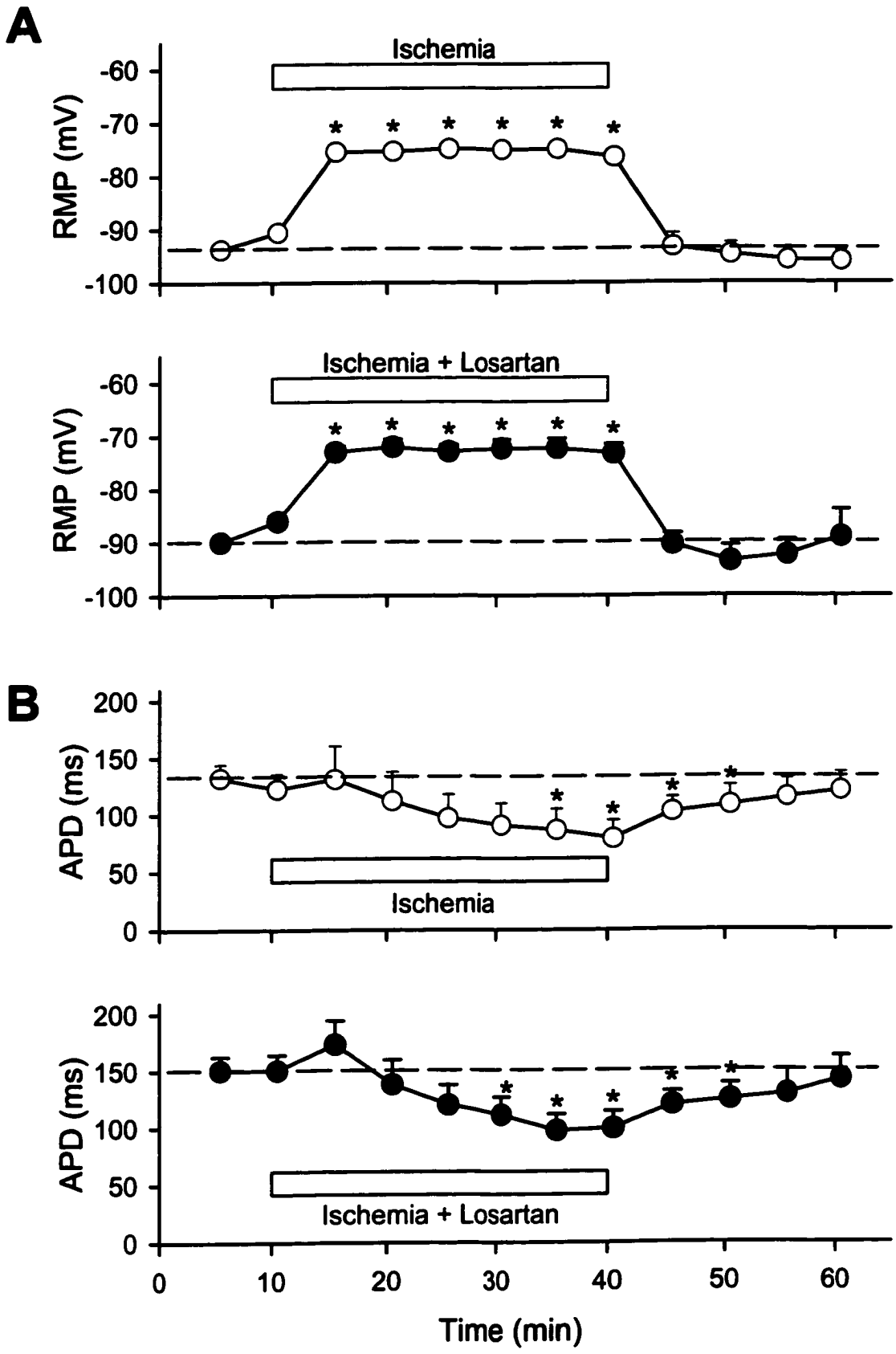

A
Ischemia
RMP (mV)
-60
-70
-80
-90
-100
Ischemia + Losartan
RMP (mV)
-60
-70
-80
-90
-100
B
APD (ms)
200
150
100
50
0
Ischemia
APD (ms)
200
150
100
50
0
Ischemia + Losartan
0
10
20
30
40
50
60
Time (min)

Figure 25

Figure 25. Losartan did not alter RMP or APD during ischemia and reperfusion. **Panel A:** In both control and losartan-treated cells, ischemia was associated with significant membrane depolarization. Both treatment groups exhibited a similar recovery of RMP during reperfusion. Parallel alterations in APD were also observed in control and drug-treated cells (**Panel B**). APD decreased gradually during ischemia in both groups, but was not significantly altered from pre-ischemic values by late reperfusion (*denotes significant difference from pre-ischemic values, $P < 0.05$; control group, $n = 21$; losartan group, $n = 18$).

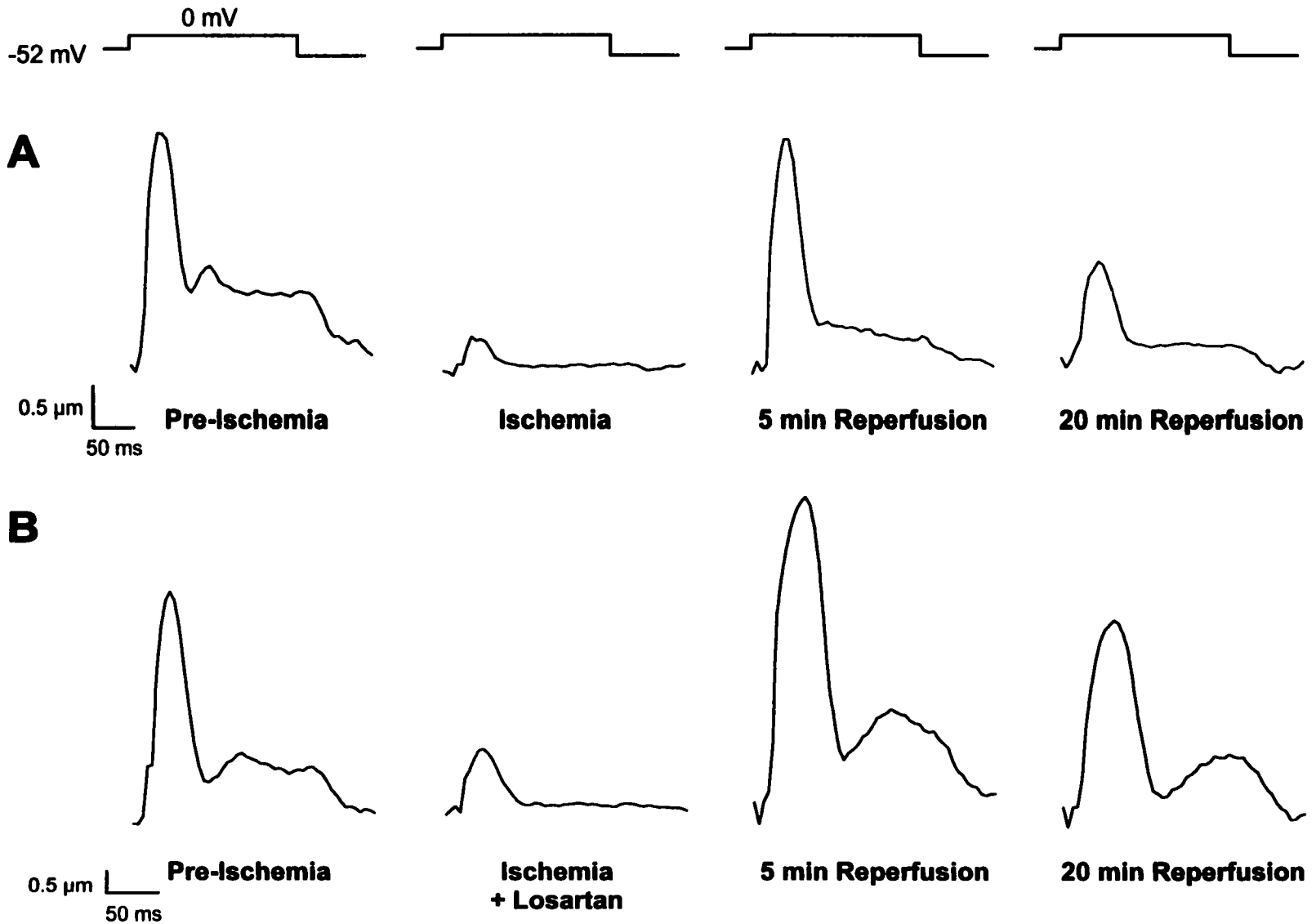

0 mV
-52 mV
A
0.5 µm
50 ms
Pre-Ischemia
Ischemia
5 min Reperfusion
20 min Reperfusion
B
0.5 µm
50 ms
Pre-Ischemia
Ischemia
+ Losartan
5 min Reperfusion
20 min Reperfusion

Figure 26

Figure 26. Losartan treatment increased post-ischemic contractions elicited by voltage steps from –52 mV to 0 mV.

Representative recordings of contraction are shown as elicited by the test step (**top**). Control cells (**Panel A**) exhibited a marked reduction in contractions during ischemia, recovery of contractions in early reperfusion, and contractile depression in late reperfusion. Contractions were also reduced during ischemia in losartan-treated cells (**Panel B**). However, losartan treatment caused a slight overshoot of contractions in early reperfusion and prevented contractile depression in late reperfusion.

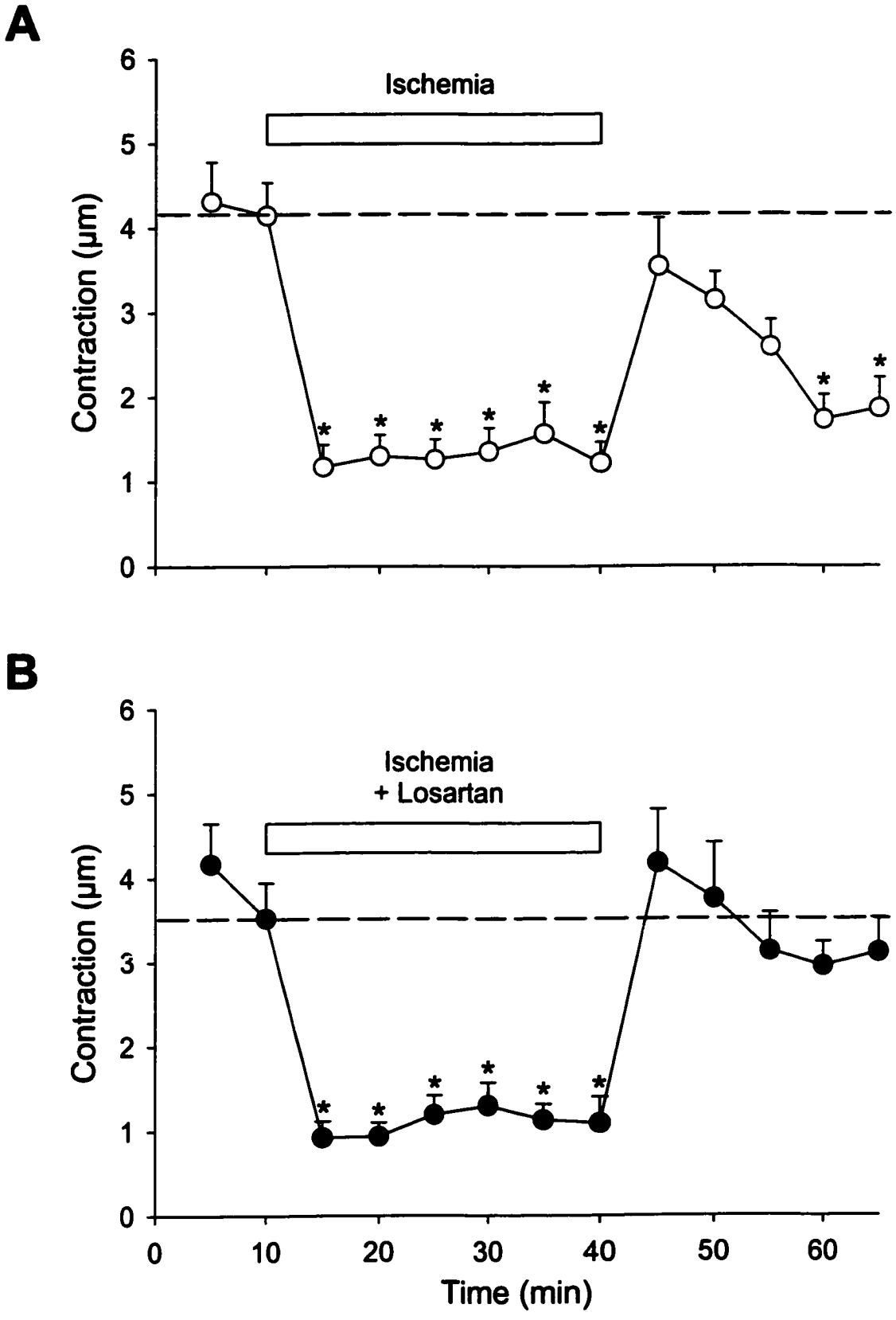

A
Ischemia
Contraction (µm)
0
1
2
3
4
5
6
B
Ischemia
+ Losartan
Contraction (µm)
0
1
2
3
4
5
6
0
10
20
30
40
50
60
Time (min)

Figure 27

Figure 27. Losartan prevented stunning in voltage-clamped cells.

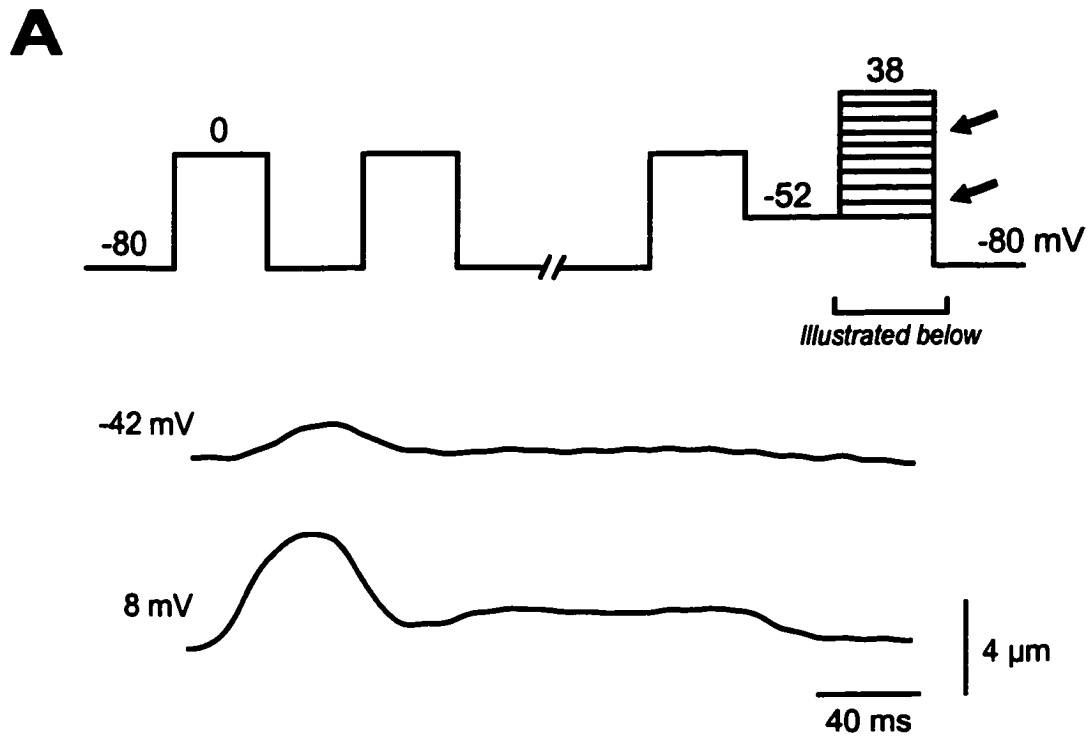
Panel A: In control cells, mean contraction amplitude was reduced during ischemia, and nearly recovered to pre-ischemic levels in early reperfusion. In late reperfusion, voltage clamp-elicited contractions were significantly reduced. In losartan-treated myocytes (**Panel B**), contractions were significantly decreased during ischemia, but recovered to levels slightly above pre-ischemia in early reperfusion. Stunning was prevented in the losartan treatment group (* denotes significant difference from pre-ischemic values, $P < 0.05$; control group, $n = 21$; losartan group, $n = 14$).

(Fig. 27B) also showed decreases in contraction during ischemia that were similar to control cells. However, losartan-treated myocytes exhibited a slightly greater recovery of contraction in early reperfusion than control cells, and no significant contractile depression in late reperfusion. Thus, since losartan protects against stunning even when the membrane potential is controlled, it appears that losartan may protect against stunning by a mechanism that does not involve alterations in action potential configuration.

B. Does Losartan Attenuate Stunning by Altering the Voltage Dependence of Contraction?

It has previously been reported that pharmacological agents can alter the voltage dependence of contraction in isolated myocytes (Mason and Ferrier, 1999). Therefore, the increase in amplitude of contractions following losartan treatment in ischemia could result either from increased maximum cell shortening or from a shift in the voltage dependence of contraction. To differentiate between these possibilities, contraction-voltage relationships were determined with the voltage-clamp protocol shown at the top of Figure 28 (described in methods). With this protocol, contractions were elicited by sequential test steps from –52 mV to allow activation of CICR and VSRM components of contraction. Representative recordings of contraction are shown in Figure 28A for two selected test steps. Under pre-ischemic conditions, contractions showed a sigmoidal relationship with voltage which plateaued near +20 mV (Fig 28B).

Normalized contraction-voltage relationships were compared in pre-ischemia, ischemia (30 min), and late reperfusion (20 min). In control myocytes, maximum contraction was significantly depressed during ischemia and late reperfusion, without a


A
38
0
-52
-80
-80 mV
Illustrated below
-42 mV
8 mV
4 µm
40 ms

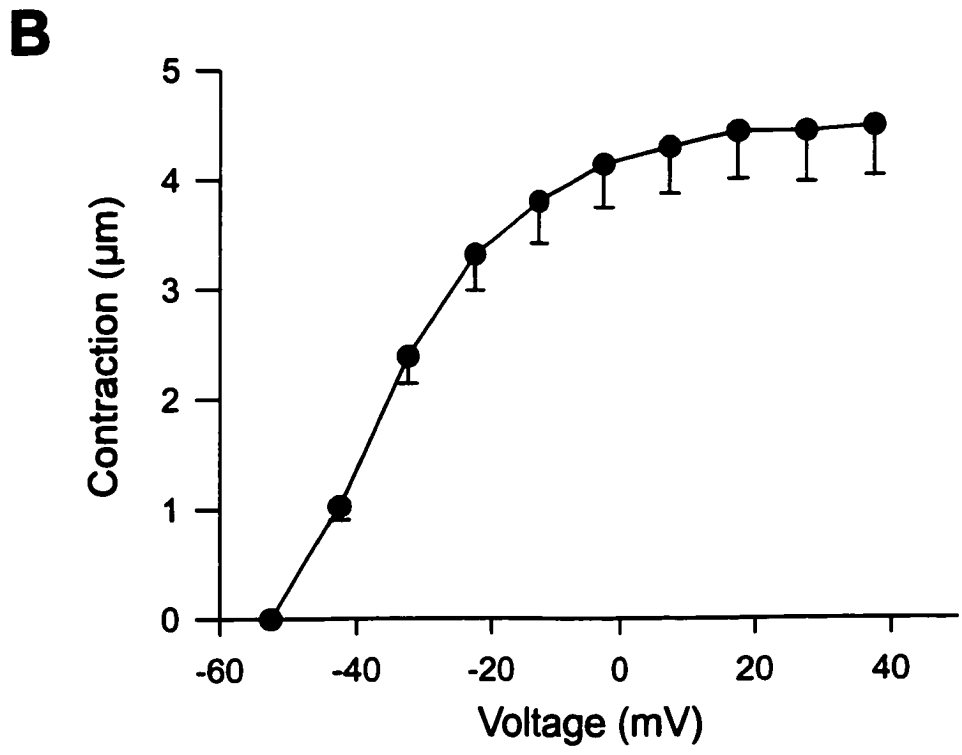

B
Contraction (µm)
5
4
3
2
1
0
-60
-40
-20
0
20
40
Voltage (mV)

Figure 28

Figure 28. Under pre-ischemic conditions, contractions showed a sigmoidal voltage-dependence.

The voltage-clamp protocol used is illustrated at the top of **Panel A**. Also shown in **Panel A** are representative recordings of contractions in pre-ischemia for two selected test potentials. The mean contraction-voltage relationship in pre-ischemia is shown in **Panel B** (n=22).

shift in the voltage dependence of contraction (Fig 29A). Losartan-treated cells exhibited reduced contractility during ischemia which was similar to that observed for untreated cells (Fig 29B). However, in late reperfusion, the mean contraction-voltage relationship was not significantly different from pre-ischemia in either amplitude or voltage dependence. Thus, losartan treatment increased maximum contraction in late reperfusion but did not shift the voltage dependence of contraction.

C. Does Losartan Treatment Affect Incidence of Transient Inward Current (I_{TI})?

To gain further insight into the mechanisms underlying the protective actions of losartan, the next set of experiments examined effects of drug treatment on the arrhythmogenic current, I_{TI}. I_{TI} was elicited by the voltage protocol shown in Figure 30A (described in methods), and was only observed in the first 10 min of reperfusion. Figures 30B and 30C show representative traces of I_{TI} and associated contractions elicited by repolarizing test steps from +20 mV. These recordings were made in a control cell after 5 min of reperfusion. I_{TI} appeared as oscillatory downward deflections in current, and was accompanied by aftercontractions. The contraction-voltage relationship for aftercontractions (Fig 30D) reached a peak at –50 mV, and was essentially the mirror image of the current-voltage plot for I_{TI} (Fig 30E). This current-voltage relationship was very similar to that described for I_{TI} in previous studies (Kass et al., 1978; Cordeiro et al., 1992,1994).

I_{TI} was observed in 60% of control cells (Fig. 31). However, in losartan-treated cells the incidence of I_{TI} was significantly reduced to 20%. This observation suggests that losartan treatment during ischemia not only protects against contractile depression in

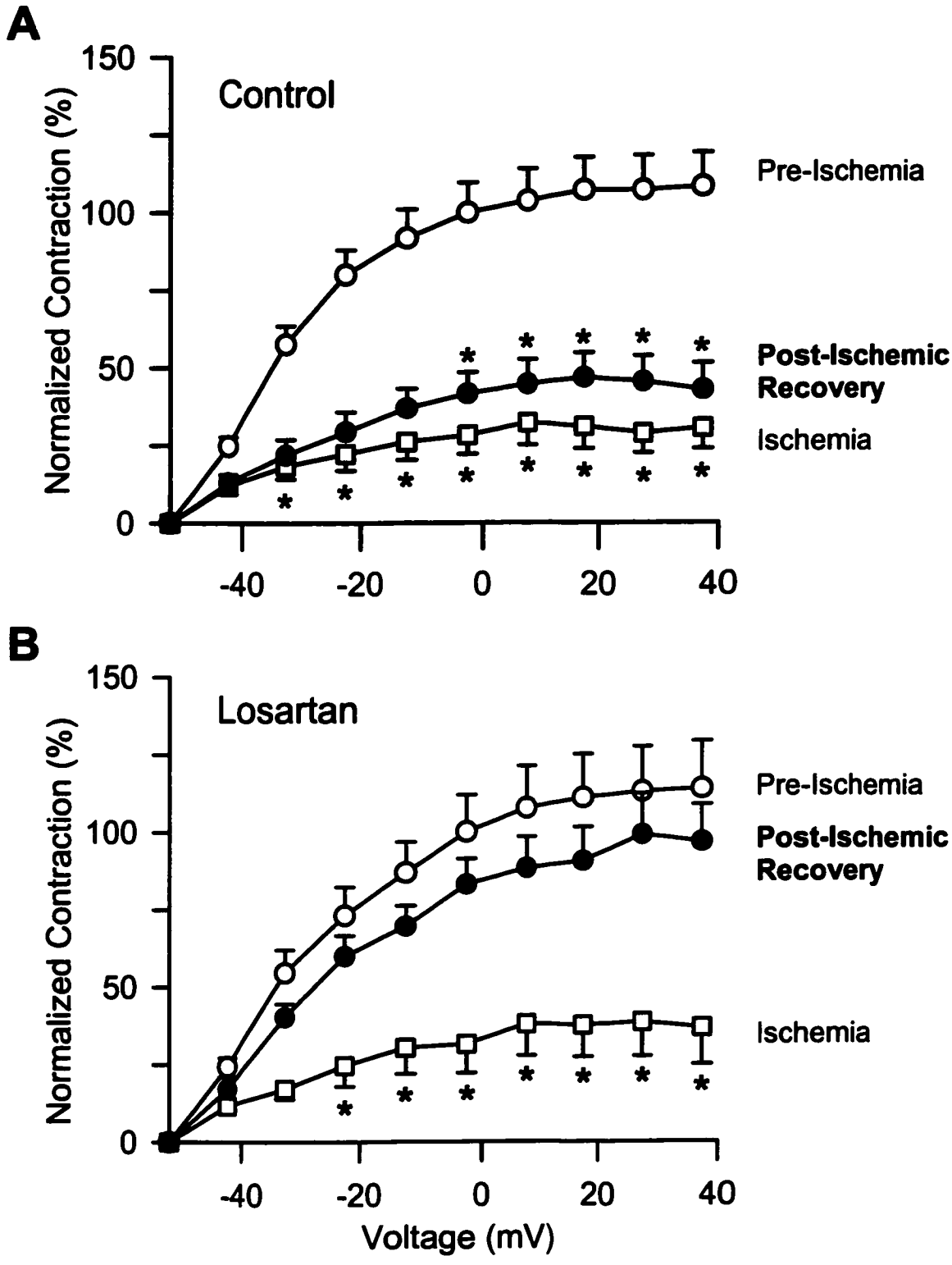

A
Control
Normalized Contraction (%)
150
100
50
0
-40
-20
0
20
40
Pre-Ischemia
Post-Ischemic Recovery
Ischemia
B
Losartan
Normalized Contraction (%)
150
100
50
0
-40
-20
0
20
40
Voltage (mV)
Pre-Ischemia
Post-Ischemic Recovery
Ischemia

Figure 29

Figure 29. Losartan dramatically improved recovery of contraction during reperfusion without altering the voltage-dependence of contraction.

The voltage-clamp protocol used was the same as that shown in Figure 28. The mean contraction-voltage relationship at 30 min of ischemia was significantly reduced from pre-ischemia in both control and losartan-treated cells (**Panel A:** Control cells, n=22; **Panel B:** Losartan-treated cells, n=14). Following 20 min of reperfusion, control cells showed a significant decrease in contractions that was almost completely prevented in losartan-treated cells. Losartan did not alter the voltage dependence of contraction during ischemia or reperfusion (* denotes significant difference from pre-ischemic values, $P < 0.05$).

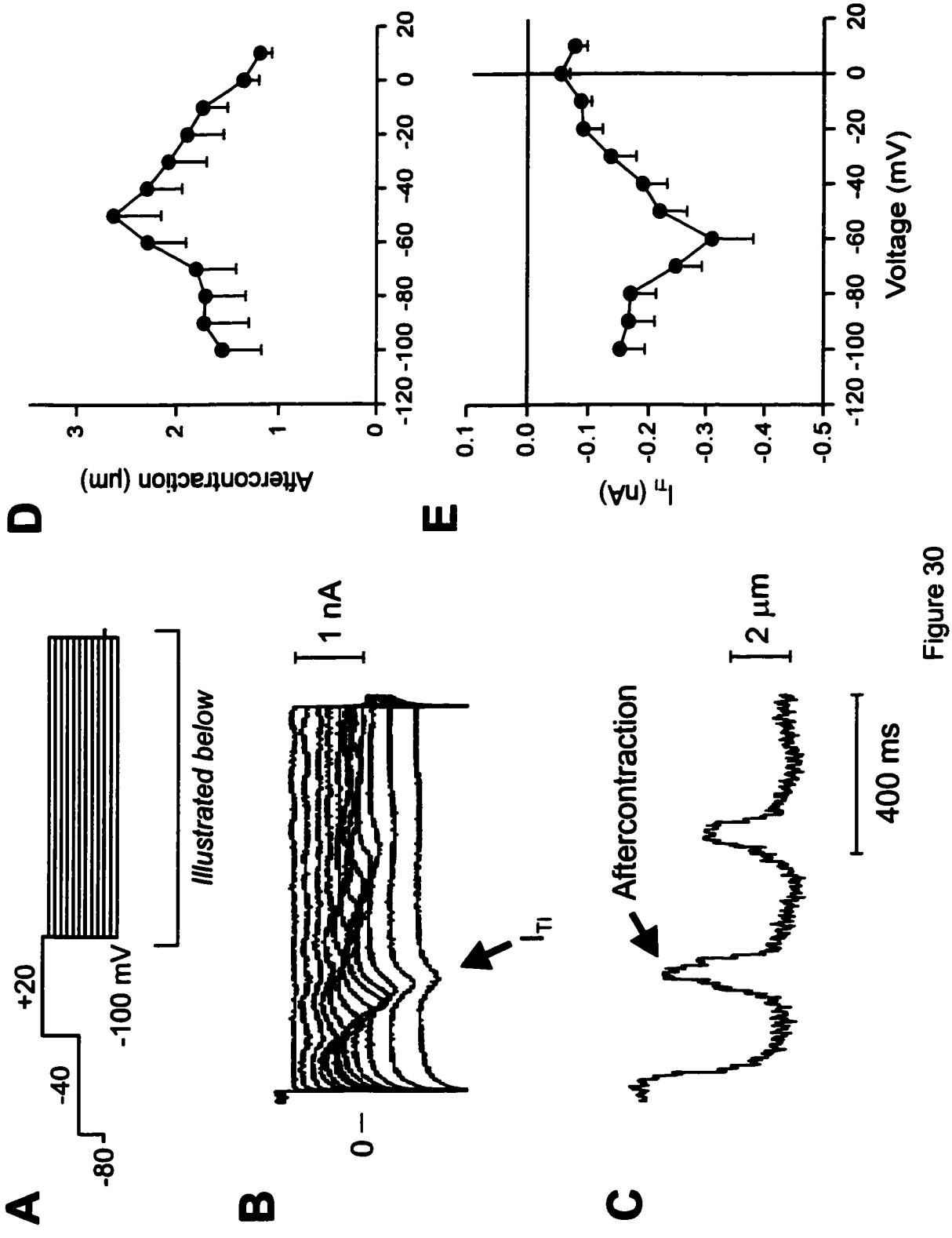

A
+20
-40
-80
-100 mV
Illustrated below
B
1 nA
0
I_{Tl}
C
Aftercontraction
2 µm
400 ms
D
Aftercontraction (µm)
3
2
1
0
-120 -100 -80 -60 -40 -20 0 20
E
$I_{\bar{T}}$ (nA)
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-120 -100 -80 -60 -40 -20 0 20
Voltage (mV)

Figure 30

Figure 30. I_{TI} and aftercontractions were induced by reperfusion in control cells. The voltage-clamp protocol used is shown in **Panel A**. Representative recordings of both I_{TI} (**Panel B,** traces for +10 mV to –100 mV steps, top to bottom) and aftercontractions (**Panel C**, step to 0 mV) are shown from an untreated myocyte at 5 min reperfusion. **Panel D** shows the contraction-voltage relationship determined for aftercontractions in untreated cells (n=13). The current-voltage relationship obtained for I_{TI} is shown in **Panel E**.

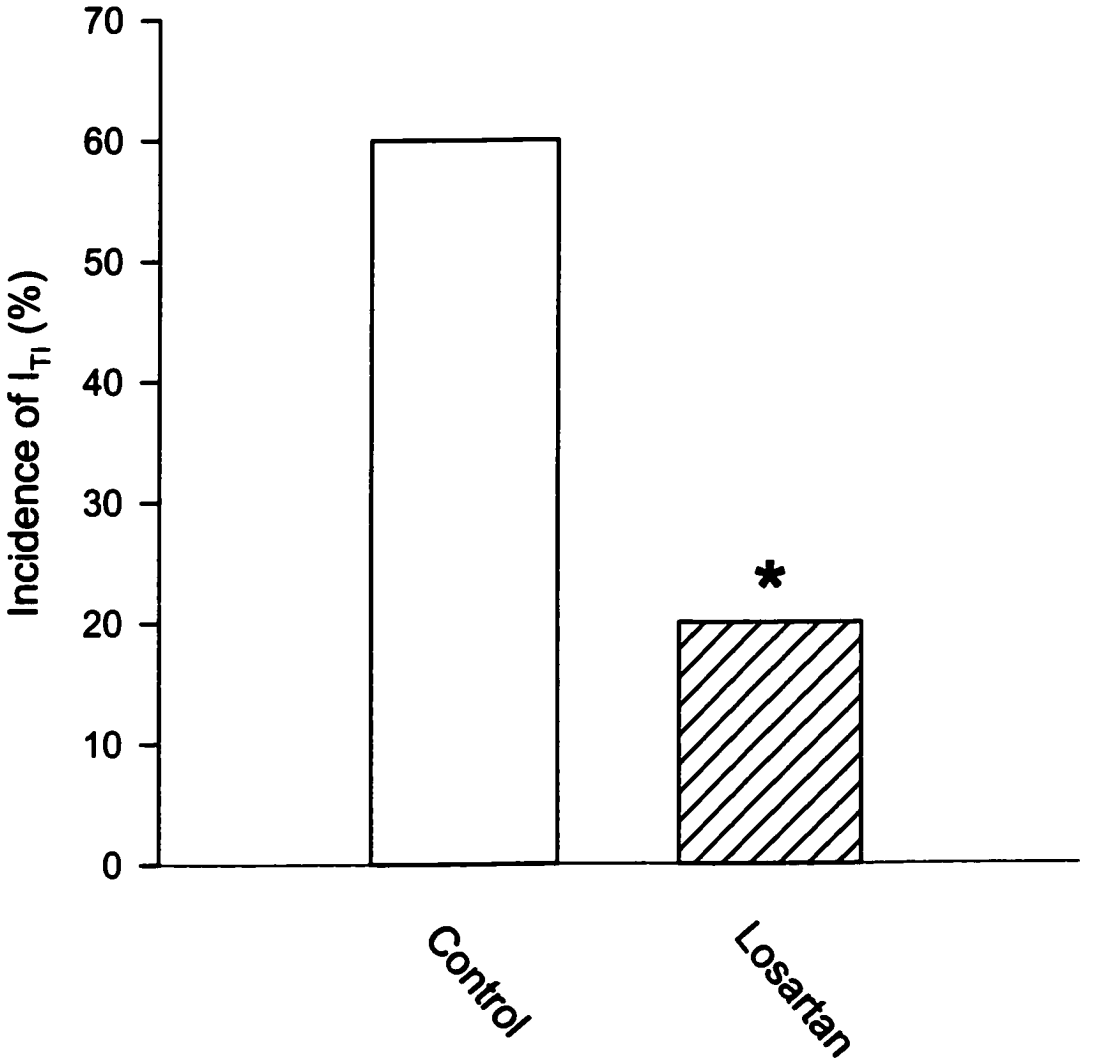


Figure 31

Figure 31. Losartan treatment reduced incidence of I_{TI}.

I_{TI} was observed in 60% of control cells during early reperfusion. Treatment with losartan during ischemia significantly reduced I_{TI} incidence to 20% (* denotes significant difference from control, $P < 0.05$; control group, n=20; losartan group, n = 15).

reperfusion but also I_{TI} generation. Since alterations in Ca^{2+} homeostasis are believed to contribute to the development of both I_{TI} and stunning, it seems reasonable to hypothesize that losartan may protect against Ca^{2+} overload during ischemia and reperfusion.

D. Do The Protective Actions of Losartan During Ischemia and Reperfusion Result From Alterations in Ca^{2+} Homeostasis?

i) Does Losartan Alter the Magnitude of L-Type Ca^{2+} Current?

The effects of losartan on $I_{Ca\text{-}L}$ during ischemia and reperfusion were examined with the voltage-clamp protocol shown at the top of Figure 12 (described in methods). Representative recordings of current are shown in Figure 32 for control and losartan-treated cells (A and B, respectively), as elicited by the test step illustrated at the top of the page. In both treatment groups, $I_{Ca\text{-}L}$ magnitude was reduced during ischemia and did not appear to recover in reperfusion.

Figure 33 shows mean measurements of $I_{Ca\text{-}L}$ magnitude during ischemia and reperfusion. In both control (Fig. 33A) and losartan-treated myocytes (Fig. 33B), ischemia was associated with a reduction in $I_{Ca\text{-}L}$ magnitude that reached statistical significance near the end of the ischemic period. $I_{Ca\text{-}L}$ magnitude remained depressed in the two treatment groups during reperfusion. In early reperfusion, myocytes exhibited a slight further reduction in $I_{Ca\text{-}L}$ magnitude that did not recover to pre-ischemic levels with continued reperfusion. These observations suggest that the protective actions of losartan during ischemia and reperfusion are not mediated through effects on $I_{Ca\text{-}L}$ magnitude.

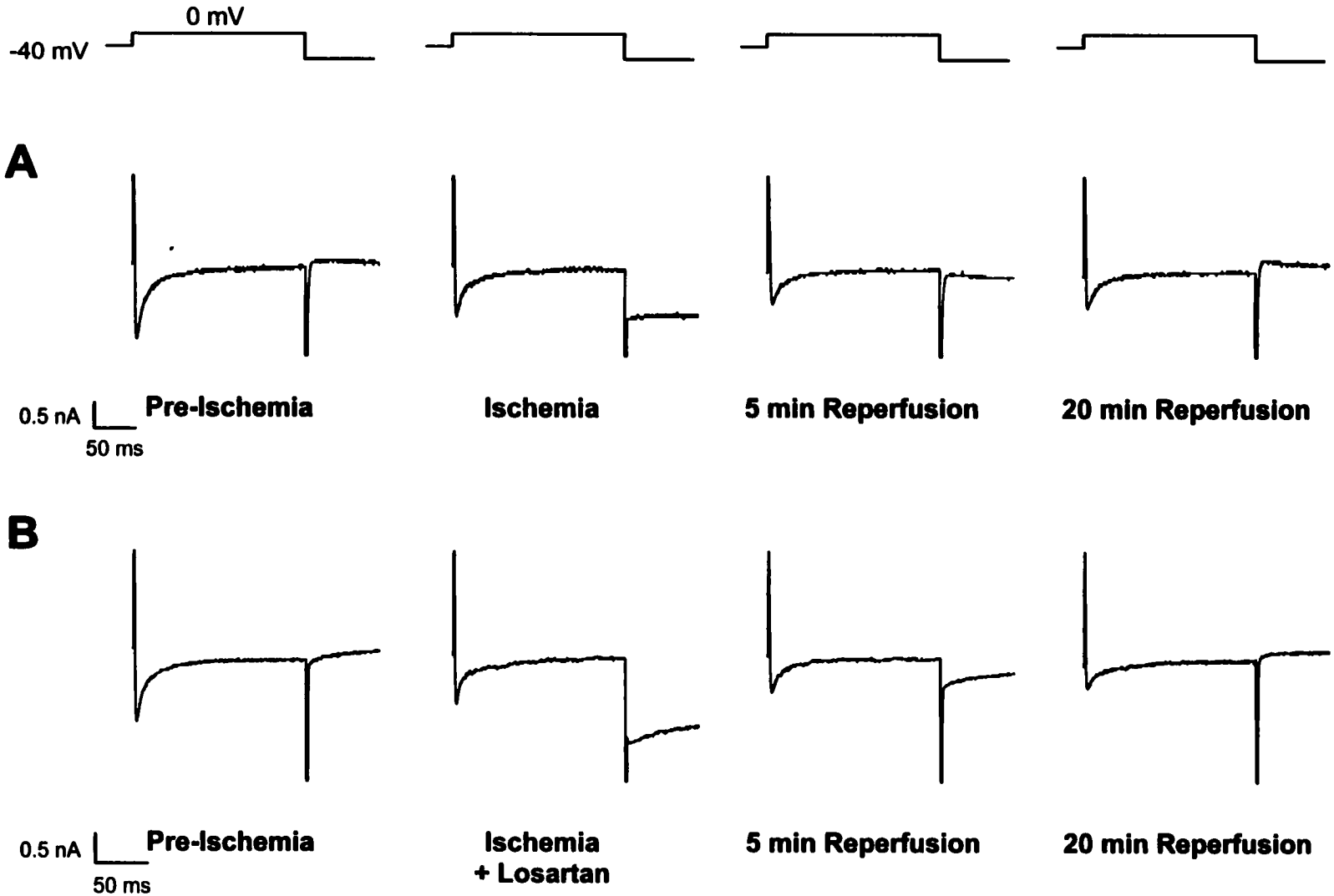

0 mV
-40 mV
A
0.5 nA
50 ms
Pre-Ischemia
Ischemia
5 min Reperfusion
20 min Reperfusion
B
0.5 nA
50 ms
Pre-Ischemia
Ischemia
+ Losartan
5 min Reperfusion
20 min Reperfusion

Figure 32

Figure 32. $I_{Ca\text{-}L}$ was reduced during ischemia and reperfusion in control and losartan-treated cells.

The voltage-clamp protocol used to elicit $I_{Ca\text{-}L}$ is shown at the top of Figure 12. Representative recordings show that, in control cells (**Panel A**), $I_{Ca\text{-}L}$ was reduced during ischemia and did not recover in reperfusion. $I_{Ca\text{-}L}$ was similarly reduced in the losartan treatment group.

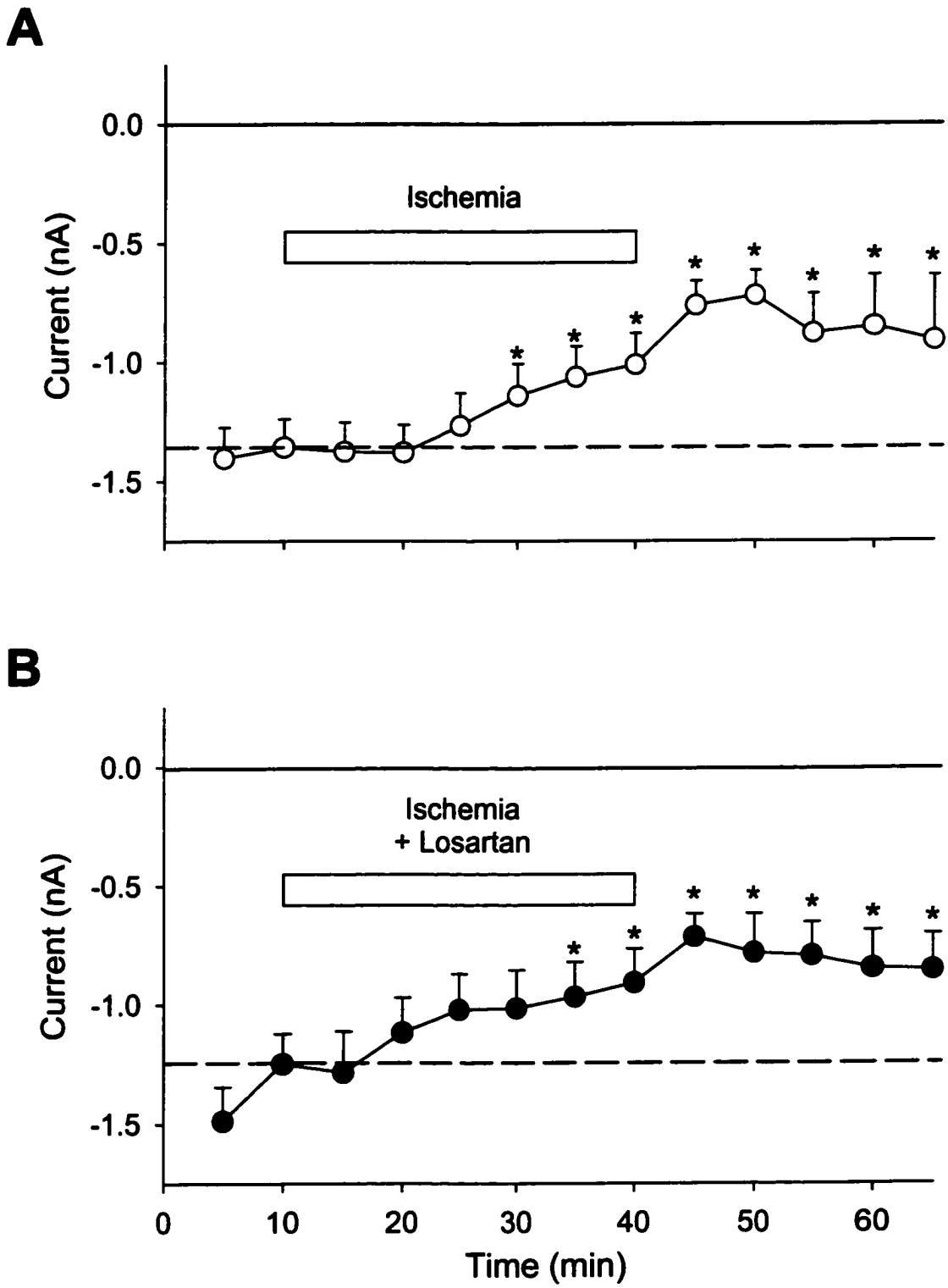

A
0.0
-0.5
-1.0
-1.5
Current (nA)
Ischemia
B
0.0
-0.5
-1.0
-1.5
Current (nA)
Ischemia
+ Losartan
0
10
20
30
40
50
60
Time (min)

Figure 33

Figure 33. Losartan did not alter the effect of ischemia and reperfusion on $I_{Ca\text{-}L}$. Mean measurements of $I_{Ca\text{-}L}$ were reduced from pre-ischemic levels during control ischemia, and further reduced in early reperfusion (**Panel A**). In late reperfusion, $I_{Ca\text{-}L}$ magnitude remained significantly reduced from pre-ischemic levels. In losartan-treated cells (**Panel B**), $I_{Ca\text{-}L}$ was similarly reduced (* denotes significant difference from pre-ischemic levels, $P < 0.05$; control group, $n = 21$; losartan group, $n = 14$).

ii) Does Losartan Prevent Ca^{2+} Overload During Ischemia and Reperfusion?

To determine whether losartan exerts effects on diastolic $[Ca^{2+}]_i$ or Ca^{2+} transients during ischemia and reperfusion, $[Ca^{2+}]_i$ was recorded in field-stimulated myocytes. Representative traces and mean $[Ca^{2+}]_i$ measurements for time control experiments were described previously in Figures 15A and 16A. In these cells, diastolic $[Ca^{2+}]_i$ increased gradually with time and Ca^{2+} transients were slightly decreased. Representative recordings of Ca^{2+} transients and mean measurements of $[Ca^{2+}]_i$ are presented for control and losartan-treated cells in Figures 34 and 35, respectively. During control ischemia, the magnitude of Ca^{2+} transients was unchanged as indicated by representative traces (Fig. 34A) and mean data (Fig. 34B, shaded region). Diastolic $[Ca^{2+}]_i$ was, however, significantly elevated from pre-ischemic levels in the last 15 min of ischemia. In early reperfusion, diastolic $[Ca^{2+}]_i$ partially recovered and Ca^{2+} transients were briefly very large. Reperfusion was also associated with a gradual reduction in the magnitude of Ca^{2+} transients and a gradual increase in diastolic $[Ca^{2+}]_i$ that reached significance by the end of the protocol.

In losartan-treated myocytes, representative traces (Fig. 35A) and mean data (Fig. 35B) show that, as in control cells, ischemia was associated with elevated diastolic $[Ca^{2+}]_i$ but no change in the magnitude of Ca^{2+} transients was observed. However, in early reperfusion, the overshoot in Ca^{2+} transients observed in control cells appeared to be prevented. With continued reperfusion, Ca^{2+} transients appeared to increase. Diastolic $[Ca^{2+}]_i$ was elevated from pre-ischemic levels in late reperfusion in losartan-treated cells.

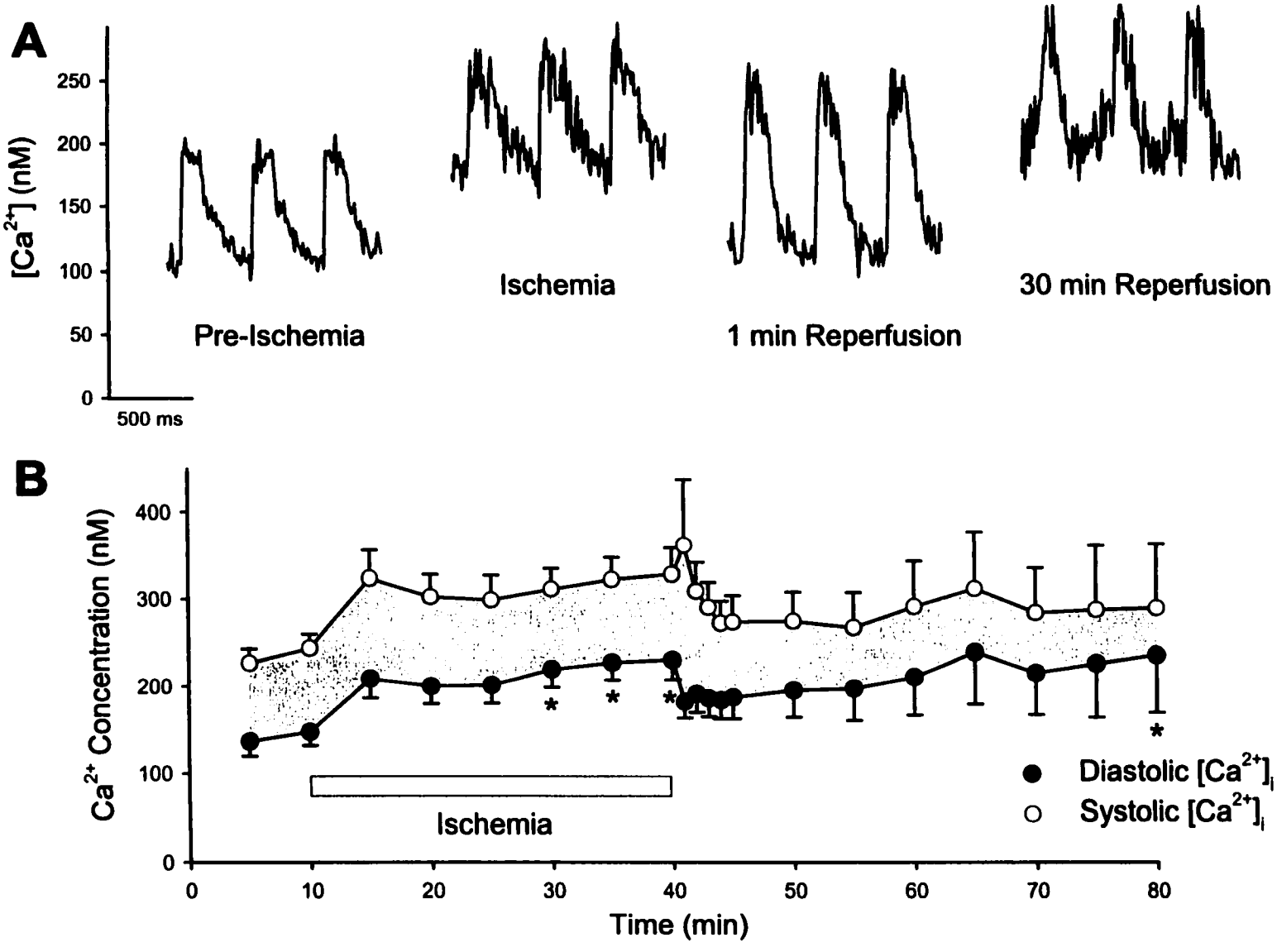

A
[Ca2+] (nM)
250
200
150
100
50
0
500 ms
Pre-Ischemia
Ischemia
1 min Reperfusion
30 min Reperfusion
B
Ca2+ Concentration (nM)
400
300
200
100
0
0
10
20
30
40
50
60
70
80
Time (min)
Ischemia
Diastolic [Ca2+]i
Systolic [Ca2+]i

Figure 34

Figure 34. Exposure to ischemia and reperfusion modified both diastolic $[Ca^{2+}]_i$ and Ca^{2+} transients.

Panel A shows representative recordings from a control cell, and mean data are presented in **Panel B.** Diastolic $[Ca^{2+}]_i$ was increased during ischemia, although the magnitude of Ca^{2+} transients was unchanged. Diastolic $[Ca^{2+}]_i$ partially recovered in early reperfusion and Ca^{2+} transients exhibited a brief overshoot. However, by late reperfusion, Ca^{2+} transients became slightly reduced and diastolic $[Ca^{2+}]_i$ was significantly elevated (* denotes significant difference from pre-ischemic values, $P < 0.05$, $n = 15$).

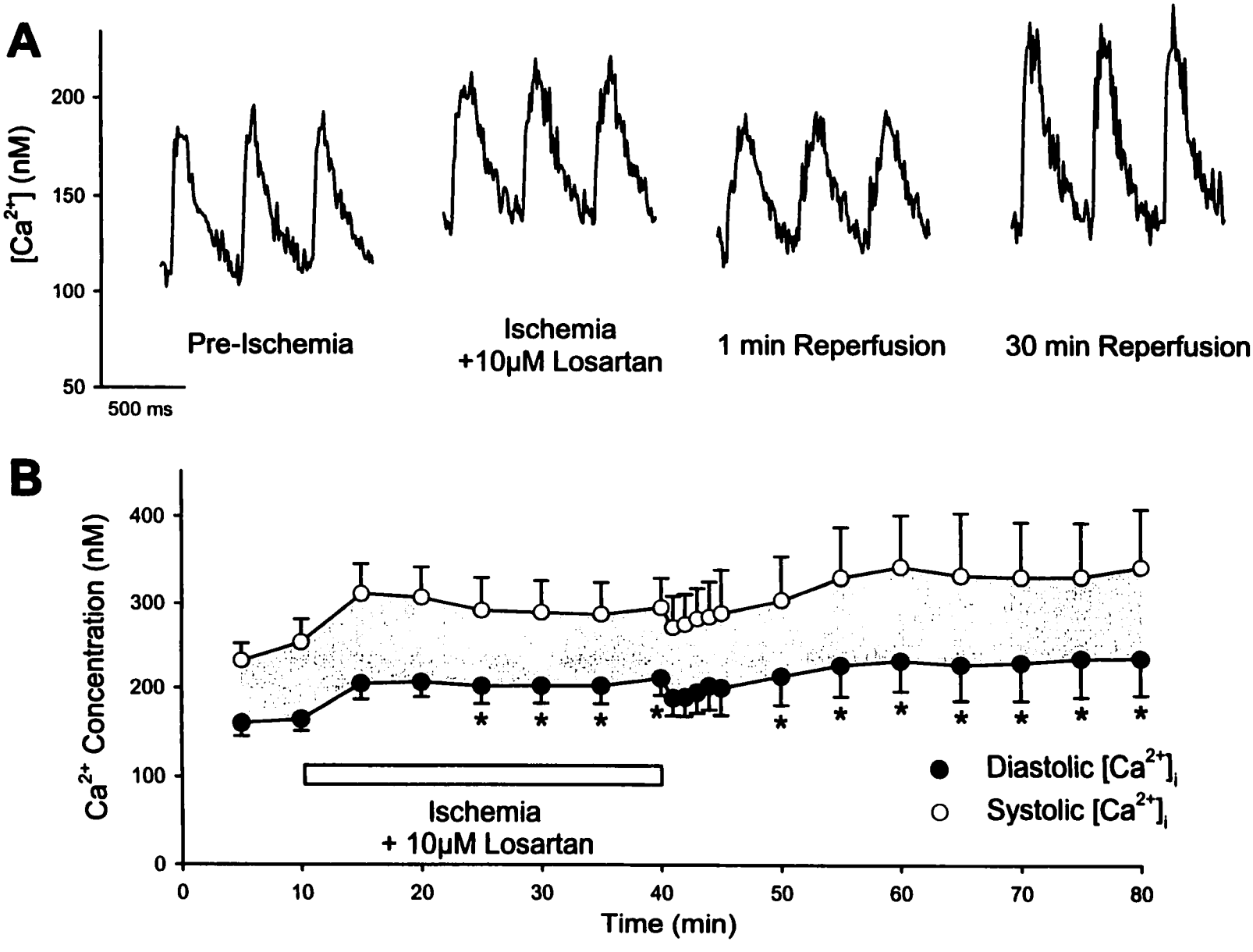

A
[Ca2+] (nM)
200
150
100
50
500 ms
Pre-Ischemia
Ischemia
+10μM Losartan
1 min Reperfusion
30 min Reperfusion
B
Ca2+ Concentration (nM)
400
300
200
100
0
Ischemia
+ 10μM Losartan
Diastolic [Ca2+]i
Systolic [Ca2+]i
0
10
20
30
40
50
60
70
80
Time (min)

Figure 35

Figure 35. Losartan treatment during ischemia attenuated the effects of ischemia and reperfusion on Ca^{2+} homeostasis.

Representative recordings (**Panel A**) and mean data (**Panel B**) show that losartan-treated cells exhibited an increase in diastolic $[Ca^{2+}]_i$ during ischemia but no change in the magnitude of Ca^{2+} transients. However, drug treatment prevented that the post-ischemic overshoot of Ca^{2+} transients observed in control cells, and increased the magnitude of Ca^{2+} transients in late reperfusion. Diastolic $[Ca^{2+}]_i$ was significantly elevated in late reperfusion (* denotes significant difference from pre-ischemic levels, $P < 0.05$; n=11).

In Figure 36, normalized Ca^{2+} transients are compared in control and drug-treated cells by plotting each with equivalent time controls. In both control and losartan-treated myocytes (Fig. 36A and B, respectively), Ca^{2+} transients were unchanged from the level of time controls throughout ischemia. In early reperfusion, control myocytes exhibited a significant overshoot of Ca^{2+} transients, an effect that was completely abolished following losartan treatment. With continued reperfusion, time controls and control myocytes exhibited a similar and gradual reduction in the magnitude of Ca^{2+} transients. However, Ca^{2+} transients were gradually increased in the losartan treatment groups, an effect that reached significance by the end of reperfusion (Fig. 36B).

Figure 37 compares normalized diastolic $[Ca^{2+}]_i$ measurements in control and drug-treated cells with time controls (A and B, respectively). During ischemia, control myocytes exhibited a marked increase in diastolic $[Ca^{2+}]_i$ relative to time controls. This effect was largely blocked in losartan-treated myocytes as no significant elevation of diastolic $[Ca^{2+}]_i$ was observed. In reperfusion, diastolic $[Ca^{2+}]_i$ measurements were very similar in the three groups of myocytes.

iii) Does Losartan Increase SR Ca^{2+} Stores?

The above observations suggest that the protective actions of losartan may involve attenuation of the increase in diastolic $[Ca^{2+}]_i$ levels during ischemia, prevention of Ca^{2+} transient overshoot in early reperfusion, and increased magnitude of Ca^{2+} transients in late reperfusion. To determine whether alterations in SR Ca^{2+} stores may contribute to these actions, SR Ca^{2+} stores were assessed using rapid application of caffeine. Representative recordings of caffeine-elicited Ca^{2+} transients are shown in

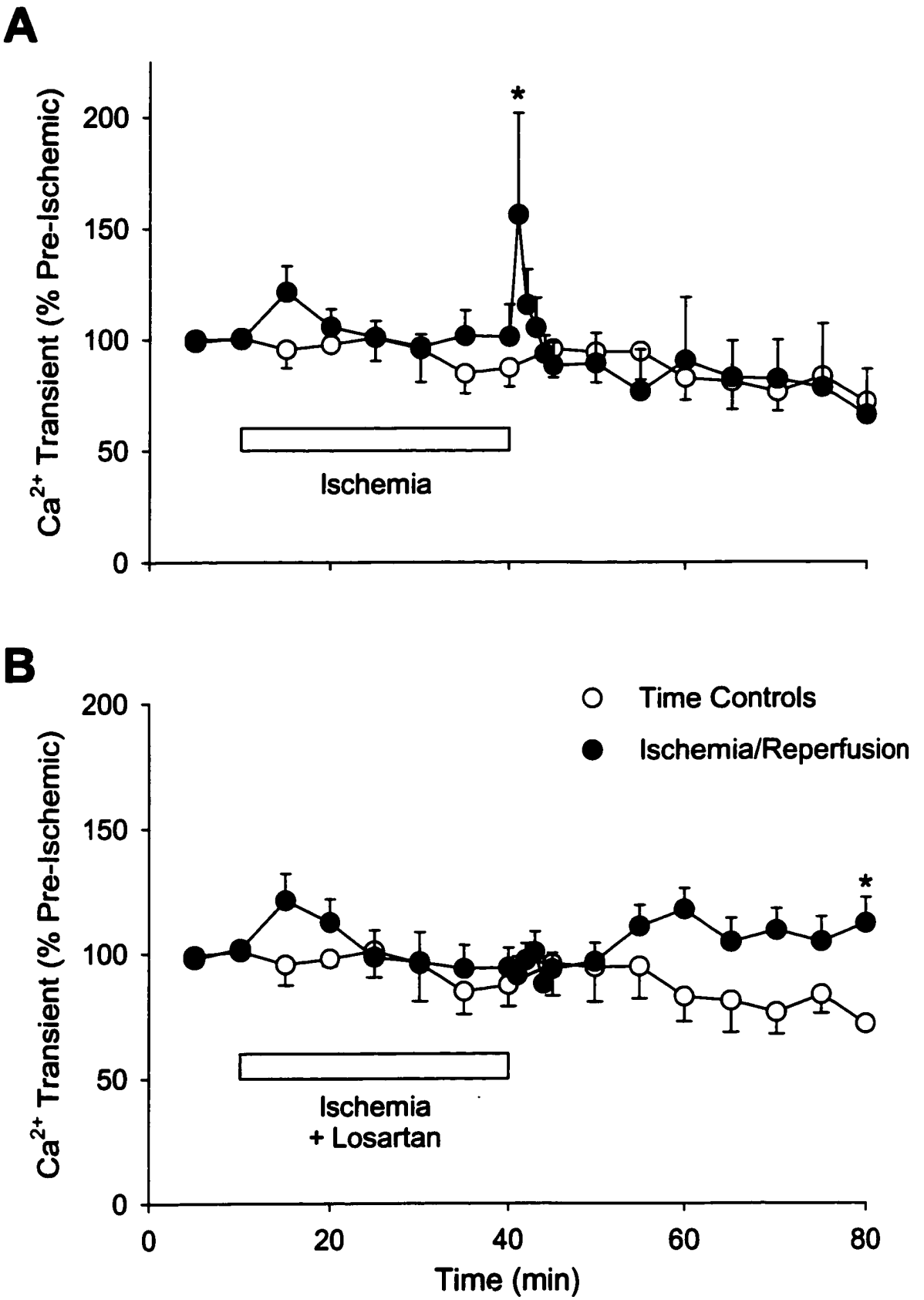

A
B
Ca2+ Transient (% Pre-Ischemic)
200
150
100
50
0
*
Ischemia
Time Controls
Ischemia/Reperfusion
Ischemia
+ Losartan
0
20
40
60
80
Time (min)

Figure 36

Figure 36. Losartan prevented overshoot of Ca^{2+} transients in early reperfusion and increased the magnitude of transients in late reperfusion.

In control cells (**Panel A**), normalized Ca^{2+} transients were unchanged in magnitude during ischemia. In early reperfusion, Ca^{2+} transients exhibited a significant overshoot relative to time controls, but were unaltered with continued reperfusion. **Panel B** shows that, in losartan-treated cells, transients were also unchanged from time values during ischemia. However, no overshoot of Ca^{2+} transients was observed in early reperfusion, and with further reperfusion, transients increased in magnitude (* denotes significant difference from time controls, $P < 0.05$; control group, $n = 15$; losartan group, $n = 11$).

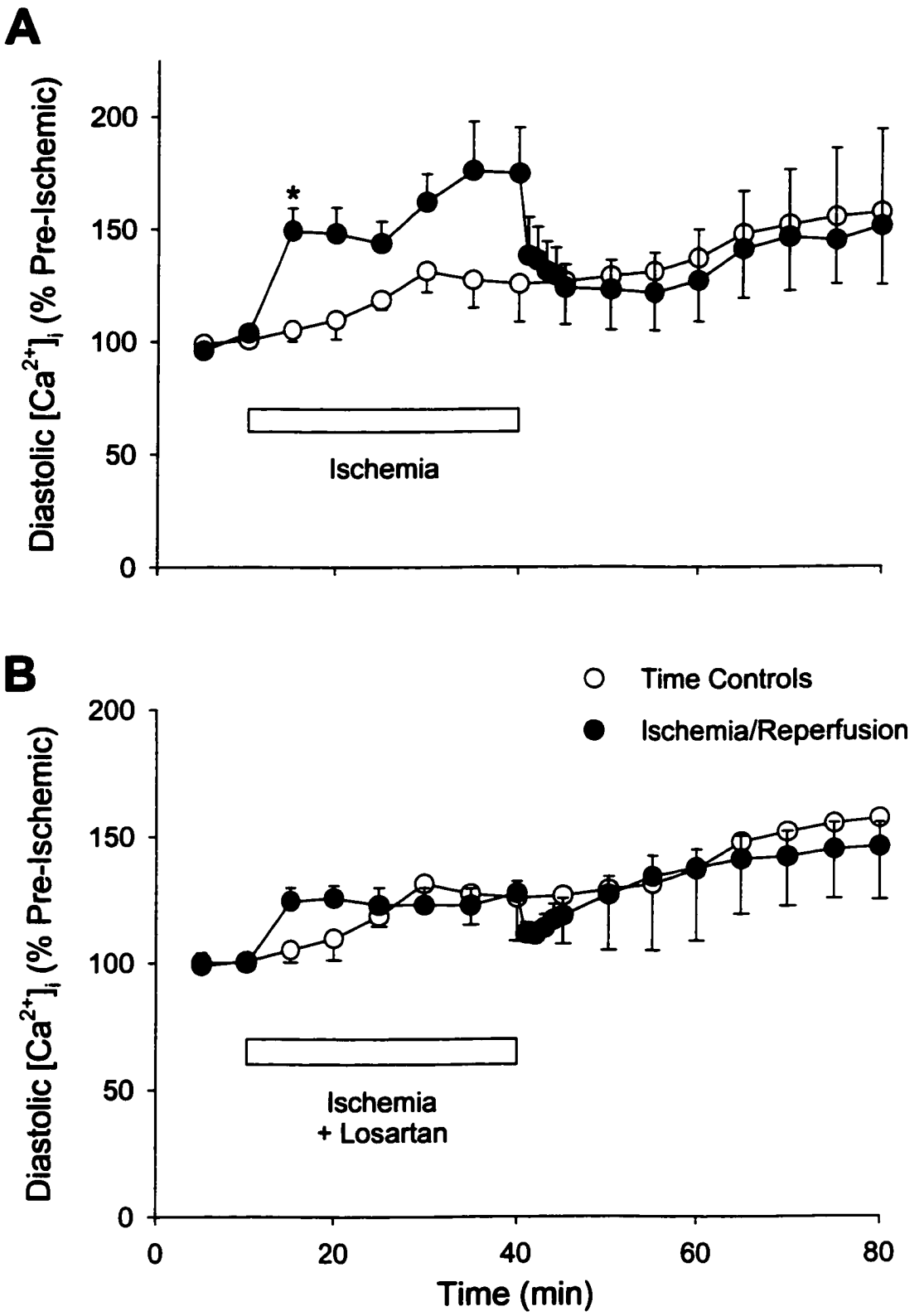

A
Diastolic [Ca2+]i (% Pre-Ischemic)
200
150
100
50
0
*
Ischemia
B
Time Controls
Ischemia/Reperfusion
Diastolic [Ca2+]i (% Pre-Ischemic)
200
150
100
50
0
Ischemia
+ Losartan
0
20
40
60
80
Time (min)

Figure 37

Figure 37. Losartan treatment attenuated elevations in diastolic $[Ca^{2+}]_i$ during ischemia.

Relative to time controls, myocytes exposed to control ischemia exhibited significant elevations in diastolic $[Ca^{2+}]_i$ (**Panel A**). However, in losartan-treated cells, significant elevation of diastolic $[Ca^{2+}]_i$ was not observed (**Panel B**). In reperfusion, diastolic $[Ca^{2+}]_i$ was not altered from time control values in either treatment group (* denotes significant difference from time contols, $P < 0.05$; control group, $n = 15$; losartan group, $n = 11$).

Figure 38. In time control experiments (Fig. 38A), no major change in the magnitude of caffeine transients was observed during the protocol. Similarly, in myocytes exposed to control ischemia (Fig. 38B), no obvious change in caffeine-elicited Ca^{2+} transients was observed during ischemia or reperfusion. However, in losartan-treated cells (Fig. 38C), the magnitude of caffeine transients appeared to be increased during ischemia, and further elevated during reperfusion.

Mean measurements of the magnitude of caffeine-elicited Ca^{2+} transients are plotted in Figure 39. Normalized caffeine transients exhibited a slight and similar reduction during time control and control ischemia / reperfusion experiments. However, losartan-treated cells exhibited a trend toward increased magnitudes of caffeine-elicited Ca^{2+} transients during ischemia and reperfusion. These observations suggest that losartan may increase SR Ca^{2+} stores during ischemia and reperfusion.

iv) Is ischemia required to observe these actions of losartan?

Since losartan treatment appeared to cause an increase in SR Ca^{2+} stores in late reperfusion relative to both time controls and control ischemia / reperfusion values, it was hypothesized that losartan might also increase SR stores under normoxic conditions. To test this hypothesis, myocytes were treated with 10 μM losartan for 30 min followed by 40 min of washout. Figure 40 shows that normalized caffeine-elicited Ca^{2+} transients increased during the treatment period. In washout, a further increase in magnitude was observed as caffeine transients were significantly elevated to 190% of pre-treatment levels. Therefore, it appears that losartan treatment may increase SR Ca^{2+} stores to an even greater extent under normoxic conditions than during ischemia and reperfusion.

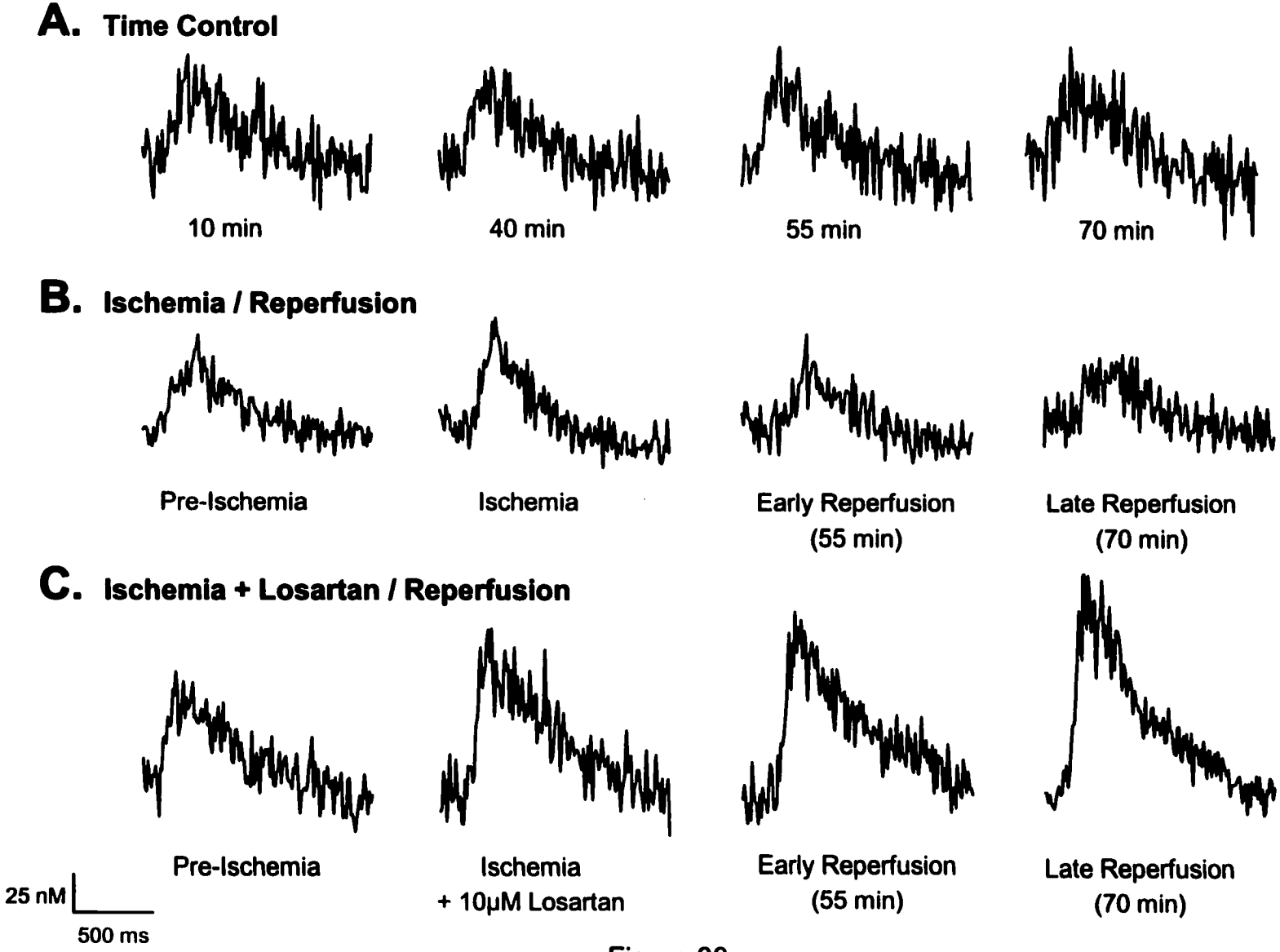

A. Time Control
10 min
40 min
55 min
70 min
B. Ischemia / Reperfusion
Pre-Ischemia
Ischemia
Early Reperfusion
(55 min)
Late Reperfusion
(70 min)
C. Ischemia + Losartan / Reperfusion
Pre-Ischemia
Ischemia
+ 10μM Losartan
Early Reperfusion
(55 min)
Late Reperfusion
(70 min)
25 nM
500 ms

Figure 38

Figure 38. Caffeine-elicited Ca^{2+} transients were increased in losartan-treated myocytes.

In time controls (**Panel A**) and cells exposed to control ischemia and reperfusion (**Panel B**), caffeine-elicited Ca^{2+} transients were relatively unchanged during the experiment. However, in losartan-treated cells (**Panel C**), caffeine transients were elevated during ischemia and were further increased in reperfusion.

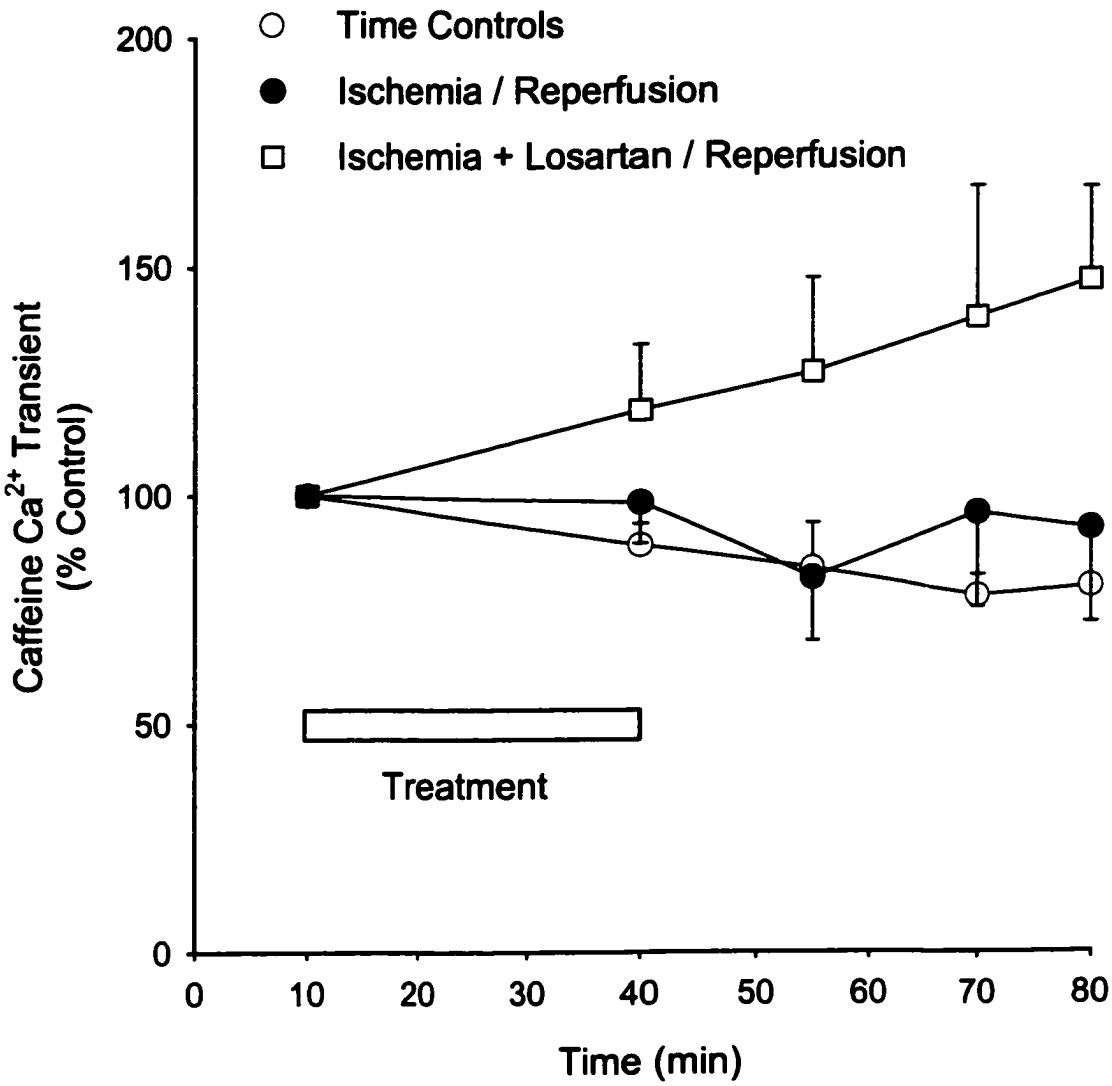

Time Controls
Ischemia / Reperfusion
Ischemia + Losartan / Reperfusion
Caffeine Ca2+ Transient (% Control)
200
150
100
50
0
Treatment
0
10
20
30
40
50
60
70
80
Time (min)

Figure 39

Figure 39. Losartan may increase SR Ca^{2+} stores during ischemia and reperfusion. Caffeine-elicited Ca^{2+} transients were used as a relative estimate of SR Ca^{2+} stores. Normalized caffeine-elicited Ca^{2+} transients were unchanged during control ischemia and reperfusion relative to time controls. In cells treated with losartan, caffeine transients were steadily, but not significantly, elevated during ischemia and reperfusion (time controls, n = 6; control ischemia, n = 13; ischemia + losartan, n = 15).

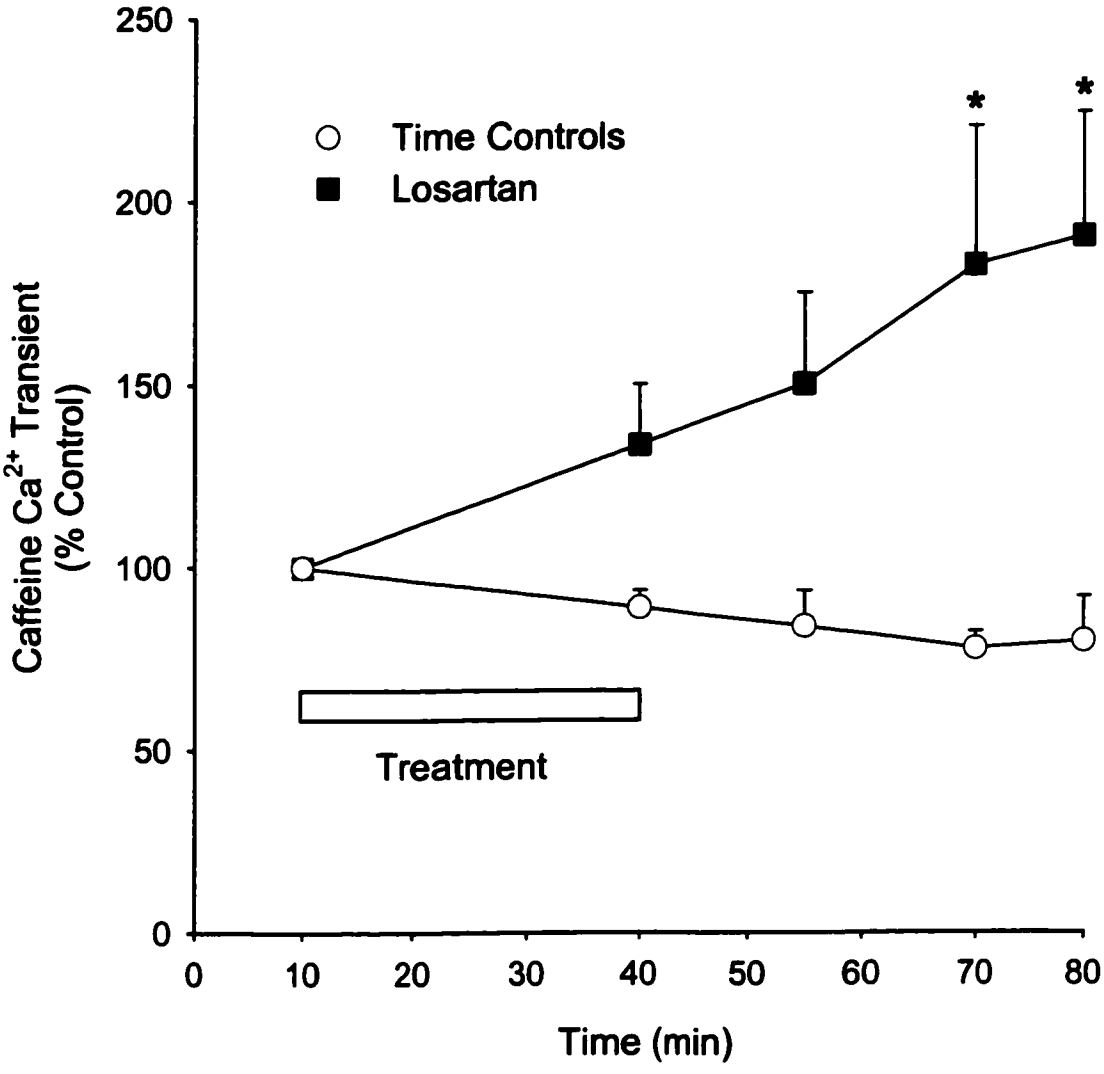

250
200
150
100
50
0
Caffeine Ca^{2+} Transient
(% Control)
Time Controls
Losartan
*
*
Treatment
0
10
20
30
40
50
60
70
80
Time (min)

Figure 40

Figure 40. Losartan treatment increases SR Ca^{2+} stores in the absence of ischemia and reperfusion.

When losartan was applied under normoxic conditions, caffeine-elicited Ca^{2+} transients were slightly increased after 30 min of drug treatment. In washout, caffeine transients were significantly elevated from pre-treatment levels, suggesting that SR Ca^{2+} stores were increased. Caffeine transients were not altered in time control experiments (* denotes significant difference from pre-treatment levels, $P < 0.05$; time controls, $n = 6$; losartan group, $n = 7$).

The effect of losartan treatment on $[Ca^{2+}]_i$ was also examined under normoxic conditions. Figure 41A shows mean data for measurements of Ca^{2+} transients. Time controls exhibited a slight but steady decrease in the magnitude of Ca^{2+} transients during 80 min of recording. However, losartan-treated cells exhibited relatively unchanged Ca^{2+} transients during the treatment period, and a steady increase in transient magnitude during washout of the drug. Despite this apparent effect of losartan, Ca^{2+} transients in the losartan treatment group were not significantly altered from pre-treatment or time control values, perhaps as a result of the small sample size in this experiment.

Figure 41B shows mean measurements of diastolic $[Ca^{2+}]_i$ in the presence and absence of losartan treatment. In time control experiments, diastolic $[Ca^{2+}]_i$ was slightly reduced during 80 min of recording. No significant difference from time control values was observed during treatment or washout. These observations suggest that under normoxic conditions, losartan may act to increase SR stores and Ca^{2+} transients without an effect on diastolic $[Ca^{2+}]_i$.

E. Are the Protective Effects of Losartan Mediated Via Blockade of AII?

The final series of experiments examined the role of AII in the protective actions of losartan during ischemia and reperfusion. In these experiments, myocytes were exposed to either 0.1 μM AII or 10 μM losartan plus 0.1 μM AII during ischemia. For comparison, these results will be displayed parallel to previously discussed data from cells treated with control ischemia and ischemia plus losartan.

Figure 42 shows the mean contraction amplitudes for the four treatment groups during ischemia and reperfusion. These contractions were elicited under the voltage-

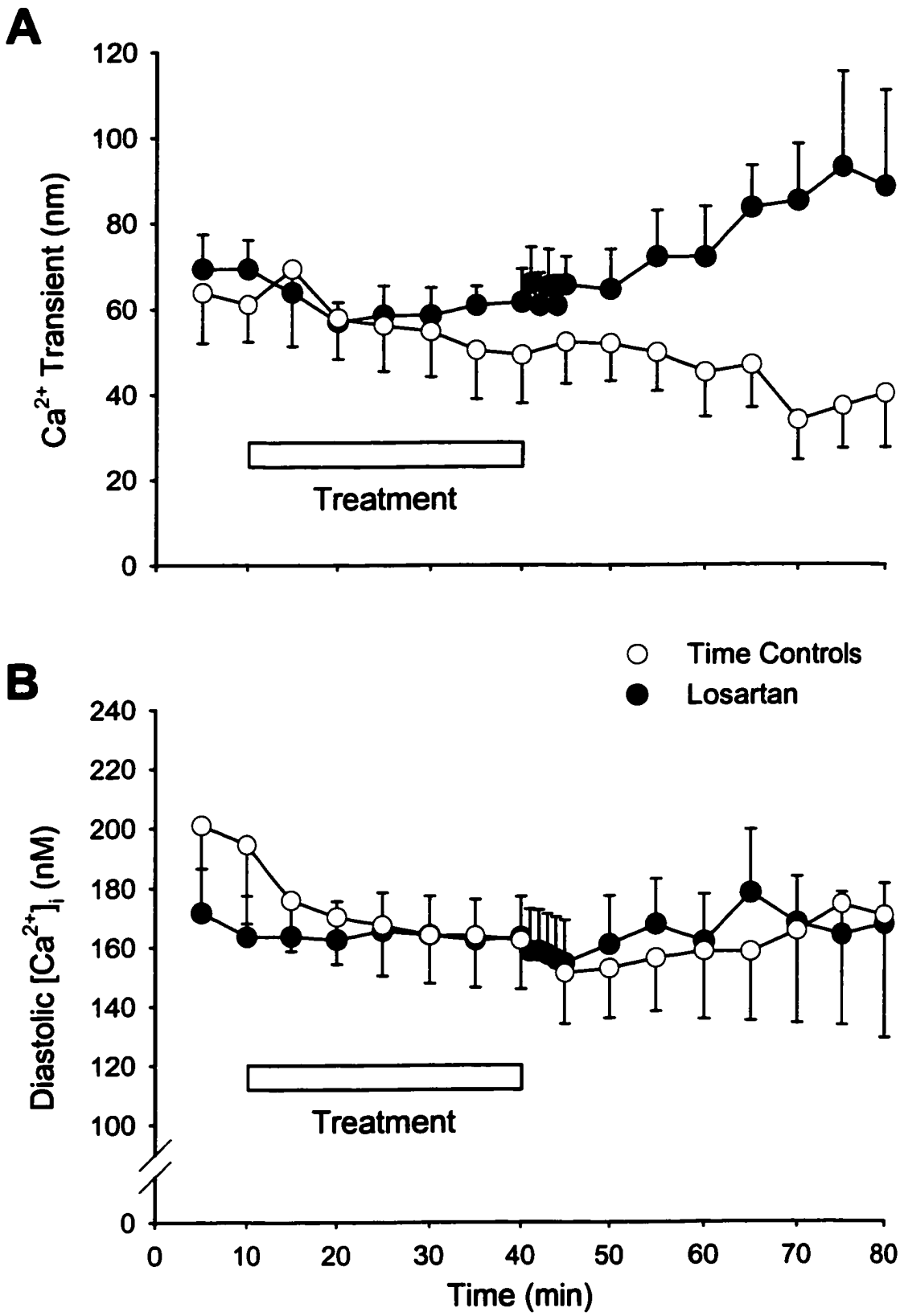

A
120
100
80
60
40
20
0
Ca2+ Transient (nm)
Treatment
B
Time Controls
Losartan
240
220
200
180
160
140
120
100
0
Diastolic [Ca2+]i (nM)
Treatment
0
10
20
30
40
50
60
70
80
Time (min)

Figure 41

Figure 41. Losartan treatment in normoxia tends to increase Ca^{2+} transients during washout.

Panel A shows mean measurements of Ca^{2+} transients in the absence of ischemia and reperfusion. Time controls exhibited a gradual decrease in the magnitude of Ca^{2+} transients during 80 min or recording. Ca^{2+} transients were relatively unchanged during losartan treatment, but exhibited a steady increase during washout. This effect was not statistically significant. Losartan treatment had no effect on diastolic $[Ca^{2+}]_i$ (**Panel B**). Time controls exhibited a very slight decrease in diastolic $[Ca^{2+}]_i$ during the experiment. (time controls, n = 6; losartan group, n = 7).

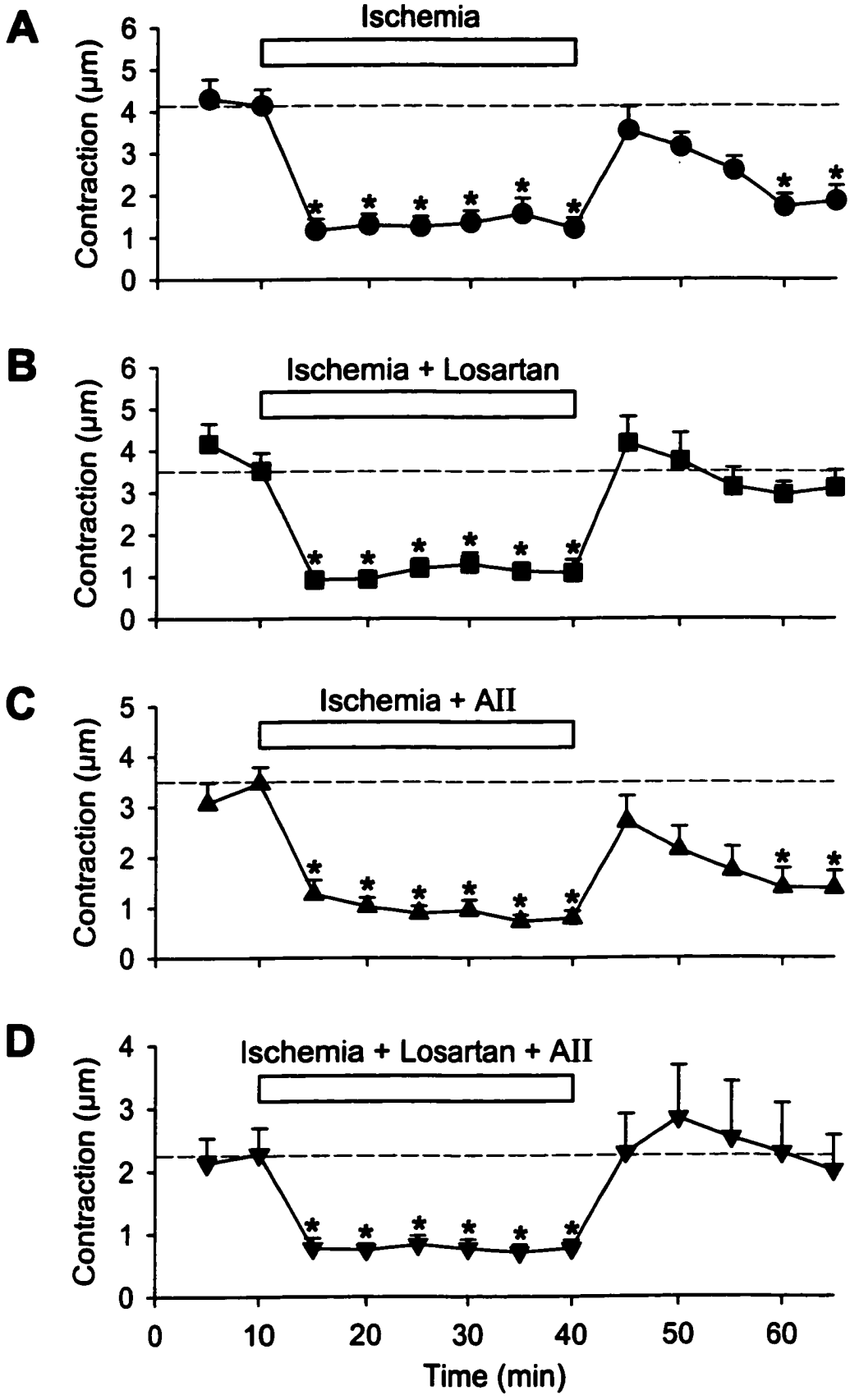

A
Ischemia
Contraction (µm)
B
Ischemia + Losartan
Contraction (µm)
C
Ischemia + AII
Contraction (µm)
D
Ischemia + Losartan + AII
Contraction (µm)
Time (min)

Figure 42

Figure 42. Losartan treatment attenuated stunning in the presence and absence of exogenous AII.

The voltage-clamp protocol used was the same as that shown in Figure 12, but with a test step from –52 to 0 mV (**top**). In control cells (**Panel A**), contractions decreased significantly during ischemia, recovered to near pre-ischemic levels in early reperfusion, and became significantly depressed in late reperfusion. Cells exposed to 10 μmol/L losartan (**Panel B**) or losartan plus AII (**Panel D**) during ischemia also showed contractile depression during ischemia but exhibited improved contractile function in reperfusion relative to pre-ischemic values. Cells treated with 0.1 μmol/L AII (**Panel C**) during ischemia showed significant contractile depression during ischemia and stunning in late reperfusion which was similar to untreated cells (* denotes $p<0.05$, control group, n = 21; losartan group, n = 14; AII group, n = 20, losartan + AII group, n = 14)

clamp protocol used in Figure 27. Myocytes exposed to control ischemia, ischemia + losartan, ischemia plus AII, and ischemia plus losartan and AII (Figs. 42A, B, C, and D, respectively) exhibited similar and significant contractile depression during ischemia. However, contractile recovery in reperfusion was strongly dependent on drug treatment during ischemia. Interestingly, reperfusion-induced contractile depression was abolished in myocytes exposed to losartan in the presence or absence of exogenous AII. However, myocytes treated with AII alone exhibited significant post-ischemic contractile depression similar to that observed in control cells.

To determine whether an effect of AII on contraction amplitude was masked by a shift in the voltage dependence of contraction, contraction-voltage relationships were examined using the voltage-clamp protocol shown in Figure 28. Figure 43 shows previously described contraction-voltage relationships for control and losartan-treated myocytes (A and B, respectively) and also data for cells treated with AII alone and losartan plus AII (C and D, respectively). Contraction-voltage curves were depressed in control and AII-treated cells during both ischemia and late reperfusion. However, cells treated with losartan in both the presence and absence of exogenous AII exhibited decreased contraction amplitude during ischemia, but relatively normal contraction-voltage relationships in late reperfusion. Thus, treatment with exogenous AII, in the presence or absence of losartan, did not appear to alter maximal cell shortening or shift the voltage-dependence of contraction. Similarly, the effects of losartan on contraction-voltage relationships were not influenced by the presence or absence of exogenous AII.

The effects of AII treatment on $I_{Ca\text{-}L}$ magnitude were also examined. $I_{Ca\text{-}L}$ was elicited by a voltage-step from –40 mV to 0 mV. In all treatment groups, $I_{Ca\text{-}L}$ decreased

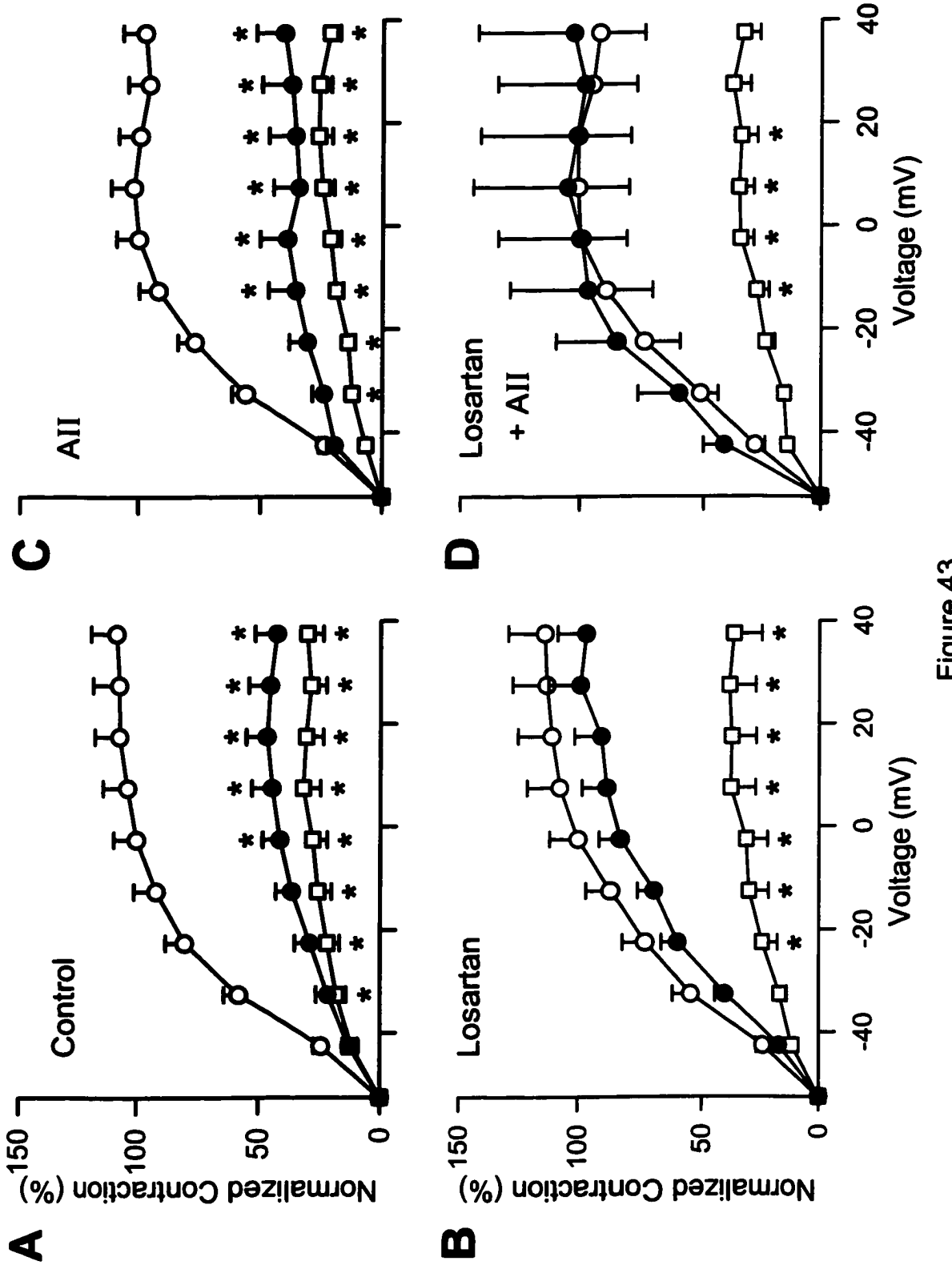

A
Control
Normalized Contraction (%)
150
100
50
0
B
Losartan
-40
-20
0
20
40
Voltage (mV)
C
AII
D
Losartan
+ AII

Figure 43

Figure 43. Exogenous AII did not alter the post-ischemic recovery of contraction-voltage relationships or protective effects of losartan.

The voltage-clamp protocol used was the same as in Figure 28. Control cells (**Panel A**, n=22) and AII-treated cells (**Panel C**, n=20) exhibited significantly reduced contraction-voltage relationships in ischemia and following 20 min of reperfusion. Cells treated with losartan (**Panel B**, n=14) and losartan plus AII (**Panel D**, n=13) also showed contractile depression in ischemia, but in late reperfusion, contraction-voltage relationships recovered to pre-ischemic levels. None of the treatment groups exhibited a shift in the voltage-dependence of contraction during ischemia or reperfusion (* denotes significant difference from pre-ischemic values, $P < 0.05$).

in magnitude during ischemia and remained depressed in reperfusion. No significant differences in $I_{Ca\text{-}L}$ magnitude were observed between any of the treatment groups. However, in myocytes treated with AII alone (Fig. 44C), $I_{Ca\text{-}L}$ depression appeared to be slightly more pronounced than in myocytes exposed to control ischemia and reperfusion (Fig. 44A). When cells were exposed to losartan as well as AII (Fig. 44D), depression of $I_{Ca\text{-}L}$ was less marked, although statistically significant. Thus, it appeared that losartan attenuated the effect of AII on $I_{Ca\text{-}L}$.

Finally, the effect of exogenous AII on I_{TI} incidence was examined in the presence and absence of losartan. Figure 45 shows that AII treatment alone during ischemia had no effect on the incidence of I_{TI} (57%) relative to control cells (60%). In myocytes treated with losartan plus AII, I_{TI} incidence was reduced to 36%, although this effect was not statistically significant. As described previously, treatment with losartan alone significantly reduced I_{TI} incidence to 20%. No significant difference in I_{TI} incidence was observed between the two losartan treatment groups.

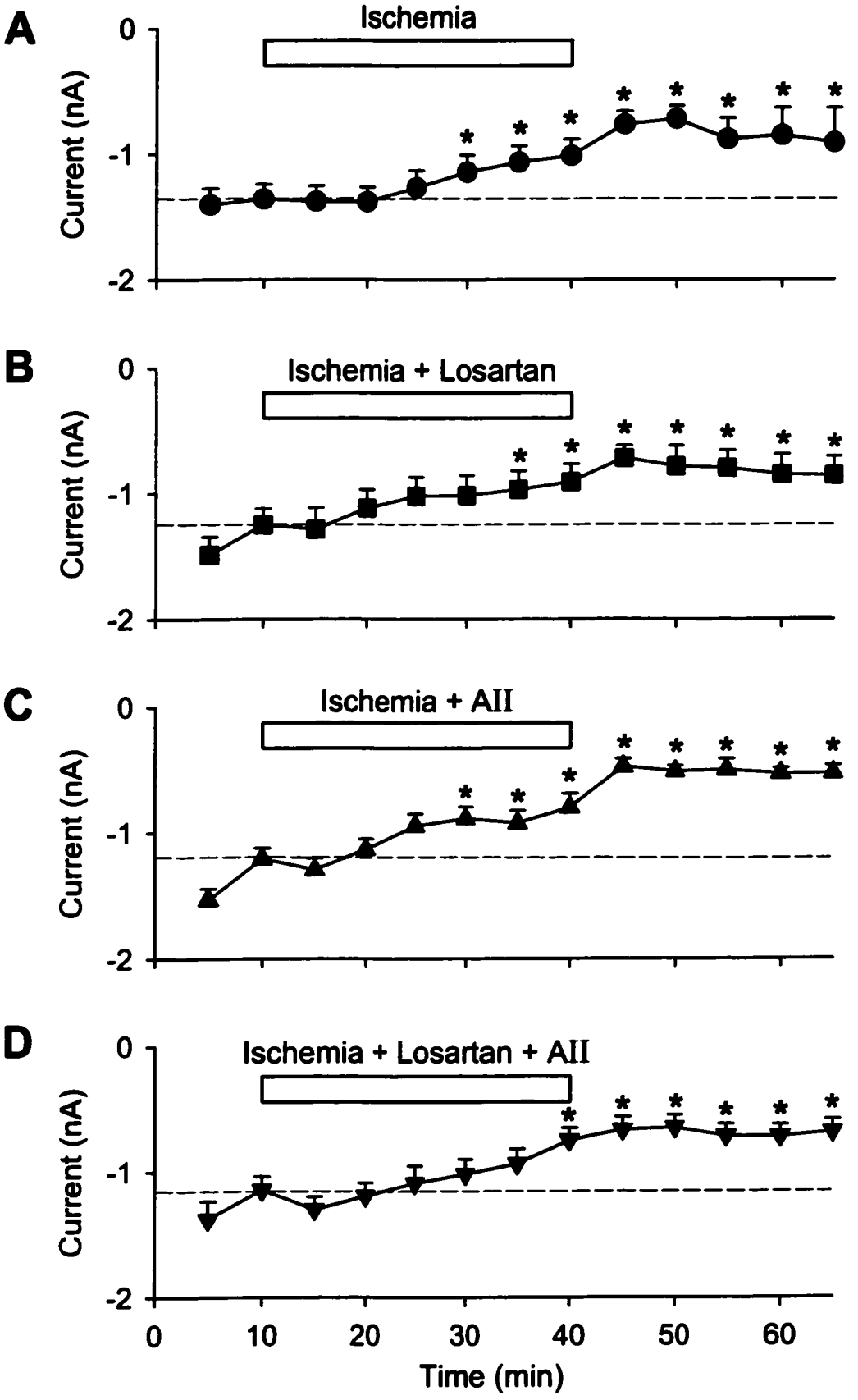

A
Ischemia
B
Ischemia + Losartan
C
Ischemia + AII
D
Ischemia + Losartan + AII
Current (nA)
0
-1
-2
0
10
20
30
40
50
60
Time (min)

Figure 44

Figure 44. Losartan attenuates effects of AII on $I_{Ca\text{-}L}$ during ischemia and reperfusion.

$I_{Ca\text{-}L}$ was elicited with a voltage-clamp step from –40 mV to 0 mV. In control cells (**Panel A**), magnitude of $I_{Ca\text{-}L}$ was decreased during ischemia, and further reduced in reperfusion. A similar time course was observed for losartan-treated cells (**Panel B**). Treatment with AII during ischemia (**Panel C**) appeared to potentiate depression of $I_{Ca\text{-}L}$ during reperfusion. This effect was attenuated by the addition of losartan (**Panel D**) (* denotes significant difference from pre-ischemic values, $P < 0.05$, control group, $n = 21$; losartan group, $n = 14$; AII group, $n = 20$, losartan + AII group, $n = 14$).

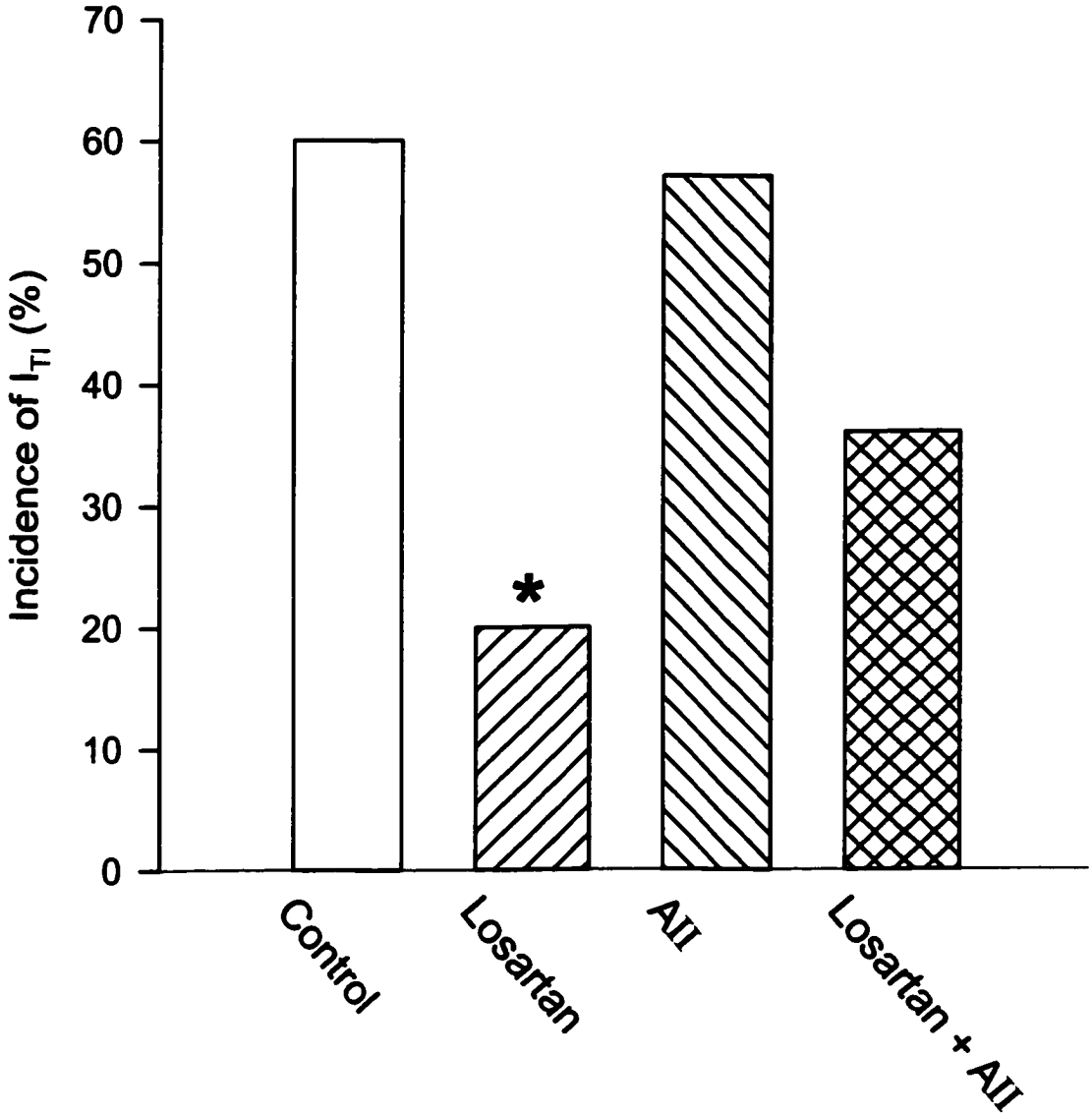

70
60
50
40
30
20
10
0
Incidence of I_{TI} (%)
*
Control
Losartan
AII
Losartan + AII

Figure 45

Figure 45. Losartan treatment reduced incidence of I_{TI} in the presence and absence of exogenous AII.

Cells treated with losartan showed a significant reduction in incidence of I_{TI} relative to control myocytes. The occurrence of I_{TI} also was reduced in cells treated with losartan + AII, although this effect was not significant. AII treatment during ischemia had no effect on I_{TI} incidence (* denotes significant difference from control, $P < 0.05$, control group, n = 21; losartan group, n = 14; AII group, n = 20, losartan + AII group, n = 14).

DISCUSSION – PART I

1. Development of a Cellular Model of Stunning

The first objective of Part I of this thesis was to develop a cellular model of stunning in which myocytes exhibited significant contractile depression associated with normal electrical activity. To define such a model, the first set of experiments examined both action potentials and contractions in isolated myocytes during ischemia and reperfusion. The results showed that contractions in myocytes became reduced in magnitude during 30 min of simulated ischemia, recovered to control levels in early reperfusion, and were reduced in magnitude with continued reperfusion. Measurements of action potentials indicated that APD decreased and RMP increased during ischemia, but that both parameters recovered to control levels by the time post-ischemic contractile depression was observed. However, it is possible that there were alterations in action potential configuration in late reperfusion which were not accounted for by measurements of APD and RMP. Nevertheless, in voltage-clamp studies, myocytes exhibited stunning even when the membrane voltage was controlled. Therefore, stunning in isolated myocytes was not the consequence of altered electrical activation of the sarcolemma. In later experiments, it was demonstrated that contractions in post-ischemic myocytes were significantly depressed from both pre-ischemic levels and also the levels of time controls. Therefore, it appears that the ischemia / reperfusion protocol can be used to model stunning, since it produces post-ischemic myocytes which exhibit both contractile depression and normal electrical activity (Hanich et al., 1993).

The conditions of simulated ischemia used in this study may be of key importance in producing stunning in isolated mycoytes. In many studies, myocardial ischemia has been simulated by exposing myocytes to anoxia or hypoxia with or without metabolic inhibition. However, exposure of isolated myocytes to these conditions does not consistently produce post-ischemic contractile depression (Stern et al., 1985; Silverman et al., 1991; Boekstegers et al., 1992). This observation may result from the fact that hypoxia and metabolic inhibition are only two of many alterations that occur during true myocardial ischemia. It might be expected, therefore, that protocols which more accurately reproduce ischemia would be more likely to produce stunned myocytes. In a recent study by Maddaford et al. (1999), myocytes were exposed to an "ischemic" solution that simulated acidosis and hyperkalemia in addition to hypoxia and substrate deprivation (metabolic inhibition). Following 45 min of these simulated ischemia conditions, myocytes exhibited significant contractile depression for 2.5 min of reperfusion before returning to control levels. To produce more protracted post-ischemic contractile depression resembling stunning, these simulated ischemic conditions were additionally altered in the present study to include hypercapnia and lactate accumulation. As well, a 90% N_2 / 10% CO_2 gas phase was layered over the surface of the experimental chamber to further reduce oxygen availability. Myocytes exposed to 30 min of these "ischemic" conditions exhibited significant contractile depression during 40 min of reperfusion. Therefore, it appears that lactate accumulation, hypercapnia, and marked reductions in oxygen availability during simulated ischemia are required to produce stunning in isolated myocytes.

A limitation of all studies that simulate ischemia and reperfusion is that assumptions must be made on how to mimic these conditions. An alternative approach for developing a cellular model of stunning would be to examine myocytes isolated from post-ischemic hearts. This type of model would be advantageous since the complex conditions of ischemia / reperfusion are produced *in situ*. In one study, it was observed that myocytes isolated from stunned hearts exhibited depressed contractile function that was similar to stunned myocardium (Lew et al., 1994). However, subsequent studies have not supported this observation (Chandrashekhar et al., 1999; Straznicka et al., 2000). As well, Lew et al (1994) did not examine the electrical activity of post-ischemic myocytes, and therefore cannot rule out the possibility that contractions were depressed as a result of altered action potential configuration. Therefore, it remains unclear whether myocytes isolated from post-ischemic hearts can be used to model stunning.

The ischemia / reperfusion protocol utilized in the present study produces a pattern of contractile change in reperfusion which resembles that observed in intact heart experiments. In early reperfusion, field-stimulated myocytes exhibited a brief recovery and overshoot of contractile function as is observed in the intact myocardium (Kusuoka and Marban, 1992; du Toit and Opie, 1992). Following this overshoot, contractions were reduced in magnitude and exhibited maximal contractile depression after 10 min of reperfusion. In intact post-ischemic hearts, stunning is also maximal at approximately this timepoint (Kusuoka and Marban, 1992; du Toit and Opie, 1992). With continued reperfusion, contractile function may persist in the intact heart for many hours. In the cellular model, contractions remained depressed relative to time controls for the duration of the 40 min reperfusion period. However, it was not possible to assess contractile

function past this point, since cell death became more frequent after 80 min of recording. Therefore, it is unclear whether contractions in post-ischemic myocytes would recover to the level of time controls with further reperfusion. However, due to the limitations associated with isolated myocyte experiments, it may not be possible to ever completely model the timecourse of contractile function in myocardial stunning at the cellular level.

2. EC Coupling in Stunning

A. Calcium Transients and SR Stores

The lesion in EC coupling responsible for stunning is the subject of much debate. One area of contention is whether Ca^{2+} availability is reduced in stunning. A number of studies have observed normal Ca^{2+} transients in intact post-ischemic hearts (for review see Bolli and Marban; 1999). However, studies that have examined *in vitro* preparations of stunned myocardium have observed abnormalities in SR proteins. Several studies have observed reductions in the activity and protein levels of the SR Ca^{2+} ATPase (Zucchi et al., 1996; Smart et al., 1997; Osada et al., 1998; Temsah et al., 1999; Osada et al., 2000; c.f. Luss et al., 1997), an effect that would be expected to reduce SR stores and the magnitude of the Ca^{2+} transient (Valdivia et al., 1997). Therefore, it is unclear whether SR function is altered in intact post-ischemic myocytes and whether these alterations affect the magnitude of the Ca^{2+} transient. The cellular model described in the present study is well suited to addressing this question, since SR Ca^{2+} stores and Ca^{2+} transients can both be examined in contracting myocytes. SR Ca^{2+} content was assessed by rapid exposure to caffeine. Caffeine is known to trigger SR Ca^{2+} release by activating the SR Ca^{2+} release channel (O'Neill and Eisner, 1990), and thus, caffeine-elicited Ca^{2+}

transients are routinely used to estimate SR Ca^{2+} content (Bers, 2001). Caffeine transients were unchanged from the levels of time controls throughout ischemia and reperfusion, suggesting that stunning does not involve reduced SR Ca^{2+} stores in this model.

The observation that SR Ca^{2+} stores appear to be normal in stunning does not necessarily prove that overall SR function is normal in late reperfusion. For example, if SR Ca^{2+} uptake and release are both reduced in stunning, as has been suggested in some studies (Osada et al., 1998; Temsah et al., 2000), it is possible that no net change in Ca^{2+} content would be observed. However, additional insight into the nature of SR function in stunning is provided by the results of field-stimulation experiments in which $[Ca^{2+}]_i$ was recorded. If SR Ca^{2+} uptake is reduced in late reperfusion, it might be expected that diastolic $[Ca^{2+}]_i$ would be increased. Similarly, changes in the magnitude of Ca^{2+} transients might be expected if SR Ca^{2+} release is altered in stunning. However, stunning was associated with both normal diastolic $[Ca^{2+}]_i$ and normal Ca^{2+} transients in the cellular model. Therefore, the present study provides no evidence to suggest that stunning is related to alterations in SR function. Instead, it appears that Ca^{2+} availability is normal in the cellular model of stunning. These results question the interpretation of previous studies that have reported abnormalities in SR proteins based on observations of isolated SR vesicles. One possible explanation for the disagreement between *in vitro* and *in situ* studies is that the SR is isolated in *in vitro* studies using a procedure which may produce an unrepresentative sample of the whole-tissue SR (Rapundalo et al., 1986).

B. Alterations in $I_{Ca\text{-}L}$: Implications for Ca^{2+} Homeostasis

Although Ca^{2+} transients appear to be of normal magnitude during stunning, it is possible that the sources of Ca^{2+} that comprise the Ca^{2+} transient are altered in reperfusion. In voltage-clamp experiments, $I_{Ca\text{-}L}$ magnitude decreased during ischemia and remained significantly depressed throughout reperfusion. This observation suggests that increased Ca^{2+} influx and/or increased Ca^{2+} release from stores must compensate for the reduction in $I_{Ca\text{-}L}$ to maintain a normal Ca^{2+} transient in stunning. Based on experiments which measured SR Ca^{2+} stores and Ca^{2+} transients, it seems unlikely that activity of the SR Ca^{2+} release channel is facilitated in late reperfusion. However, it is possible that the gain of CICR is increased in reperfusion, meaning that the ability of $I_{Ca\text{-}L}$ to trigger SR Ca^{2+} release via CICR is enhanced. Alternatively, Ca^{2+} entry by reverse-mode Na^{+}-Ca^{2+} exchange may be increased to compensate for a decrease in $I_{Ca\text{-}L}$. Activation of Ca^{2+}-dependent proteases and production of oxygen-derived free radicals, events that are well documented in reperfusion, have been shown to increase activity of the $NaCa_{ex}$ (Hilgemann, 1990; Lyu et al., 1991; Goldhaber, 1996; Chesnais et al., 1999). Reverse-mode Na^{+}-Ca^{2+} exchange may be specifically increased in reperfusion (Tani and Neely, 1989; Elias et al., 2001) since elevations in $[Na^{+}]_i$ recover slowly (Pike et al., 1990; Tani and Neely, 1990). Interestingly, Ca^{2+}-dependent proteases and free radicals are also known to decrease Ca^{2+} currents (Cerbai et al., 1991; Romanin et al., 1991; Tokube et al., 1996; Kameyama et al., 1998). Therefore, it is possible that Ca^{2+}-dependent proteolysis and free radical generation may have been responsible for both the decrease in $I_{Ca\text{-}L}$ and the maintained Ca^{2+} transient observed during stunning.

The reduced magnitude of $I_{Ca\text{-}L}$ in early reperfusion is also of interest since Ca^{2+} entry in the initial moments of reperfusion is believed to be an important trigger for stunning (Shine et al., 1978; Tani and Neely, 1990). As has been observed in studies of intact hearts (Carrozza et al., 1992; Harada et al., 1994; Varadarjan et al., 2001), isolated myocytes exhibited a large overshoot in the magnitude of Ca^{2+} transients in early reperfusion. Since $I_{Ca\text{-}L}$ magnitude was significantly reduced at this time, the large Ca^{2+} transient apparently results from increased Ca^{2+} entry via another route for Ca^{2+} influx and/or facilitated SR Ca^{2+} release. It is unclear whether SR Ca^{2+} release is altered in early reperfusion. However, it is generally accepted that Ca^{2+} entry by the $NaCa_{ex}$ in early reperfusion is an important contributor to reperfusion injury. Pharmacological inhibition of reverse-mode Na^{+}-Ca^{2+} exchange protects against reperfusion injury (Ladilov et al., 1999; Elias et al., 2001). As well, transgenic overexpression of the $NaCa_{ex}$ delays post-ischemic contractile recovery (Cross et al., 1988). The results of the present study suggest that, since $I_{Ca\text{-}L}$ is reduced, post-ischemic Ca^{2+} entry via the $NaCa_{ex}$ may make an even more important contribution to reperfusion injury than previously hypothesized.

C. CICR and the VSRM in Stunning

Contractions elicited by CICR and the VSRM were similarly altered during ischemia and reperfusion. Both types of contraction were reduced during ischemia, but briefly recovered to pre-ischemic levels in early reperfusion. With continued reperfusion, however, CICR and VSRM contractions both became significantly depressed. These observations suggest that stunning does not result from a selective downregulation of

either mechanism of EC coupling. It is somewhat surprising that post-ischemic CICR contractions were not depressed to a greater extent than VSRM contractions, since $I_{Ca\text{-}L}$ was observed to be reduced in reperfusion. One possible explanation for this observation is that the gain of CICR is increased in reperfusion to compensate for reductions in $I_{Ca\text{-}L}$. An increase in the gain of CICR could also at least partly explain why Ca^{2+} transients remain normal in stunning despite depression of $I_{Ca\text{-}L}$.

D. Myofilament Responsiveness in Stunning

In the present study, Ca^{2+} transients recovered in late reperfusion, suggesting that defects downstream of Ca^{2+} cycling make a major contribution to stunning. Indeed, a number of recent studies have suggested that stunning results from reduced myofilament responsiveness to Ca^{2+} (Kusuoka et al., 1987; Kusuoka et al., 1990; Carrozza et al., 1992; Hofmann et al., 1993; Gao et al., 1995). This reduction in myofilament responsiveness may result from a decrease in Ca^{2+} sensitivity and/or reduced maximal force-generating capacity of the myofilaments (Duncker et al., 1998). The results of the present study can provide some insight into the nature of contractile depression in post-ischemic myocytes. In the intact myocardium, myofilament sensitivity is determined by examining force-Ca^{2+} relationships. Although force was not measured in the present study, a ratio of Δ cell length / Δ $[Ca^{2+}]_i$ during the action potential can be used as a relative estimate of myofilament responsiveness. The mean of this ratio is plotted versus time in Figure 46. During simulated ischemia, isolated myocytes exhibited reduced contractions but the magnitude of Ca^{2+} transients was maintained (see Figure 20). Therefore, Δ cell length / Δ $[Ca^{2+}]_i$ was markedly reduced. This finding is in agreement with previous reports that

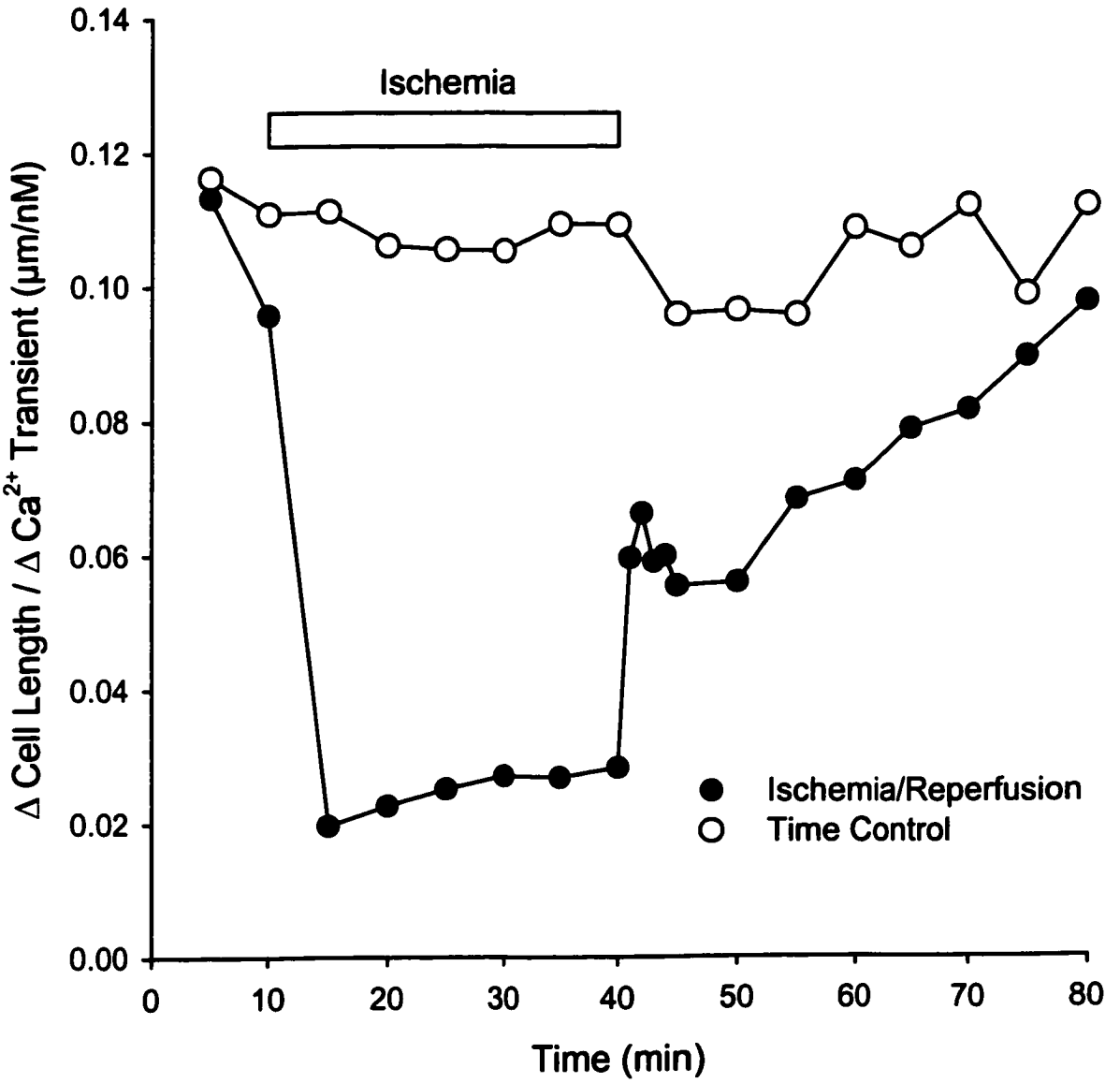

Ischemia
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δ Cell Length / Δ Ca2+ Transient (µm/nM)
0
10
20
30
40
50
60
70
80
Time (min)
Ischemia/Reperfusion
Time Control

Figure 46

Figure 46. Evaluation of Myofilament Responsiveness During Ischemia / Reperfusion.

To estimate changes in myofilament responsiveness, a ratio of Δ cell length / Δ $[Ca^{2+}]_i$ was determined at all timepoints during the ischemia / reperfusion protocol. This ratio was calculated using mean changes in cell length and $[Ca^{2+}]_i$ during the action potential ((diastolic length - systolic length) / (systolic $[Ca^{2+}]_i$ - diastolic $[Ca^{2+}]_i$)). Values for Δ cell length / Δ $[Ca^{2+}]_i$ were markedly decreased during ischemia. Upon reperfusion, the ratio rapidly increased but remained below the level of time controls. Following this initial rebound, calculated values of Δ cell length / Δ $[Ca^{2+}]_i$ were reduced during the next 10 min of reperfusion and then recovered steadily toward time values during the remainder of reperfusion. These results appear to confirm the finding that stunning is associated with reduction in myofilament responsiveness.

have suggested that myofilament responsiveness is reduced during ischemia as a result of intracellular acidosis and inorganic phosphate accumulation (Lee and Allen, 1991).

Changes in Δ cell length / Δ $[Ca^{2+}]_i$ calculated in myocytes during early reperfusion appear to roughly correspond to measurements of myofilament responsiveness in the reperfused myocardium. In the intact heart, myofilament Ca^{2+} sensitivity has been reported to rapidly but briefly recover in early reperfusion, following a rapid decrease in $[H^+]_i$ and P_i (Mosca et al., 1996; Hendrikx et al., 1994; Harada et al., 1994). Similarly, isolated myocytes exhibited a rapid but incomplete recovery of Δ cell length / Δ $[Ca^{2+}]_i$, as both Ca^{2+} transients and contractions increased in magnitude upon reperfusion. Following this initial rebound, Δ cell length / Δ $[Ca^{2+}]_i$ decreased slightly during the next 10 min of reperfusion as Ca^{2+} transients returned to the level of time controls while contractions became markedly depressed (see Figure 20). With further reperfusion, the Δ cell length / Δ $[Ca^{2+}]_i$ relationship gradually recovered toward the values of time controls. These observations appear to support the hypothesis that stunning involves a decrease in myofilament responsiveness to Ca^{2+} (Kusuoka et al., 1987; Kusuoka et al., 1990; Carrozza et al., 1992; Hofmann et al., 1993; Gao et al., 1995).

A shortcoming of previous studies of the stunned myocardium is that myofilament responsiveness has generally been assessed at only a selected point in reperfusion (Kusuoka et al., 1990; Kusuoka et al., 1987; Hofmann et al., 1993; Gao et al., 1995; Carrozza et al., 1992). Therefore, the present study provides new insight into the time course and importance of post-ischemic alterations in myofilament responsiveness.

The small downward dip in the Δ cell length / Δ $[Ca^{2+}]_i$ relationship observed at approximately 5-10 min of reperfusion corresponded closely with a similar depression in the contraction-time relationship (Figure 20) when contractions were maximally reduced. This observation suggests that, at this timepoint in the protocol, reduced myofilament responsiveness to Ca^{2+} may be an important contributor to contractile depression. However, in later reperfusion, changes in Δ cell length / Δ $[Ca^{2+}]_i$ and contraction amplitudes were not parallel. While values of Δ cell length / Δ $[Ca^{2+}]_i$ recovered steadily towards control levels, cell shortening appeared to reach a new steady state after 20 min of reperfusion during which little additional recovery was observed. This finding raises the possibility that mechanisms other than reduced myofilament responsiveness may partly determine the timecourse of recovery from stunning. However, since Ca^{2+} transients appear to be normal in late reperfusion, it is unclear what these mechanisms could be.

The time course of post-ischemic changes in the Δ cell length / Δ $[Ca^{2+}]_i$ relationship also raises questions concerning the mechanisms which are responsible for restoration of myofilament responsiveness. It has previously been hypothesized that resynthesis of damaged contractile proteins, such as troponin I, could determine the rate of recovery from stunning (Bolli, 1990; Gao et al., 1997). However, in the present study, the recovery of Δ cell length / Δ $[Ca^{2+}]_i$ occurs far more quickly than would be expected if resynthesis of damaged proteins is essential. Instead, these results suggest that perhaps contractile proteins are simply inactivated in early reperfusion and become reactivated, not resynthesized, as reperfusion progresses. Support for this hypothesis comes from the observation that degradation of contractile proteins is not a prerequisite for stunning

(Thomas et al., 1999; Luss et al., 2000). Clearly, much additional work is needed to determine the mechanisms responsible for the decrease in myofilament responsiveness during stunning.

Although reductions in the Δ cell length / Δ $[Ca^{2+}]_i$ relationship strongly suggest that myofilament responsivess is decreased in post-ischemic myocytes, it is unclear whether stunning resulted from a decrease in Ca^{2+} sensitivity and/or maximum force-generating capacity of the myofilaments. This issue also remains unresolved in the intact post-ischemic heart (for review see Bolli and Marban, 1999). To address this question, future studies in isolated myocytes could examine the complete cell length - $[Ca^{2+}]_i$ relationship by experimentally altering $[Ca^{2+}]_i$.

E. Changes in Cell Length During Reperfusion

The results of this study also provide some insight into the nature of diastolic dysfunction during reperfusion. In the intact heart, reperfusion triggers an increase in diastolic $[Ca^{2+}]_i$ (Harada et al., 1994; Varadarajan et al., 2001). Reperfusion of the ischemic heart also causes protracted myocardial contracture and a resulting increase in diastolic pressure (Schaff et al., 1981). In isolated myocytes, diastolic length was also rapidly decreased upon reperfusion and did not recover to the level of time controls. Interestingly, diastolic $[Ca^{2+}]_i$ levels recovered rapidly from ischemic elevations upon reperfusion. This observation appears to contradict the suggestion that diastolic elevation of $[Ca^{2+}]_i$ might trigger post-ischemic contracture (Gao et al., 1995). Instead, this finding suggests that other mechanisms may be responsible for this phenomenon. A recent study by Eberli et al (2000) has reported that reductions in [ATP] / [ADP] can prevent normal

myofilament relaxation by causing actin-myosin crossbridges to lock in the rigor state. Since ATP levels are reduced in reperfusion (Marban et al., 1994), it is conceivable that this mechanism is responsible for impaired relaxation in post-ischemic myocytes. Support for this view comes from the observation that interventions which improve recovery of ATP levels attenuate post-ischemic elevations in diastolic pressure (Cargnoni et al., 1999). Therefore, the marked decrease in post-ischemic diastolic cell length observed in the present study may result from reductions in [ATP] / [ADP].

F. Summary and Significance

The results of this study provide a number of insights into the nature of EC coupling in stunning. In summary, it was observed that SR Ca^{2+} stores, diastolic $[Ca^{2+}]_i$, and Ca^{2+} transients are not altered from time control values in late reperfusion, suggesting that SR function and overall Ca^{2+} cycling may be normal in stunning. However, $I_{Ca\text{-}L}$ magnitude appears to be reduced in stunning, although CICR and VSRM components of contraction are similarly depressed in late reperfusion. One explanation for this observation is that the gain of CICR is increased during stunning to compensate for reductions in $I_{Ca\text{-}L}$ and maintain a normal Ca^{2+} transient. Alternatively, reductions in $I_{Ca\text{-}L}$ could be counteracted by increased Ca^{2+} entry by reverse-mode Na^{+}-Ca^{2+} exchange.

The observation that Ca^{2+} transients are normal in post-ischemic myocytes suggests that reductions in myofilament responsiveness may be important in the cellular model of stunning. Examination of the Δ cell length / Δ $[Ca^{2+}]_i$ relationship in reperfusion supports this hypothesis, and suggests that myofilament responsiveness recovers steadily in late reperfusion.

If confirmed in future investigations, the results of this study could have significant therapeutic implications. This is the first study to examine $I_{Ca\text{-}L}$ in stunning. The results suggest that therapeutic interventions that prevent reduction of $I_{Ca\text{-}L}$ magnitude during ischemia and reperfusion could protect against stunning by increasing the magnitude of Ca^{2+} transients. Such strategies would be expected to very effective if the gain of CICR is, in fact, increased during reperfusion.

A number of studies have examined the benefits of $I_{Ca\text{-}L}$ blockade in the prevention of reperfusion injury. Some of these studies have observed that Ca^{2+} channel blockers applied just prior to reperfusion reduce post-ischemic Ca^{2+} accumulation and myocardial damage (Przyklenk and Kloner, 1988; Smart et al., 1997). The present results suggest that, since $I_{Ca\text{-}L}$ is reduced during ischemia and reperfusion, the usefulness of $I_{Ca\text{-}L}$ antagonists in early reperfusion is limited. Instead, therapies designed to reduce post-ischemic Ca^{2+} entry by the $NaCa_{ex}$ may be of greater benefit.

This is also the first study to examine both SR function and Ca^{2+} transients in intact post-ischemic myocytes. The observation that the SR appears to function normally during stunning is in agreement with the findings of intact heart. Also of interest is the observation that myofilament responsiveness recovered more quickly than expected in late reperfusion. If confirmed, this finding could require a re-evaluation of current hypotheses regarding the mechanisms responsible for stunning.

G. Possible Limitations of the Study

There are several limitations of a cellular model of stunning. One obvious criticism is that cells are free of membrane attachments in a myocyte model, and thus,

exhibit unloaded cell shortening. Therefore, it might be expected that contractile function would be different in *in situ* and isolated myocytes. Although contraction amplitudes appear to be similarly altered in the two models, it is possible that other characteristics of myocyte contraction, such as velocity of shortening or relaxation, could be differently affected. In future studies, measurement of these parameters might reveal such limitations of the cellular model.

In the cellular model of stunning, myocytes are perfused with saline buffers to simulate ischemia and reperfusion. In the intact animal, blood components such as neutrophils and platelets are believed to play an important role in the development of stunning (Kraemer et al., 1989; Westlin and Mullane, 1989; Williams, 1996). Neutrophil and platelet adhesion are enhanced in reperfusion leading to inflammatory injury (Inauen et al., 1990). As well, neutrophils release oxygen radicals and proteases causing further cell injury (Hansen, 1995). Since isolated myocytes are not exposed to the effects of neutrophils and platelets, reperfusion injury produced in the cellular model is somewhat different from that observed in the intact animal. Therefore, it is conceivable that alterations in EC coupling observed in this model do not resemble those of the post-ischemic myocardium. The validity of the cellular model is supported, however, by the observation that the simulated ischemia / reperfusion protocol produces changes in contraction magnitude, APD, RMP, diastolic $[Ca^{2+}]_i$, and Ca^{2+} transients which resemble those observed in intact hearts.

Despite the possible limitations of a cellular model of stunning, there are also many advantages to such a model. Intact post-ischemic hearts frequently exhibit vascular abnormalities that can contribute to contractile dysfunction (Gao et al., 1995). An

isolated myocyte model of stunning, however, enables contractile dysfunction to be assessed in the absence of such vascular effects. The well-controlled environment of a cellular model could also allow an examination of the exact relationship between specific ischemic alterations and the pathogenesis of stunning. In addition, an isolated myocyte model could be useful in investigating alterations in EC coupling which may contribute to stunning. Unlike in intact heart models of stunning, voltage-clamp techniques can be used in the cellular model to investigate transmembrane currents. SR function can also be assessed in the intact post-ischemic myocyte using this model. Therefore, a cellular model of stunning used in combination with existing models could prove to be very useful in determining the mechanisms that underlie this condition.

H. Summary and Conclusions

In Part I of this thesis, a cellular model of stunning was developed in which myocytes exposed to simulated ischemia and reperfusion exhibited contractile depression associated with normal electrical activity. This model was used to examine the hypothesis that Ca^{2+} homeostasis is altered in stunning. The hypothesis was not disproven. Stunned myocytes exhibited normal diastolic $[Ca^{2+}]_i$, Ca^{2+} transients, and SR Ca^{2+} stores but reduced $I_{Ca\text{-}L}$. Since contractions elicited by the VSRM and CICR were equally depressed in late reperfusion, the gain of CICR may be increased in stunning. Alternatively, increased Ca^{2+} influx by the $NaCa_{ex}$ may compensate for reductions in $I_{Ca\text{-}L}$. The results also support the view that contractile depression in stunning results, at least in part, from reduced responsiveness of the myofilaments to Ca^{2+}.

DISCUSSION - PART II

3. Losartan Protects Against Stunning by a Direct Action on Cardiomyocytes

The first objective of Part II of this thesis was to determine whether losartan protects against stunning in isolated myocytes. In this investigation, the effects of losartan were examined in the cellular model of stunning described above. The results showed that treatment with 10 μM losartan during ischemia prevented significant post-ischemic contractile depression (stunning).

Several studies have previously reported that losartan protects against stunning in the intact myocardium (Paz et al., 1998; Yang et al., 1998; Wang and Sjoquist, 1999; Zhu et al., 1999). Paz et al. (1998) observed that, following losartan treatment, this improvement in contractile function was associated with increased coronary perfusion. However, Wang and Sjoquist (1999) observed no such correlation. Therefore, based on these studies, it is unclear whether the protective actions of losartan on contraction are mediated by effects on the vasculature. In a cellular model, however, contractile function is assessed in the absence of vascular effects. Thus, the results of the present study suggest that losartan attenuates stunning, at least in part, by direct actions on cardiomyocytes.

4. Insights Into the Mechanisms Underlying the Protective Actions of Losartan

As discussed in the introduction, a multitude of interventions and drugs have been reported to protect against stunning by a variety of mechanisms (for review see Maxwell and Lip, 1997). However, it is not clear how losartan attenuates stunning by actions on

on myocytes. Therefore, the objective of the remaining experiments in this thesis was to identify mechanisms by which losartan might attenuate stunning.

Measurement of action potentials indicated that losartan treatment did not alter RMP or APD during ischemia and reperfusion. As well, losartan protected against stunning even when the membrane voltage was controlled using voltage-clamp protocols. This action was mediated without an effect on the voltage-dependence of contraction. These observations suggest that losartan attenuates stunning by a mechanism that does not involve alterations in action potential configuration. Instead, losartan may alter EC coupling in post-ischemic myocytes.

A. Effects of Losartan on I_{TI}

The first clues in identifying the mechanisms by which losartan alters post-ischemic EC coupling were provided by the observation that losartan treatment decreased incidence of I_{TI} in early reperfusion. Since I_{TI} is believed to cause triggered arrhythmias through the generation of oscillatory afterpotentials (Ferrier, 1977), these results suggest that losartan may suppress triggered arrhythmias in reperfusion. Losartan has also been previously reported to protect against re-entrant arrhythmias during ischemia and reperfusion (Thomas et al., 1996). These actions might explain anti-arrhythmic effects of losartan reported in studies in animal models of ischemia and reperfusion (Lee et al., 1997; Zhu et al., 2000) and in humans (Pitt et al., 1997).

Conditions of Ca^{2+} overload, such as those that occur in early reperfusion, can trigger I_{TI} in response to spontaneous SR Ca^{2+} release (Fabiato and Fabiato, 1972; Lederer and Tsien, 1976; Ferrier, 1977; Lappe and Lakatta, 1980). Ca^{2+} overload is also

believed to be an important trigger of re-entrant arrhythmias (ter Keurs et al., 2001) and stunning (Dunker et al., 1998). Therefore, it was hypothesized that losartan may attenuate stunning by inhibiting Ca^{2+} overload during ischemia and reperfusion.

B. Effects of Losartan on Ca^{2+} Homeostasis

Measurements of $[Ca^{2+}]_i$ in field-stimulated myocytes showed that, in control cells, diastolic $[Ca^{2+}]_i$ was significantly elevated during ischemia relative to time controls. Losartan treatment inhibited this increase. As well, losartan treatment during ischemia prevented overshoot of Ca^{2+} transients in early reperfusion. Interestingly, losartan also increased the magnitude of transients in late reperfusion relative to time controls. It seems, then, that the protective actions of losartan on $[Ca^{2+}]_i$ may be complex.

Losartan could theoretically reduce Ca^{2+} overload during ischemia and early reperfusion by a number of possible mechanisms. For example, interventions that attenuate acidosis, Na^+ loading, or Ca^{2+} influx all may protect against elevations in $[Ca^{2+}]_i$ (Kitakaze et al., 1988; Przyklenk et al., 1988; Tani and Neely, 1989; Tani and Neely, 1990). However, few of these possible mechanisms could also explain the increase in Ca^{2+} transients that was observed in late reperfusion following losartan treatment. Presumably, only an increase in either Ca^{2+} influx or SR release could account for this effect. Both of these possibilities were examined. When $I_{Ca\text{-}L}$ was recorded during the experiment, no difference in magnitude was observed between control and losartan-treated cells. However, assessment of SR Ca^{2+} stores by caffeine application showed that, following losartan treatment, there was a strong trend toward increased SR stores. Although this effect was not statistically significant, it is important to note that SR stores

were largest at the end of reperfusion (Fig. 39A) when Ca^{2+} transients were significantly elevated (Fig. 36B). Therefore, it is possible that losartan treatment during ischemia increases Ca^{2+} transients in late reperfusion by increasing SR Ca^{2+} stores and release.

The protective actions of losartan during ischemia and early reperfusion could also be related to increased SR Ca^{2+} content. Increased uptake of Ca^{2+} into the SR during losartan treatment could attenuate elevation of diastolic $[Ca^{2+}]_i$ during ischemia. However, it is more difficult to explain how this mechanism could account for the actions of losartan in early reperfusion. An increase in SR stores might be expected to exacerbate the post-ischemic overshoot of Ca^{2+} transients, however, no overshoot was observed in drug-treated cells. One explanation for this apparent contradiction is that Ca^{2+} entry, not SR release, has been suggested to be the predominant contributor to the post-ischemic rise in $[Ca^{2+}]_i$ (Tani and Neely, 1989; Goldhaber, 1996; Ladilov et al., 1999). Therefore, increased SR stores and Ca^{2+} release may only minimally alter the magnitudes of Ca^{2+} transients in early reperfusion. Instead, post-ischemic Ca^{2+} entry may have been reduced in losartan-treated cells. By attenuating elevations in $[Ca^{2+}]_i$ during ischemia, it is likely that losartan treatment also attenuated oxidative stress. Interestingly, oxidative stress has been reported to increase activity of the $NaCa_{ex}$ in early reperfusion (Goldhaber and Liu, 1994; Goldhaber, 1996). Therefore, losartan treatment may have indirectly decreased oxidative stress and Na^+-Ca^{2+} exchange that prevented post-ischemic overshoot of Ca^{2+} transients.

C. Effects of Losartan on Myofilament Responsiveness

Prevention of Ca^{2+} overload during ischemia and early reperfusion is a well-documented strategy for attenuation of stunning (for review see Bolli and Marban, 1999). Ca^{2+} overload has been suggested to contribute to the pathogenesis of stunning by triggering Ca^{2+}-dependent proteolysis of the myofilaments, thus reducing myofilament responsiveness to Ca^{2+} (Gao et al., 1996). Therefore, one might have predicted that losartan treatment reduced stunning in myocytes by attenuating Ca^{2+} overload and resulting reductions in myofilament responsiveness. To examine this hypothesis, a ratio of Δ cell length / Δ $[Ca^{2+}]_i$ was calculated in control and losartan-treated cells as a relative estimate of myofilament responsiveness. Figure 47 shows that during ischemia, Δ cell length / Δ $[Ca^{2+}]_i$ was markedly reduced in both the presence and absence of losartan. Upon reperfusion, contractions increased in losartan-treated cells, but transients were unchanged in magnitude. Thus, Δ cell length / Δ $[Ca^{2+}]_i$ values rapidly recovered to the level of time controls. Following this initial recovery, however, ratio values quickly decreased and remained well below time control values for the remainder of reperfusion. In control cells, reperfusion had much different effects on values of Δ cell length / Δ $[Ca^{2+}]_i$. In early reperfusion, control cells exhibited only a partial recovery of Δ cell length / Δ $[Ca^{2+}]_i$, but ratio values gradually increased with continued reperfusion.

The above calculations of Δ cell length / Δ $[Ca^{2+}]_i$ suggest that, following losartan treatment, myofilament responsiveness recovered in early reperfusion but decreased in late reperfusion. Therefore, changes in myofilament responsiveness are not responsible for attenuation of contractile depression in losartan-treated cells after the first few

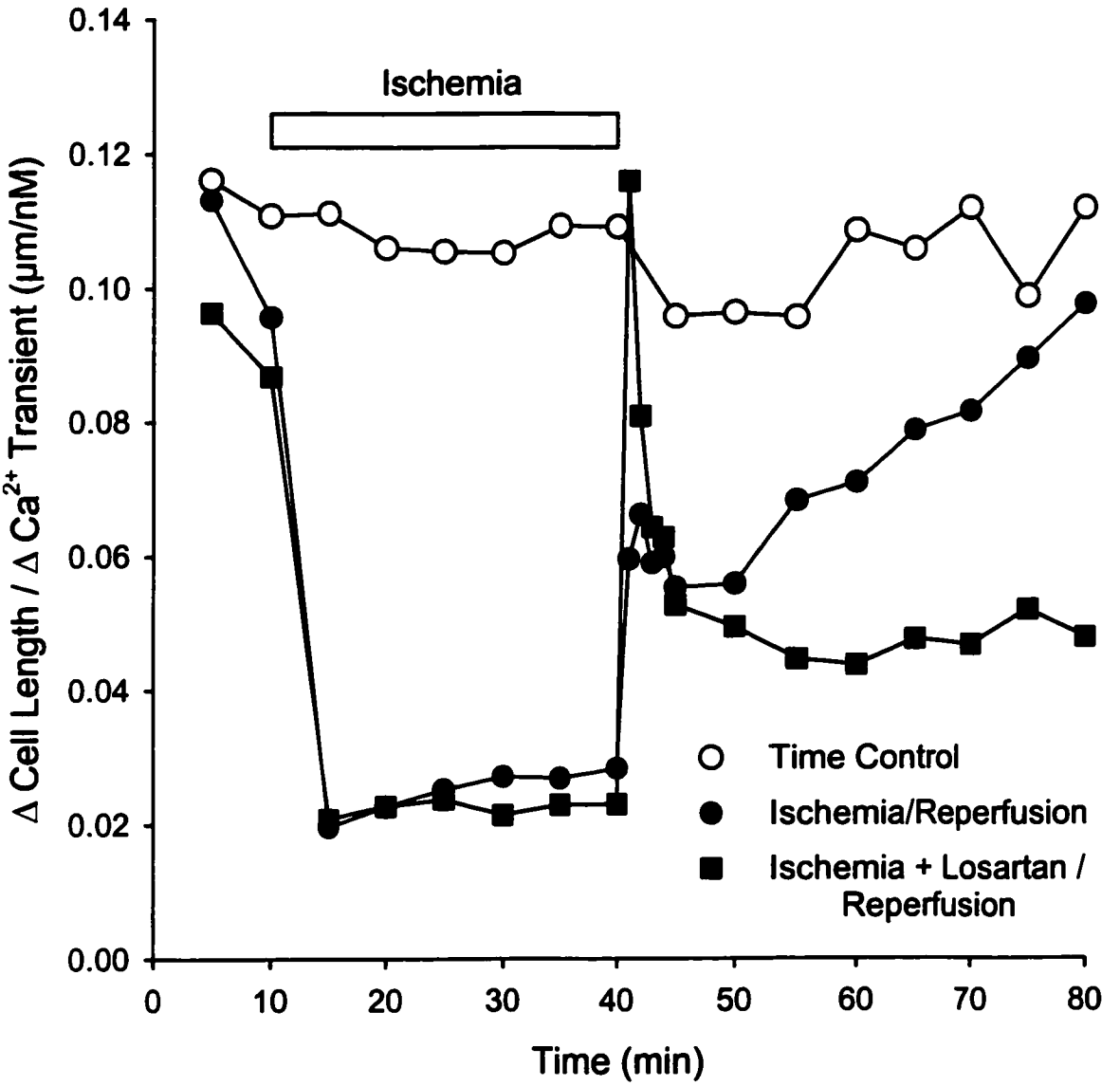

Ischemia
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δ Cell Length / Δ Ca2+ Transient (µm/nM)
0
10
20
30
40
50
60
70
80
Time (min)
Time Control
Ischemia/Reperfusion
Ischemia + Losartan / Reperfusion

Figure 47

Figure 47. Losartan Treatment did not Attenuate Depression of Myofilament Responsiveness in Late Reperfusion.

Changes in myofilament responsiveness to Ca^{2+} were estimated using a ratio of Δ cell length / Δ $[Ca^{2+}]_i$. During ischemia, control and losartan-treated cells exhibited reduced contractions but maintained Ca^{2+} transients. Therefore, values of Δ cell length / Δ $[Ca^{2+}]_i$ were similarly depressed in the two treatment groups. Control cells exhibited a partial recovery of Δ cell length / Δ $[Ca^{2+}]_i$ in early reperfusion. However, in losartan-treated cells ratio values were dramatically increased upon reperfusion to the level of time controls. With continued reperfusion, control cells exhibited a gradual recovery of Δ cell length / Δ $[Ca^{2+}]_i$. In losartan-treated cells, ratio values declined rapidly after the first few minutes of reperfusion and remained markedly depressed relative to time controls.

minutes of reperfusion. Instead, it appears that increases in the magnitude of Ca^{2+} transients may actually compensate for reductions in myofilament responsiveness to increase contraction magnitude. It is important to note, however, that these results do not necessarily contradict the theory that stunning can be triggered by Ca^{2+}-dependent proteolysis of the myofilaments. Losartan could have effects on myofilament responsiveness that are not related to its actions on Ca^{2+} homeostasis during ischemia and reperfusion. In this case, these actions could conceal the fact that losartan may inhibit myofilament proteolysis by reducing Ca^{2+} overload. To address this question, an appropriate experiment would examine the effects of losartan on myofilament responsiveness under normoxic conditions.

D. Some Actions of Losartan May be Independent of AII Blockade

Effects of losartan on SR Ca^{2+} stores could theoretically result from AT_1 receptor blockade. Binding of AII to AT_1 receptors activates PLC which releases diacyl glycerol and inositol triphosphate (IP_3) (Griendling et al., 1987). IP_3, in turn, releases Ca^{2+} from the SR in cardiomyocytes (Fukuta et al., 1998). Therefore, in the present study, AII could be produced endogenously by the local cardiac RAS, and could act to reduce SR Ca^{2+} stores during the experiment. Losartan treatment could theoretically attenuate this decrease in SR Ca^{2+} content by blocking the actions of endogenous AII.

Blockade of AT_1 receptors might be expected to have protective actions during ischemia and reperfusion based on studies which have suggested a role of the RAS in reperfusion injury. Application of exogenous AII before or during ischemia has been reported to provoke myocardial stunning (Linz and Scholkens, 1987; Scholkens and Linz,

1988) and arrhythmogenesis (Linz et al., 1986; Scholkens and Linz, 1988). Although it is unclear whether these actions of AII are mediated by IP_3-triggered Ca^{2+} release, it could be predicted that losartan might attenuate stunning and arrhythmias by blocking the actions of AII.

However, a recent study by Thomas et al. (1996) showed that losartan may have an intrinsic anti-arrhythmic action that is independent of AT_1 receptor blockade. To examine whether the effects of losartan in the present study were also independent of AII blockade, two additional groups of myocytes were exposed to exogenous AII, and losartan plus AII during ischemia. If the actions of losartan resulted from AT_1 receptor blockade, it might have been expected that application of exogenous AII would promote I_{TI} and worsen contractile recovery in reperfusion. However, treatment with AII did not alter the occurrence of I_{TI} and did not alter the voltage dependence or amplitude of post-ischemic contractions. This might be explained if endogenous AII activated AT_1 receptors maximally, and therefore, no further effect could occur with addition of exogenous agonist. However, treatment with exogenous AII was observed to reduce $I_{Ca\text{-}L}$ in reperfusion, and this action appeared to be blocked in the losartan plus AII group. Thus, endogenous AII likely was not exerting maximal effects. These observations suggest that the protective actions of losartan did not result from AII blockade. Further evidence for this hypothesis is that cells treated with AII and losartan together showed the same protection of post-ischemic contractility as cells treated only with losartan. Since losartan is a competitive antagonist, effects mediated by receptor blockade should be partially reversed by increased competition of agonist for binding sites. Thus, these

observations provide further evidence that losartan has protective actions which are independent of AII blockade.

E. Site of Action of Losartan

If the actions of losartan do not result from blockade of the actions of AII, then the search for the site of action must continue. Further insight into the mechanism of action of losartan was provided by $[Ca^{2+}]_i$ measurements in ischemia / reperfusion experiments. As described above, losartan treatment was observed to attenuate changes in $[Ca^{2+}]_i$ resulting from ischemia and early reperfusion. In late reperfusion, Ca^{2+} transients and caffeine transients were increased in losartan-treated cells, but were not altered following control ischemia or in time controls. This observation suggested that the actions of losartan observed in late reperfusion might also be observed under normoxic conditions. To test this hypothesis, losartan was administered in the absence of ischemia and reperfusion. As in ischemia / reperfusion experiments, losartan treatment in normoxia steadily increased SR Ca^{2+} content during treatment and washout. Similarly, Ca^{2+} transients also began to increase in magnitude during the treatment period, and were further increased during washout. However, both Ca^{2+} content and Ca^{2+} transients were increased to a greater degree when losartan was applied during normoxia than during ischemia. As was suggested in the case of ischemia / reperfusion experiments, the increase in SR Ca^{2+} stores following losartan treatment in normoxia may have been responsible for the observed increase in Ca^{2+} transients.

These observations suggest that losartan increases SR Ca^{2+} content by a mechanism that is partially inhibited during ischemia and reperfusion. One candidate site

of action is the SR Ca^{2+} ATPase. If losartan increased activity of the SR Ca^{2+} ATPase during normoxia, it might be expected that SR content and Ca^{2+} transients would be markedly elevated. During ischemia and reperfusion, these actions might be less pronounced because activity of the Ca^{2+} ATPase is decreased by reductions in [ATP] / [ADP] (Griese et al., 1988). Interestingly, losartan was not observed to alter diastolic $[Ca^{2+}]_i$ during ischemia / reperfusion or normoxia. Therefore, if losartan acts to increase SR Ca^{2+} uptake, various proteins involved with Ca^{2+} homeostasis may act to compensate for alterations in diastolic $[Ca^{2+}]_i$. Indeed, Eisner et al. (2000) have recently suggested that alterations in SR Ca^{2+} stores alter both Ca^{2+} transients and Ca^{2+} fluxes across the sarcolemma.

Other possible sites of action of losartan seem less feasible. Reducing SR Ca^{2+} release, for example, would be expected to increase SR stores but would likely decrease the magnitude of Ca^{2+} transients. Increasing Ca^{2+} influx could also conceivably lead to an increase in Ca^{2+} stores. However, such an action would be expected to cause an increase in diastolic $[Ca^{2+}]_i$ during ischemia when the SR Ca^{2+} ATPase is inhibited (Griese et al., 1988). Therefore, the SR Ca^{2+} ATPase seems to be the most likely site of action that could explain the actions of losartan observed in this thesis.

Very few studies in the literature have reported protective actions of losartan on Ca^{2+} homeostasis, and fewer still have observed actions that were independent of AT_1 receptor antagonism (Jaiswal et al., 1991; Bertolino et al., 1994; Chansel et al., 1994). However, the findings of a study by Jaiswal et al (1991) are very relevent to the present discussion on the mechanism of action of losartan. They showed that losartan stimulates release of prostacyclin in vascular smooth muscle and neuronal cells by an action

independent of AII receptor blockade. Interestingly, prostacyclin has been shown to attenuate myocardial stunning (Hohlfeld et al., 1991) and to have anti-arrhythmic effects during ischemia and reperfusion (Fiedler and Mardin, 1986). As well, prostacylin has also been observed to stimulate SR Ca^{2+} uptake by activating the SR Ca^{2+} ATPase (Doni et al., 1994; Karczewski et al., 1998). Karczewski et al. (1998) reported that prostacylcin mediated this action by increasing phosphorylation of phospholamban, the regulator of the SR Ca^{2+} pump. These findings could explain the effects of losartan observed in the present study. In future experiments, it would be interesting to test the hypothesis that losartan increases SR Ca^{2+} stores in cardiomyocytes by stimulating prostacyclin release, and thus increasing activity of the SR Ca^{2+} ATPase.

In other future experiments, it would be useful to examine the effects of the active P450-derived metabolite of losartan, EXP-3174 (Yun et al., 1995). In the present study, losartan was examined in a flow-through system and, therefore, most of the effects of losartan are likely attributable to losartan itself. However, it is possible that some losartan was metabolized to EXP-3174 since P450 enzymes may be present in cardiomyocytes (Xiao et al., 1998). Since EXP-3174 is a non-competitive AT_1 receptor antagonist, it is important to rule out any contribution of the metabolite in experiments in which losartan and AII were in competition for the AT_1 receptor. Future experiments could accomplish this task by comparing the actions on losartan and EXP-3174. Experiments with other structurally unrelated AT_1 antagonists would also be useful in determining which cardiovascular actions of losartan are dependent on AII blockade. It would be particularly beneficial to examine the dependence of losartan-induced changes

in $[Ca^{2+}]_i$ on AT_1 receptor antagonism as this relationship was not examined in the present study.

A final goal of future experiments would be to determine the mechanism responsible for the time course of action of losartan. In the present study, losartan was observed to exert effects in reperfusion and washout up to 40 min after treatment. In fact, the actions of losartan on Ca^{2+} transients and caffeine transients were much larger after losartan was removed from the superfusate. One explanation for this observation is that losartan simply requires time to exert its effects. In this case, the actions of losartan may result from irreversible binding at its site of action, so that drug effects are not reversed upon washout. Alternatively, it is conceivable that removal of losartan from the superfusate is a key component of the actions of the drug. This possibility seems unlikely, but could be examined by comparing the actions of losartan during 30 min treatment / 40 min washout with those in cells treated with the drug for 70 min.

F. Summary and Conclusions

In Part II of this thesis, the effects of losartan were examined in a cellular model of stunning. The hypothesis was not disproven. Losartan treatment during ischemia protected against stunning by an action that appeared to be independent of AT_1 receptor blockade. This protective action likely involved attenuation of Ca^{2+} overload during ischemia and early reperfusion and an increase in Ca^{2+} transients during late reperfusion. This action did not result in improvement of myofilament responsiveness in late reperfusion. Losartan also attenuated incidence of I_{TI} during early reperfusion but did not alter magnitude of $I_{Ca\text{-}L}$ during the experiment. The actions of losartan on $[Ca^{2+}]_i$ may

have resulted from increased SR Ca^{2+} sequestration during ischemia and reperfusion, an action that was more pronounced following drug treatment in normoxia. It is hypothesized that the precise mechanism by which losartan increases SR Ca^{2+} stores may involve elevated activity of the SR Ca^{2+} ATPase.

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