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CHANGES IN ELECTRICAL ACTIVITY AND CONTRACTILE FUNCTION DURING SIMULATED ISCHEMIA AND REPERFUSION IN GUINEA PIG VENTRICULAR MYOCYTES.

by

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Submitted in partial fulfillment of the requirements for the degree

of Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

June, 1995

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THIS THESIS IS DEDICATED TO MY PARENTS, RENEE AND STAN, WHO ALWAYS TAUGHT ME TO "JUST DO YOUR BEST".

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ABSTRACT

The effect of pharmacological agents on changes in electrical and contractile activity were examined in a novel cellular model of simulated ischemia and reperfusion in guinea pig ventricular myocytes. Electrical activity was recorded with both conventional and voltage clamp techniques using intracellular microelectrodes filled with 2.7 M KCl. Contractions were monitored with a video edge detector. In the absence of drug, exposure of myocytes to simulated ischemia caused depolarization of the membrane potential, abbreviation of the action potential duration (APD) and inhibition of contraction. Under voltage clamp, the calcium current (I_{Ca}) declined gradually in ischemia. Signs of Ca^{++} overload, including oscillatory afterpotentials (OAP), transient inward current (I_{Ti}) and aftercontractions occurred in 73% of myocytes in reperfusion. Upon reperfusion, APD and I_{Ca} recovered slowly; however, contractions recovered quickly and temporarily exceeded control. Amiloride, a Na^+/H^+ exchange inhibitor, during ischemia and reperfusion lowered incidence of I_{Ti} in early reperfusion. Nifedipine, a Ca^{++} channel blocker, and lidocaine, a Na^+ channel blocker, had no effect on I_{Ti}. Induction of OAP, I_{Ti} and aftercontraction in reperfusion was associated with reduced peak I_{Ca}. Further analysis of contractions demonstrated that the L-current associated contraction, the Na^+-Ca^{++} exchange contraction and the contraction initiated by the novel release mechanism were inhibited by simulated ischemia. Reperfusion in the absence of drug caused a transient overshoot in the magnitude of all three contractions. The transient overshoot of the three different contractions could be selectively inhibited by different pharmacological interventions. The effects of the nucleoside adenosine (ADN) on cardiac cellular electrical and contractile activity were determined during ischemia and reperfusion. ADN had no effect under control conditions. When ADN was present during ischemia, abbreviation of APD was greater and recovery was delayed. In ischemia, I_{Ca} declined equally, and contractions were abolished in control and ADN-treated myocytes. ADN abolished contractile overshoot and reduced incidence of I_{Ti} and aftercontractions to 37.5%. The effects of exogenous ADN were inhibited by both the non-selective ADN antagonist 8-phenyltheophylline and the A_1-selective antagonist cyclopentyltheophylline. Exogenous ADN in ischemia may protect the myocardium in reperfusion via A_1 receptors.

ABBREVIATIONS USED

ADN	adenosine
APD	action potential duration
APD_{90}	action potential duration at 90% repolarization
A_1 receptors	adenosine receptor subtype 1
A_2 receptors	adenosine receptor subtype 2
A_3 receptors	adenosine receptor subtype 3
ATP	adenosine trisphosphate
Ca^{++}	calcium
CICR	calcium-induced calcium release
CPT	8-cyclopentyltheophylline
8-PT	8-phenyltheophylline
H^+	protons
I_{Ca}	calcium current
$I_{K,ATP}$	ATP-sensitive potassium channels
I_{Na}	sodium current
I_{TI}	transient inward current
I-V	current-voltage
K^+	potassium
$K^+_{Ach,ADN}$	ADN/acetylcholine sensitive K^+ channels
KB	Kraftbruhe
kHz	kilohertz

MDP	maximum diastolic potential
min	minutes
mM	millimolar
mm Hg	millimeters of mercury
Mn^{++}	manganese
MΩ	megaohms
msec	milliseconds
mV	millivolts
nA	nanoamps
Na^{+}	sodium
OAP	oscillatory afterpotentials
Po_2	partial pressure of oxygen
SR	sarcoplasmic reticulum
µM	micromolar
µm	micrometer

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PUBLICATIONS

Portions of this thesis have previously been published.

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2. **Cordeiro JM**, Howlett SE, Ferrier GR (1994) Simulated ischemia and reperfusion in isolated guinea pig ventricular myocytes. Cardiovasc Res **28:** 1794-1802.

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* denotes peer-reviewed abstract

INTRODUCTION

The occurrence of sudden cardiac death in humans has been observed since the earliest of times. Hippocrates noted that sudden death was often accompanied by chest pains. He observed that "sharp pains irradiating soon towards the clavicle and towards the back, are fatal". Hippocrates also observed that "those who are constitutionally very fat are more apt to die quickly than those who are thin". From these early times, sudden death had been observed in a number of individuals; however, the link between sudden death and coronary artery occlusion was not fully established. One of the first clinical reports that linked sudden death to a myocardial infarction was made by Amatus Lusitanus in 1560. He attributed the death of an individual to an obstruction in the heart. Amatus Lusitanus wrote as follows: "A reverend abbot from the Isle of Croma, when he was in good health and talking to several persons, said that he suddenly felt pain in his heart and with his hand moved rapidly toward the region of the heart, he fell, though slowly, to the earth and rapidly lost all his animal faculties. Not only was the pulse at the metacarpium and the temples missing, but even no motion upon the heart could be perceived."

Sudden cardiac death has been defined by the International Society of Cardiology as death occurring within one hour of the onset of symptoms or of last being seen alive. There is little dispute that sudden cardiac death from coronary artery disease is a major cause of death in the western world, as well as representing a smaller proportion of deaths in underdeveloped countries.

Post-mortem examination of sudden cardiac death patients has revealed major obstructions in one of the coronary arteries supplying the heart. It is now well established that when the blood supply to the heart is interrupted, there is a profound decrease in contractile function within minutes of the interruption and contractions eventually cease (Allen and Orchard, 1987). Both the duration and the severity of the ischemic period are important in determining the survival of the myocardium and ultimately of the patient. Because of the deleterious effects of coronary artery occlusion, it is plausible that re-establishing blood flow would be beneficial. However, clinicians have observed that reperfusion of previously ischemic myocardium does not always produce desired results. Depending on the duration and severity of the ischemic period, reperfusion can produce a variety of effects. In some cases reperfusion results in full recovery of contractile function whereas in other cases, reperfusion results in a depression of contractile activity; however, this depression is reversible. Reperfusion also may result in an acceleration in damage to the myocardium. In addition, during the early phase of reperfusion, arrhythmias often are observed. These tend to dissipate as reperfusion progresses.

The objective of this thesis was to develop a new cellular model of simulated ischemia and reperfusion in ventricular myocytes and to use this model to study changes in transmembrane electrical activity and cell shortening that occur during ischemia and reperfusion. This model can be used to examine the mechanism of action of drugs on membrane currents and contractions during

ischemia and reperfusion. The following review will initially discuss mechanisms of cardiac excitation-contraction coupling and then will focus on mechanisms of cardiac contraction during ischemic conditions and reperfusion.

1. EXCITATION-CONTRACTION COUPLING

One of the earliest experiments of cardiac excitation-contraction coupling was by Ringer (1883) who demonstrated that an isolated frog heart would not contract when the extracellular Ca^{++} was removed from the buffer. These experiments demonstrated that extracellular Ca^{++} is important for cardiac muscle contraction. It is believed that contraction in cardiac tissue is initiated by a rise in intracellular free Ca^{++}. This rise in intracellular free Ca^{++} can be derived from either Ca^{++} release from the sarcoplasmic reticulum (SR) or influx of Ca^{++} across the sarcolemma. In a series of classic experiments, Fabiato (1985a,b,c) demonstrated that Ca^{++} release from internal stores can be triggered by a sudden rise in Ca^{++} near the SR Ca^{++} release sites. This observation, whereby a small amount of Ca^{++} is capable of initiating a much larger release of Ca^{++}, has been referred to as "Ca^{++}-induced Ca^{++} release" (CICR). Subsequent experiments suggest that the Ca^{++} which triggers SR Ca^{++} release is due to influx via L-type voltage-sensitive Ca^{++} channels (Cannell et al., 1987; Nabauer et al., 1989). However, in the last few years there has been an increasing amount of evidence suggesting that CICR may be initiated by other mechanisms.

There is now considerable evidence suggesting Na^{+} influx via Na^{+}

channels may either directly or indirectly trigger Ca^{++} release from the SR (Mullins, 1979; Lipp and Niggli, 1994). Leblanc and Hume (1990) demonstrated that a Ca^{++} transient may be initiated following activation of I_{Na} in guinea pig ventricular myocytes. This Ca^{++} transient could be abolished by the Na^+ channel inhibitor tetrodotoxin suggesting that I_{Na} may be involved. Leblanc and Hume (1990) speculated that activation of I_{Na} resulted in a momentary rise in intracellular Na^+. The transient elevation in Na^+ resulted in Ca^{++} entry via reverse mode Na^+-Ca^{++} exchange which triggered SR Ca^{++} release. Similar results were also obtained by Lipp and Niggli (1994) in guinea pig ventricular myocytes. They also demonstrated that I_{Na} could initiate a Ca^{++} transient and that this Ca^{++} transient could be suppressed by extracellular substitution of Li^+ for Na^+. Lipp and Niggli (1994) concluded that a momentary rise in Na^+ concentration resulting from I_{Na} leads to Ca^{++} influx via reverse mode Na^+-Ca^{++} exchange. This influx of Ca^{++} from the Na^+-Ca^{++} exchange triggers Ca^{++} release from the SR. The Na^+-Ca^{++} exchange working in reverse mode is also capable of causing Ca^{++} influx across the sarcolemma and initiating SR Ca^{++} release (Levi et al., 1993); this Ca^{++} influx can occur independently of Na^+ channel activation. Levi et al. (1993) provided evidence for a role in direct depolarization induced Ca^{++} influx via the Na^+-Ca^{++} exchange in rat ventricular myocytes. This influx of Ca^{++} is believed to either initiate Ca^{++} release from the SR or directly activate the myofilaments.

The classical physiological notion holds that Ca^{++} influx across the

sarcolemma via L-type Ca^{++} channels triggers a larger Ca^{++} release from the SR Ca^{++} release channels (Barcenas-Ruiz and Wier, 1987; Cannell et al., 1987; Nabauer et al., 1989). However, recently there is evidence that a voltage-sensitive component of contraction associated with SR Ca^{++} release exists in guinea pig ventricular myocytes which is different from those previously described (Ferrier and Howlett, 1995). Ferrier and Howlett (1995) demonstrated that this component of contraction cannot be blocked by L-type Ca^{++} channel blockers, operates in the absence of I_{Na}, and can be inhibited by low concentrations of the SR Ca^{++}-release channel inhibitor ryanodine. In addition, contractions generated by this release mechanism have an activation threshold more negative than the activation threshold for the L-type Ca^{++} current (Ferrier and Howlett, 1995). Furthermore, contractions initiated by this release mechanism were not proportional to the magnitude of inward current. The non-proportionality between the magnitude of contraction and magnitude of inward current may suggest that this voltage-dependent release mechanism may activate a portion of cardiac contraction which is attributed to SR release of Ca^{++} (Ferrier and Howlett, 1995). Thus, there is considerable evidence to suggest that classic mechanisms of cardiac excitation-contraction coupling may need to incorporate additional mechanisms. For a review on various mechanisms for triggering sarcoplasmic reticular calcium release in the heart, see Levi et al., 1993.

In summary, contraction in cardiac tissue is critically dependent on influx

of Ca^{++} from the extracellular fluid. This influx of Ca^{++} increases the local concentration of Ca^{++} in the vicinity of the Ca^{++} release channel which, in turn, triggers SR Ca^{++} release. However, there are many different routes of Ca^{++} influx across the sarcolemma which can elicit Ca^{++} release from the SR.

2. MYOCARDIAL ALTERATIONS DURING ISCHEMIA

When the myocardial blood flow is insufficient to meet the metabolic demands of the heart, the heart is said to be ischemic. Ischemia and hypoxia produce a profound reduction in the magnitude of contraction and alterations in Ca^{++} homeostasis. In this section of the thesis, I will discuss studies which examine some of the consequences of ischemic injury. The studies to be described were performed on both multicellular and single cell preparations. These experimental manipulations produce similar, but not identical results. The use of various experimental models to study aspects of ischemia and reperfusion will be discussed in detail later (page 46).

A. Contractile Failure during Ischemia.

When the blood supply to the heart is stopped, there is a rapid decrease in the magnitude of contraction and contractions eventually stop (for review see Allen and Orchard, 1987). This decrease in contractility often occurs within minutes of ischemia. Many techniques have been used to examine contractile function during ischemia and hypoxia. Studies utilizing models of hypoxia,

particularly those on single cells, tend to maintain adequate perfusion of the tissue but remove most or all the O_2. Since occlusion of all the atmospheric oxygen is technically difficult, most of these studies employ cyanide or dinitrophenol to inhibit oxidative phosphorylation (reviewed by Lee and Allen, 1991). On the other hand, ischemia involves a decreased supply of both oxygen and substrates as well as accumulation of metabolic products such as lactate and potassium. The differences in models of ischemia versus models of hypoxia will be discussed later (page 47).

i) Ca^{++} Transient and Contractile Failure in Ischemia

Probably the most interesting aspect of contractile failure is the observation that cytosolic Ca^{++} levels actually increase during ischemia. Lee et al. (1988) used the fluorescent Ca^{++} indicator indo-1 to examine changes in Ca^{++} transient, contraction and action potential in perfused rabbit heart exposed to acute global ischemia. Results of their study showed that in ischemia there was an increase in the Ca^{++} signal during systole and a decrease in the Ca^{++} signal during diastole. The increase in the cytoplasmic Ca^{++} signal during systole suggests that SR Ca^{++} release is unaffected. The decrease in the Ca^{++} signal in diastole shows that Ca^{++} was removed from the cytosol. There was an elevation in both systolic and diastolic levels of Ca^{++} during ischemia. However, the ventricular developed pressure was markedly reduced. Since Ca^{++} transients were unaffected during global ischemia this suggests that the impaired

contractile function does not result from impaired influx and subsequent release of Ca^{++} in ischemia. A similar rise in systolic and diastolic Ca^{++} transients was observed by Camacho et al. (1993) in whole rat hearts subjected to global ischemia. In this study, it did not appear that the release of Ca^{++} from the SR was affected. However, contractile failure in isolated myocytes exposed to ischemia and reperfusion is different from contractile failure in multicellular models. These cellular models appear to exhibit inhibition of release of Ca^{++} from the SR. Goldhaber et al. (1991) have shown that the magnitudes of the systolic and diastolic Ca^{++} transients detected by fura-2 are markedly reduced by metabolic inhibition in myocytes. These results suggest that in myocytes exposed to metabolic inhibition, there is impairment of Ca^{++} influx or release resulting in the abolishment of the Ca^{++} transient.

In summary, it appears that in multicellular preparations exposed to global ischemia, Ca^{++} influx across the sarcolemma and SR Ca^{++} release do not appear to be affected during ischemia. In addition, Ca^{++} removal processes are also unaffected. However, in single myocytes, there is a decrease or abolition in the Ca^{++} transient signal.

ii) Contractile Failure and ATP Levels in Ischemia

Its seems plausible that a reduction in ATP observed during ischemia would result in reduced energy supply necessary for contraction. Murry et al. (1985) showed that ATP levels in early ischemia (0-5 min) declined about 35%

in canine hearts made ischemic by occlusion of the circumflex artery. However, Koretsune et al. (1991) showed that cytosolic ATP levels do not decline significantly in ferret hearts made globally ischemic. Their study, which measured both intracellular ATP levels and left ventricular developed pressure, showed that when complete contractile failure had occurred, there was no change in ATP levels. This study is in contrast to studies performed on single cardiac myocytes which employ metabolic poisons such as cyanide and 2-deoxyglucose rather than conditions observed during ischemia. Both anoxia and metabolic poisons cause a rapid fall in intracellular ATP and activation of ATP-sensitive K+ channels ($I_{K,ATP}$)(Stern et al., 1988; Lederer et al., 1989). Abbreviation of the action potential by $I_{K\text{-}ATP}$ may play an important role in rapid contractile failure in anoxia or in response to metabolic poisons (Stern et al., 1988; Nichols and Lederer, 1990).

In summary, it appears that ATP levels in whole heart preparations made globally ischemic do not change significantly at the time contractile failure is observed. However, in single cell preparations exposed to metabolic poisons, contractile failure occurs when ATP levels within the cell decline. It is unclear whether contractile failure produced by metabolic inhibition is directly related to low ATP levels. Alternatively, contractile failure may be an indirect consequence of low ATP levels which activate $I_{K\text{-}ATP}$ and thereby limit Ca^{++} influx or alter SR Ca^{++} release or sequestration via SR Ca^{++} ATP-ases into the SR.

iii) Abbreviation of Action Potential Duration during Ischemia.

It is believed that shortening of the action potential duration limits the influx of Ca^{++} and thus may contribute to contractile failure in ischemia. It is well established that myocardial ischemia is associated with a number of electrophysiological and contractile changes. Previous experiments in both canine (Samson and Scher, 1960) and porcine hearts (Kleber et al., 1978) have demonstrated a decreased amplitude of the action potential and significant reduction of action potential duration within minutes of ischemia. It was subsequently discovered that the abbreviation in action potential duration during ischemia was most likely due to activation of an outward K^+-current. This class of K^+-channels increases significantly when cardiac cells are exposed to hypoxia or cyanide, and decreases when ATP is injected intracellularly (Noma, 1983). These channels were subsequently named ATP-sensitive K^+ channels (Noma, 1983). ATP-sensitive K^+ channels are believed to be the most abundant K^+ channels in the heart (Noma and Shibasaki, 1985); however under normal conditions, when ATP levels within the cell are high, these channels remain inactive (for reviews see Nichols and Lederer, 1991; Edwards and Weston, 1993).

It is unclear as to whether activation of ATP-sensitive K^+ channels during ischemia exerts a protective effect on the heart. Cole et al. (1991) determined the effects of agents that influence activity of ATP-sensitive K^+ channels in a guinea pig right ventricular free wall model of simulated ischemia and

reperfusion. Results of their study showed that when pinacidil, an ATP-sensitive K^+ channel agonist, was added throughout 30 min of ischemia, the electrical and mechanical activity in the ventricular wall preparation recovered completely upon reperfusion. In contrast, when glyburide, an ATP-sensitive K^+ channel blocker, was present during 20 min of ischemia there was a marked reduction in function during reperfusion. The mechanism by which activation of ATP-sensitive K^+ channels protect against ischemia and reperfusion injury is believed to be by limiting Ca^{++} influx during the action potential (Cole, 1993). Activation of these channels shortens the duration of the action potential, thereby limiting the amount of Ca^{++} entering through L-type Ca^{++} channels. This reduction in Ca^{++} influx and accumulation in the heart may be a mechanism for protecting the myocardium when blood flow is decreased.

Studies have shown that activation of ATP-sensitive K^+ channels is protective in ischemia and reperfusion; however, a number of studies have shown that activation of ATP-sensitive K^+ channels may be deleterious. Bekheit et al. (1990) examined the effects of glyburide, an ATP-sensitive K^+ channel blocker, on ischemia-induced electrical changes and increases in extracellular K^+ in canine hearts. Their study utilized an in vivo model of regional ischemia produced by ligation of the left anterior descending coronary artery. Results of this study demonstrated that glyburide diminished the shortening of the action potential duration during ischemia. As shortening of the APD is believed to underlie malignant reentrant-type arrhythmias, prolongation of APD with

glyburide may protect against life-threatening arrhythmias.

As mentioned previously, ATP-sensitive K^+ channels activate as ATP levels in the cell decline. Interestingly, Elliott et al. (1989) showed that the measured ATP concentration at the time of action potential shortening is still near normal levels. This result suggests that some mechanism other than ATP-sensitive K^+ channels contributes to the decline in action potential duration. Alternatively, since ATP-sensitive K^+ channels are the most abundant K^+ channels in the myocardium (Noma and Shibasaki, 1985), perhaps activation of only a small percentage of these channels will result in a significant abbreviation in the duration of the action potential. Nichols et al. (1991) indicated that less than a 1% increase in the open probability of ATP-sensitive channels will result in a significant shortening of the action potential duration. This is consistent with the notion that activation of only a few ATP-sensitive channels will shorten the action potential.

There is evidence suggesting that ATP-sensitive K^+ channels may be modulated by a number of substances. It has previously been shown that adenosine (ADN) acting at A_1 ADN receptors is capable of enhancing the activity of ATP-sensitive K^+ channels (Kirsch et al., 1990). These studies were conducted in membrane patches excised from rat ventricular myocytes and showed that ADN or the ADN analogue N^6-cyclohexyladenosine could enhance the activity of ATP-sensitive K^+ channels via an inhibitory G protein. Furthermore, the actions of ADN on A_1 receptors were via a membrane-delimited

pathway rather than a second messenger travelling through the cytosol. However, Kirsch et al. (1990) only found enhancement of ATP-sensitive K^+ channels by ADN in 4 out of 10 excised patches. It would be expected that with the high density of ATP-sensitive K^+ channels, a higher success rate would be expected. One explanation may be that the density of A_1 ADN receptors is very low (Linden et al., 1985). Another substance capable of modulating ATP-sensitive K^+ channels is lactate (Keung and Li, 1991). In their study, Keung and Li (1991) found that lactate in the intracellular solution at a concentration of 20-40 mM was capable of activating ATP-sensitive K^+ channels. In addition, the activation of ATP-sensitive K^+ channels occurred in the presence of 2-5 mM ATP (Keung and Li, 1991).

In summary, ATP-sensitive K^+ channels are a class of K^+ channels that exist in the myocardium. When cytosolic ATP levels decrease in ischemia, these channels are activated and function to limit APD and decrease Ca^{++} influx. However, activation of these channels may promote induction of reentrant type arrhythmias. It appears that these channels can be modulated by certain substances, namely ADN and lactate. Both ADN and lactate are substances which accumulate during conditions of myocardial ischemia. This modulation may provide at least part of the explanation for the discrepancy between cytosolic ATP levels and action potential duration shortening during ischemia.

iv) Reduction in pH during Ischemia.

In the whole heart preparation made globally ischemic, contractile failure does not appear to be due to a decline in cytosolic ATP levels (Koretsune et al., 1991) or a loss of the Ca^{++} transient (Lee et al., 1988; Camacho et al., 1993). These results suggest that some other mechanism contributes to contractile failure. As already discussed, there is decline in cytosolic ATP levels during ischemia (Koretsune et al., 1991). Associated with the breakdown of ATP is the accumulation of inorganic phosphate and protons (H^+) (Dennis et al., 1991). In addition, the accumulation of lactate through glycolysis may contribute to the addition of more protons (Dennis et al., 1991). Regardless of the source of protons, the accumulation of H^+ affects every step in the excitation-contraction coupling pathway including the delivery of Ca^{++} to the myofilaments and the response of the myofilaments to Ca^{++} (for review see Orchard and Kentish, 1990). Thus, accumulation of protons results in inhibition of the myofilaments to Ca^{++} and may contribute to contractile failure during ischemia.

B. Ca^{++} Gain in Ischemia.

Under normoxic conditions, cytosolic Ca^{++} levels within the myocardium are maintained in the nanomolar range (Barcenas-Ruiz and Weir, 1987). It is believed that during an action potential, influx of Ca^{++} across the sarcolemma triggers a much larger release of Ca^{++} from the SR thro ·gh ryanodine sensitive Ca^{++} release channels (Fabiato, 1985). It is this rise in Ca^{++} that initiates the contraction-relaxation process in the myofilaments (Bers, 1991). As the cardiac

cell repolarizes, Ca^{++} is both extruded from the cell and taken up by the sarcoplasmic reticulum. It has been shown that an amount of Ca^{++} equivalent to that which enters via voltage-sensitive Ca^{++} channels is extruded via the Na^{+}-Ca^{++} exchange (Barry and Bridge, 1993). Similarly, the Ca^{++} that is released from the SR is sequestered back into the SR (Barry and Bridge, 1993). Therefore, low cytosolic Ca^{++} levels are maintained by a number of very efficient exchangers and pumps located either on the sarcolemma, to extrude Ca^{++} or in the SR, to sequester Ca^{++} into intracellular stores.

Under conditions of ischemia and hypoxia, the mobilization of Ca^{++} is quite different. When the energy supply is stopped, the efficiency of ATP-dependent pumps to maintain the ionic gradients is compromised and cells accumulate Ca^{++}. The accumulation of free intracellular Ca^{++} has been implicated in cell damage in many tissues and pathological conditions (Shen and Jennings, 1972; Nayler et al., 1979). It is now apparent that Ca^{++} plays a critical role in the amount of myocardial injury during ischemia (for reviews see Murphy et al., 1987; Silverman and Stern, 1994). Early investigations suggested that accumulation of intracellular Ca^{++} was an important step in the transition from reversible to irreversible myocardial cell injury (Shen and Jennings, 1972). They studied the effect of ischemia produced by coronary artery ligation on $^{45}Ca^{++}$ uptake in canine myocardium. Results of their study showed that 40 minutes of ischemia followed by 10 minutes of reperfusion increased the Ca^{++} content in the myocardium 18 fold and caused irreversible cell injury. However, 10 minutes of

ischemia followed by 20 minutes of reperfusion resulted in no significant Ca^{++} accumulation and no injury. Since the work of Shen and Jennings (1972a,b), many studies have focused on changes in intracellular Ca^{++} and Na^{+} concentrations. In addition, new technical advances allow ion concentrations within myocardial tissue to be measured and permit investigators to determine the mechanism by which they occur. Results of these studies are presented in the next three sections.

i) Ca^{++} from Intracellular Stores.

The increase in cytosolic Ca^{++} during ischemia may be due to either an increase in Ca^{++} release from the SR or impaired Ca^{++} uptake. Lee et al. (1988) used the fluorescent Ca^{++} indicator indo-1 to examine changes in Ca^{++} transients, contractions and action potentials in perfused rabbit heart exposed to acute global ischemia. Results of their study showed that there was an elevation in both systolic and diastolic levels of Ca^{++}. This suggests the release of Ca^{++} from the SR and the reuptake of Ca^{++} into the SR was unaffected. Interestingly, the increase in the level of the Ca^{++} transient was accompanied by a reduction in ventricular developed pressure. A similar rise in the Ca^{++} transient was observed by Camacho et al. (1993) in whole rat hearts subjected to global ischemia. In this study, it did not appear that the release of Ca^{++} from the SR was affected; however, both systolic and diastolic Ca^{++} levels were elevated. One drawback of this study was that the Ca^{++} signal was from a whole heart and

therefore included fluorescence signals from sources such as the endothelium. In contrast, another study directly examined the SR Ca^{++} uptake in vesicles isolated from whole hearts which had previously been exposed to 15 minutes of global ischemia (Kaplan et al., 1992). The results of this study showed uptake of Ca^{++} into SR vesicles was impaired in hearts exposed to global ischemia. A similar study was performed by Hohl et al. (1992). Their study looked at SR Ca^{++} uptake into vesicles from rat ventricular myocytes exposed to simulated ischemia and reperfusion. Once again, Ca^{++} uptake was found to be reduced in myocytes exposed to simulated ischemia and reperfusion.

In summary, it appears that release of Ca^{++} from the SR is unaffected by ischemic conditions; however, it is unclear whether sequestration of Ca^{++} into the SR is depressed during ischemia. Studies which examined SR Ca^{++} uptake into vesicles showed that Ca^{++} uptake is reduced in vesicles isolated from hearts made ischemic. This observation is consistent with the idea that sequestration is an ATP-dependent process which becomes depressed as the ATP levels decline during ischemia. This could cause or contribute to increased intracellular Ca^{++} concentration in ischemia.

ii) L-type Ca^{++} Channels

It is unclear what role L-type Ca^{++} channels play in the accumulation of Ca^{++} during ischemia. It seems likely that excessive Ca^{++} influx through these voltage operated Ca^{++} channels would contribute to an increase in cytosolic Ca^{++}

during ischemia. However, Lederer et al. (1989) measured I_{Ca} in rat ventricular myocytes subjected to complete metabolic inhibition using cyanide and 2-deoxyglucose. Results from Lederer et al. (1989) showed that approximately 50% of I_{Ca} remained, even after the onset of a contracted rigor state. This suggests that the accumulation of Ca^{++} is not due exclusively to Ca^{++} influx since I_{Ca} was depressed during metabolic inhibition. Lee et al. (1988) used the fluorescent Ca^{++} indicator indo-1 to examined changes in Ca^{++} transients, contractions and action potentials in perfused rabbit heart exposed to acute global ischemia. The gradual elevation in systolic and diastolic Ca^{++} levels could be reduced by the addition of the Ca^{++} channel antagonist verapamil during ischemia, suggesting that influx of Ca^{++} through L-type Ca^{++} channels was responsible for this elevation. Similar results were obtained by Hano et al. (1991) in rat myocytes exposed to anoxic conditions. Results of their study showed, that under control conditions, rat myocytes made hypoxic by an argon gas phase gradually accumulated Ca^{++} as detected by indo-1 fluorescence. Addition of the dihydropyridine-type Ca^{++} channel blocker nicardipine, greatly attenuated the rise in cytosolic Ca^{++} levels. However, addition of another dihydropyridine compound, nifedipine, did not prevent the rise in cytosolic Ca^{++}. They concluded from these results that L-type Ca^{++} channel blockade with dihydropyridines does not attenuate the rise in cytosolic Ca^{++} in ischemia. However, nicardipine may attenuate Ca^{++} gain in ischemia through a mechanism independent from L-type Ca^{++} channel blockade.

In summary, it is unclear whether L-type Ca^{++} channel blockade reduces Ca^{++} gain in ischemia. While some investigations suggest that Ca^{++} channel blockade during ischemia reduces Ca^{++} gain, other studies have found no such effect. Many of the differences depend on the type of tissue used and whether ischemia, hypoxia or metabolic inhibition was employed.

iii) Na^{+}-Ca^{++} Exchange.

Under normoxic conditions, the Na^{+}-Ca^{++} exchange is believed to extrude one Ca^{++} from the cytoplasm in exchange for three Na^{+} (for reviews see Mullins, 1979; Eisner and Lederer, 1985). Studies have suggested that the Na^{+}-Ca^{++} exchange may function in "reverse" mode to allow Ca^{++} to enter the cell during the upstroke of the action potential (Mullins, 1979). Leblanc and Hume (1990) proposed that the momentary increase in Na^{+} concentration initiated by I_{Na} causes the Na^{+}-Ca^{++} exchange to bring Ca^{++} into the cell. It is believed that this influx of Ca^{++} is capable of triggering Ca^{++} release from the SR. Evidence also suggests that the Na^{+}-Ca^{++} exchange will function in reverse mode under conditions of high cytosolic Na^{+} levels. It is difficult to determine whether Na^{+}-Ca^{++} exchange contributes to Ca^{++} loading in ischemia, since there are no specific inhibitors of the Na^{+}-Ca^{++} exchange. However, Daly et al. (1984) isolated sarcolemmal membrane fractions from isolated rat hearts exposed to global ischemia for 60 minutes. Results of their study demonstrated that Ca^{++} transport via the Na^{+}-Ca^{++} exchange was reduced in sarcolemmal vesicles

isolated from ischemic hearts. Interestingly, sarcolemmal vesicles from hearts that were made only hypoxic by exposure to N_2 did not exhibit a decrease in Ca^{++} transport via the Na^+-Ca^{++} exchange. Haworth and Goknur (1992) studied the ATP dependence of calcium uptake by the Na^+-Ca^{++} exchange in rat myocytes. Results of their study demonstrated that Ca^{++} transport via the Na^+-Ca^{++} exchange was greatly reduced in ATP depleted cells. However, this inhibition of the Na^+-Ca^{++} exchange did not occur until cytosolic ATP levels were 25% of normal.

As mentioned previously, the accumulation of protons has been shown to inhibit excitation-contraction coupling at almost every step in the pathway (Orchard and Kentish, 1990). Ischemic conditions can also cause a decrease in intracellular pH. The effect of pH on Na^+-Ca^{++} exchange activity was studied in canine cardiac sarcolemmal vesicles (Philipson et al., 1982). Results suggest that the effect of pH is due to a direct interaction of the protons with the Na^+-Ca^{++} exchange. They concluded in their study that a reduction in intracellular but not extracellular pH markedly reduced Ca^{++} transport mediated via the Na^+-Ca^{++} exchange.

In summary, it is generally believed that under normal conditions, the Na^+-Ca^{++} exchange removes Ca^{++} from the cell following each action potential. In addition, Na^+-Ca^{++} exchange may function in reverse mode to bring Ca^{++} into the cell during the upstroke of the action potential, or when cytosolic Na^+ levels are high. However, in conditions of ischemia the Na^+-Ca^{++} exchange is inhibited.

The result of this inhibition is a reduction in Ca^{++} extrusion from the myocardial cell and an accumulation of intracellular Ca^{++}. It appears that the inhibition of the Na^+-Ca^{++} exchange is due to a decrease in intracellular pH and, to a lesser extent, a reduction in cytosolic ATP levels.

C. Na^+ Gain in Ischemia.

As previously mentioned, a rise in intracellular Na^+ will result in an increase in intracellular Ca^{++} via the Na^+-Ca^{++} exchanger. This is a result of a reduction in the Na^+ gradient which is necessary to extrude Ca^{++} (Eisner and Lederer, 1985). During ischemia, there are a number of postulated mechanisms by which Na^+ can accumulate within the myocardium.

i) Na^+-K^+-ATPase

Under normal conditions, the Na^+-K^+-ATPase removes 3 Na^+ from the cytosol in exchange for 2 K^+. It would be expected that during ischemia, the activity of the Na^+-K^+-ATPase would be reduced as a result of decreased cytosolic ATP. Accordingly, it has been shown that the Na^+-K^+-ATPase is half-maximally inhibited by ATP concentrations of 0.1 mM (Glynn and Karlish, 1976). This level of ATP represents a significant reduction from the normal intracellular ATP concentration of 5-10 mM (Weiss et al., 1989). Large decreases in cytosolic ATP levels do occur during prolonged ischemia. In adult male rat hearts subjected to global ischemia for 60 minutes, the ATP concentration

decreases to 8% of control levels (Daly et al., 1984). This reduction in cytosolic ATP levels corresponds to only a 21-24% reduction in Na^+-K^+-ATPase activity (Daly et al., 1984). These results tend to suggest that decreased Na^+ extrusion via the Na^+-K^+-ATPase during ischemic conditions is not a major route of Na^+ accumulation, until cytosolic ATP levels decline to extremely low levels.

ii) Na^+ Channels

An obvious route for Na^+ accumulation during ischemia is via Na^+ channels. A prolongation in the time Na^+ channels remain open would lead to a build up of Na^+ within the cytosol. The accumulation of Na^+, measured by nuclear magnetic resonance (NMR), was studied in rat hearts subjected to global ischemia for 10 minutes (Butwell et al., 1993). The results of the study demonstrate that Na^+ accumulates gradually during 10 min of global ischemia. Addition of 130 μM lidocaine during the ischemic period significantly decreases, but does not abolish, the gain in intracellular Na^+ (Butwell et al., 1993). In a similar study, Haigney et al. (1992) measured the intracellular Na^+ concentration using the fluorescent dye SBFI in rat myocytes exposed to hypoxia. Exposure of the myocytes to hypoxia results in a gradual rise in the intracellular Na^+ concentration. Addition of the drug R56865, a compound capable of blocking the noninactivating component of the Na^+ current, markedly reduces the rise in the SBFI fluorescent ratio (Haigney et al., 1992).

It is not entirely clear whether Na^+ accumulation is due to an increase in

Na^+ influx via I_{Na} or an increase in Na^+ influx via another pathway. However, Wu and Corr (1994) demonstrated that accumulation of long-chain acylcarnitines during ischemia caused spontaneous activation of I_{Na} and thereby increased cytosolic Na^+ in rabbit ventricular myocytes. Wu and Corr (1994) concluded that accumulation of these metabolites during ischemia contributes to Na^+ loading during ischemia. In contrast to the above studies, Mejia-Alvarez and Marban (1992) directly measured I_{Na} in both the whole cell and single channel recording configuration in guinea pig ventricular myocytes exposed to metabolic inhibition by dinitrophenol. Results of their study demonstrate that there is a modest but significant reduction in I_{Na} during metabolic inhibition.

In summary, it appears that the gain in cytosolic Na^+ can be partially attenuated by application of Na^+ channel blockers during ischemia. Interestingly, direct measurements of I_{Na} during metabolic inhibition revealed that I_{Na} is reduced (Mejia-Alvarez and Marban, 1992). These results suggest that the reduced efficiency of Na^+ extrusion overcomes the reduction in I_{Na}, resulting in net Na^+ accumulation in ischemia. Complete blockade of I_{Na} by pharmacological agents may result in reduced Na^+ influx via I_{Na}, and thus lower Na^+ accumulation.

iii) Na^+-H^+ Exchange

The pH in extracellular fluid is around 7.4 under normoxic conditions, however the pH within myocardial cells is 7.1-7.2. Within the plasma membrane, the Na^+-H^+ exchanger mediates the uphill extrusion of H^+ driven by the downhill

flow of Na^+ into the cell. Therefore, the Na^+-H^+ exchange is important in regulating the extrusion of H^+ from the cell and in mediating the uptake of Na^+. A number of studies have shown that the Na^+-H^+ exchange plays an important role in the regulation of internal pH (for reviews see Aronson, 1985; Lazdunski et al., 1985; Frelin et al., 1988).

During conditions of ischemia, it has been shown that both the internal and external pH gradually decrease over time (Lazdunski et al., 1985). If the Na^+-H^+ exchange plays an important role in the regulation of intracellular pH, then the Na^+-H^+ exchange should function to remove H^+ from the cell in exchange for Na^+ during ischemia. Vandenburg et al., (1993) studied the mechanism of intracellular pH recovery by NMR after global ischemia in Langendorff-perfused ferret heart. Results of their study show that intracellular pH decreases from about 7.1 in control to 6.75 after 10 minutes of global ischemia. Similar results were obtained by Yan and Kleber (1992) using intracellular ion-selective electrodes to measure intracellular pH in rabbit papillary muscle perfused via the septal artery. These results also demonstrate that intracellular, as well as extracellular pH, decrease during global ischemia.

Unfortunately, the contribution of the Na^+-H^+ exchanger to Na^+ loading during ischemia cannot be assessed pharmacologically because there are no specific inhibitors of Na^+-H^+ exchange. In addition, it is believed that the Na^+-H^+ exchange is stimulated by low intracellular pH and inhibited by low extracellular pH. Therefore, the precise activity rate of the Na^+-H^+ exchange during ischemia

is not known. However, Tani and Neely (1989) demonstrated that amiloride (0.5-1.0 mM), which is a non-selective inhibitor of Na^+-H^+ exchange, produced a small but significant decrease in the cytosolic Na^+ concentration following 15 minutes of global ischemia. These results suggest that intracellular acidification during ischemia leads to an accumulation of intracellular Na^+. Some of the accumulation of Na^+ appears to occur via the Na^+-H^+ exchanger, since non-selective Na^+-H^+ exchange inhibitors such as amiloride, reduced Na^+ gain in ischemia. Similar results were obtained by Murphy et al. (1991) who measured both intracellular Ca^{++} and Na^+ by NMR in rats exposed to global ischemia. Results of their study demonstrate that amiloride (1 mM) greatly attenuates the rise in both intracellular Na^+ and Ca^{++} during ischemia when compared to control. However, it is possible that the effects of amiloride may be due to inhibition of some other ion exchanger, particularly the Na^+-Ca^{++} exchange, since the effects of amiloride are not specific for the Na^+-H^+ exchanger (Lazdunski et al., 1985).

3. MYOCARDIAL ALTERATIONS DURING REPERFUSION.

Although reperfusion of previously ischemic myocardium is essential for the survival of the heart following a myocardial infarction, reperfusion can cause detrimental effects by itself. The severity and duration of the ischemic period dictate the outcome of myocardial tissue survival upon reperfusion. Many of the detrimental effects of reperfusion have been attributed to a sudden rise in

cytosolic levels of Ca^{++} (for reviews see Murphy et al., 1987; Tani, 1990; Silverman and Stern, 1994). High levels of cytosolic Ca^{++} can cause myocyte damage in many ways. Many proteases are activated by Ca^{++} which could act to destroy important intracellular structures such as the cytoskeleton (Kishimoto et al., 1983). In addition, excessively high levels of Ca^{++} upon reperfusion may cause persistent activation of the myofilaments (Haworth et al., 1981). High cytosolic Ca^{++} levels also are associated with induction in the transient inward current (I_{TI}) (Lederer and Tsien, 1976). I_{TI} is an arrhythmogenic current that is activated by high intracellular Ca^{++} (Kass et al., 1978a,b). However, the precise mechanism by which Ca^{++} accumulates upon reperfusion is unknown.

A. Ca^{++} Gain during Reperfusion.

i) Ca^{++} Transients upon Reperfusion

It is believed that the accumulation of Ca^{++} upon reperfusion is due to Ca^{++} influx from the extracellular fluid, since much of the Ca^{++} accumulation upon reperfusion is prevented by lowering the extracellular Ca^{++} concentration (Shine et al., 1978). As previously discussed (page 14), ischemic conditions cause the intracellular Ca^{++} concentration to increase. However, reperfusion may cause a secondary rise in cytosolic Ca^{++} levels (for reviews see Murphy et al., 1987; Silverman and Stern, 1994). For example, Haigney et al. (1992) measured intracellular Ca^{++} transients with indo-1 in rat ventricular myocytes exposed to hypoxic conditions produced by an argon gas phase followed by reoxygenation.

Results of their study demonstrate that, during hypoxia, the magnitude of the Ca^{++} transient gradually increases. Upon reoxygenation there is a secondary increase in the Ca^{++} transient followed by a slow return of the Ca^{++} transient to control levels (Haigney et al., 1992). In contrast to the above study, Koyama et al. (1992) exposed guinea pig ventricular myocytes to simulated ischemic conditions followed by reperfusion. Intracellular Ca^{++} transients were measured by fura-2. Koyama et al (1992) showed that during simulated ischemia, the Ca^{++} transient gradually increased during ischemia; however, upon reperfusion there was no secondary increase in the Ca^{++} transient signal, but a slow reduction in the Ca^{++} transient with continued reperfusion. Ca^{++} transients also have been measured with the bioluminescent protein aequorin in ferret papillary muscle exposed to ischemic conditions and reperfusion (Allen et al., 1989). Results of this study show that during simulated ischemia, the Ca^{++} signal measured by aequorin gradually increases. Upon reperfusion, they found a further increase in the magnitude of the Ca^{++} transients followed by a slow return of the Ca^{++} transient to control levels. These results suggest that, upon reperfusion, there is a secondary increase in the cytosolic Ca^{++} level which gradually declines with continued reperfusion (Allen et al., 1989). Kihara et al. (1989) measured changes in intracellular Ca^{++} concentration during global ischemia and reperfusion in the isolated perfused ferret heart. In this study, the Ca^{++} signal was measured with aequorin. Results of this study show that the Ca^{++} transient slowly increases during global ischemia. However, upon reperfusion there was

no temporary increase in the Ca^{++} transient signal, but a slow return of the Ca^{++} transient to control levels. It is unclear why there are differences between the results of Allen et al (1989) and those of Kihara et al. (1989) since both studies utilized the same animal species and same Ca^{++} indicator. However, differences in the effects of global ischemia in the intact heart versus simulated ischemia in a papillary muscle preparation may account for the differences in the results.

In summary, ischemic conditions cause a rise in the cytosolic Ca^{++} levels as measured by Ca^{++} indicators. Upon reperfusion, it appears that there may be a secondary rise in the magnitude of the Ca^{++} transient, although this is not seen in all experimental conditions. The differences may be due to the type of the animal species studied or whether the investigators employed hypoxia, metabolic inhibition or ischemia.

ii) Sarcolemmal Ca^{++} Channels

One obvious method by which Ca^{++} can accumulate upon reperfusion is through voltage-gated Ca^{++} channels (Tani, 1990). Excessive Ca^{++} gain through voltage-gated Ca^{++} channels coupled with decreased Ca^{++} extrusion would, in theory, lead to an accumulation in cytosolic Ca^{++}. In a study designed to assess the magnitude of L-type Ca^{++} current during reoxygenation, Benndorf et al. (1991a,b) measured the magnitude of the Ca^{++} current in guinea pig ventricular myocytes subjected to extreme hypoxia with an argon gas phase layered over the superfusion chamber. The results of their study demonstrate that, upon

reoxygenation following 15 min of hypoxia, the Ca^{++} current decreases from 20 to 100% within the first few minutes. To further investigate the role of L-type Ca^{++} channels during reperfusion, many studies have added L-type Ca^{++} channel blockers at various times throughout ischemia and reperfusion. Nayler et al. (1988) determined the effects of acute Ca^{++} channel blockade in rat hearts subjected to global ischemia. In their study, Ca^{++} channel blockade was present during both ischemia and reperfusion. Results of this study show that acute Ca^{++} channel blockade delays, but does not prevent, Ca^{++}-mediated damage upon reperfusion. A similar study was performed by Hano et al. (1991) who examined the effects of nifedipine on substrate-free anoxia in rat ventricular myocytes. Results show that nifedipine does not prevent the rise in cytosolic Ca^{++} during ischemia or the additional gain in intracellular Ca^{++} in early reperfusion (Hano et al., 1991). Interestingly, pretreatment with Ca^{++} channel blockers for 4-5 days prior to an ischemic insult decreases reperfusion-associated damage (Nayler et al., 1980). This protective effect exerted by Ca^{++} channel blockade is not due exclusively to blockade of the channel, but to an ability to slow the rate of depletion of the ATP reserves (Nayler et al., 1980). Conversely, Maruyama et al. (1991) determined the effects of the Ca^{++} agonist Bay K 8644 in guinea pig right ventricular papillary muscle subjected to simulated ischemia and reperfusion. Results of their study show that the Ca^{++} channel agonist exacerbates the effects of simulated ischemia and reperfusion.

In summary, it appears that the magnitude of I_{Ca} is reduced during the

early stages of reperfusion. Inhibition of the L-type Ca^{++} channel by pharmacologic blockade does not protect the myocardium from Ca^{++} gain in early reperfusion. However, enhancement of I_{Ca} leads to a greater degree of myocardial damage upon reperfusion. This suggests that Ca^{++} gain in early reperfusion must be due to Ca^{++} influx by some mechanism other than L-type Ca^{++} current.

iii) The Na^{+}-Ca^{++} Exchange

As discussed previously, it has been observed that both intracellular Na^{+} and Ca^{++} levels rise during ischemia (in section 2.B. and 2.C.). However, it is unclear whether the secondary rise in cytosolic Ca^{++} in early reperfusion is due to Na^{+} loading during ischemia. Tani and Neely (1989) used the drug monensin, a Na^{+} ionophore, to vary the degrees of Na^{+} loading during global ischemia in rats. Results of their study show that, as higher concentrations of monensin are added in ischemia, the amount of Na^{+} loading increases. Upon reperfusion, the degree of Ca^{++} loading and cellular damage increases with increasing concentrations of monensin (Tani and Neely, 1989). This suggests that the degree of Ca^{++} gain in reperfusion is related to the degree of Na^{+} loading and provides indirect evidence that the Na^{+}-Ca^{++} exchange may play an important role in Ca^{++} gain in early reperfusion. In another study which used a rat ventricular myocyte model of simulated ischemia and reperfusion, Nishida et al. (1993) examined the role of altering cation gradients; intracellular Ca^{++}

transients were measured with fura-2 and cell shortening was measured with a video edge detector. Under control conditions, reperfusion after 10 min ischemia results in a temporary overshoot in the Ca^{++} transient and the magnitude of cell shortening (Nishida et al., 1993). When the Ca^{++} concentration in the superfusion buffer is decreased to nominal levels 5 minutes prior to reperfusion and continued throughout reperfusion, the transient overshoot in the magnitude of contraction is prevented. In addition, the Ca^{++} transient signal does not temporarily exceed control (Nishida et al., 1993). Nishida et al. (1993) concluded from this study that intracellular Na^{+} played a pivotal role in reperfusion-induced cell injury by permitting transient Ca^{++} overload via the Na^{+}-Ca^{++} exchange. In a cellular model of hypoxia in rat ventricular myocytes, both intracellular Na^{+} and Ca^{++} were measured by fluorescence detection (Haigney et al., 1992). During hypoxia, both intracellular Na^{+} and Ca^{++} were found to increase gradually. Upon reoxygenation, there is a secondary increase in the cytosolic Ca^{++} signal, while intracellular Na^{+} declines. Furthermore, interventions which decrease the intracellular Na^{+} concentration also decrease Ca^{++} gain upon reoxygenation (Haigney et al., 1992). Haigney et al. (1992) concluded that a reduction in Na^{+} gain during hypoxia and reoxygenation reduces the gain in Ca^{++} and prevents cellular injury and hypercontracture.

In summary, the evidence presented suggests that upon reperfusion there is an increase in the cytosolic Ca^{++} content. The secondary accumulation of

Ca^{++} is the result of influx of Ca^{++} from the extracellular fluid, since reperfusion in the presence of low extracellular Ca^{++} reduces the total intracellular Ca^{++} concentration. Interventions which tend to reduce the degree of Na^+ loading during ischemia also diminish the extent of Ca^{++} loading upon reperfusion. Pharmacological interventions which block the Na^+-Ca^{++} exchange may be therapeutically useful for reducing Ca^{++} gain due to reperfusion of previously ischemic myocardium.

B. Na^+ Gain in Reperfusion.

i) Na^+-H^+ Exchange

Regulation of intracellular pH under normoxic conditions is maintained by a number of membrane transport processes and intracellular buffers. However, it is believed that under conditions of ischemia, the Na^+-H^+ exchange transport process predominates (Lazdunski et al., 1985; Frelin et al., 1988). As discussed previously (page 13), the intracellular pH drops gradually during conditions of ischemia (Koretsune et al., 1991). Many previous studies utilizing inhibitors of the Na^+-H^+ exchange have demonstrated the role of the Na^+-H^+ exchange in the recovery of intracellular pH following an ischemic insult (reviewed by Lazdunski et al., 1985). Since inhibitors of the Na^+-H^+ exchange also tend to inhibit other exchange processes, the results of studies which use these inhibitors do not provide conclusive evidence that Na^+-H^+ exchange is the only mechanism important in recovery of pH in reperfusion. Lazdunski et al (1985) speculated on

the possible role of the Na^+-H^+ exchange during cardiac ischemia and suggested that during ischemia, both the internal and external pH drop to very low values. When the external pH is very low, the Na^+-H^+ exchange is nearly inactive. During reperfusion with a perfusate at a pH close to neutrality, a pH gradient develops instantaneously across the plasma membrane of cardiac cells but the interior of the cell remains acidic. Under these conditions, maximal activity of the Na^+-H^+ exchanger is attained. As a result of this activation of the exchanger, there is a massive entry of Na^+. The high intracellular Na^+ will then be extruded via the Na^+-Ca^{++} exchange which will, in turn, cause a large entry of Ca^{++} and the associated harmful effects (Lazdunski et al., 1985). Therefore, if Lazdunski et al. (1985) are correct then selective inhibitors of the Na^+-H^+ exchange may be beneficial in protecting against ischemia and reperfusion injury.

To examine the effects of more potent and specific Na^+-H^+ exchange inhibitors during ischemia and reperfusion, Moffat and Karmazyn (1993) examined the compound methylisobutyl amiloride on myocardial contractile and biochemical changes in rat and guinea pig hearts. Global ischemia was induced for 45 min in the presence and absence of methylisobutyl amiloride. In both species, contractile recovery upon reperfusion following global ischemia was significantly faster in the presence of methylisobutyl amiloride. In addition, the elevation in resting tension was greater in non-treated hearts. The results from their study demonstrate that Na^+-H^+ exchange inhibition during ischemia results in significant improvement in contractile recovery upon reperfusion. In addition,

this protection during ischemia and reperfusion is not species-specific but appears to be universal. The protective effects of amiloride analogues appears to be due to Na^+-H^+ exchange inhibition since the compound Hoe 694, a non-amiloride analogue capable of inhibiting Na^+-H^+ exchanger, is also protective during ischemia and reperfusion (Scholz et al., 1993). In the study by Scholz et al. (1993), isolated rat hearts were subjected to global ischemia for 15 min. They found that Hoe 694 significantly reduced the incidence of ventricular fibrillation upon reperfusion following 15 min of ischemia. In addition, there was a significant increase in tissue glycogen and ATP content in Hoe 694 treated hearts. The results from their study demonstrate that Hoe 694 shows anti-arrhythmic and cardioprotective effects during ischemia and reperfusion which is probably due to Na^+-H^+ exchange inhibition.

In summary, the Na^+-H^+ exchange regulates the internal pH. During periods of ischemia, the internal pH gradually decreases. The result of the pH decrease is an accumulation of Na^+ via the Na^+-H^+ exchange. As the intracellular Na^+ rises, the Na^+-Ca^{++} exchange would function to remove the Na^+ resulting in an elevation in intracellular Ca^{++}. Addition of inhibitors of the Na^+-H^+ exchange appear to protect the myocardium from injury due to ischemia and reperfusion.

C. Reperfusion Arrhythmias.

Conditions of ischemia produced by coronary artery ligation result in the

appearance of arrhythmias (Tennant and Wiggers, 1935). In addition, reperfusion of previously ischemic myocardium also produces arrhythmias, some of which include ventricular fibrillation (Harris and Rojas, 1943; Harris, 1950). The appearance of arrhythmias following reperfusion of previously ischemic myocardium is well established (for a review see Manning and Hearse, 1984). Under experimental conditions, reperfusion arrhythmias tend to occur very soon after the start of reperfusion (Manning and Hearse, 1984). Pogwizd and Corr (1987a,b) performed a series of experiments where they measured electrical activity from 256 different recording sites from the hearts of chloralose-anaesthetized cats. From these measurements, they constructed three-dimensional computer-assisted maps of the heart during conditions of ischemia and reperfusion. Pogwizd and Corr (1987a,b) determined that approximately 75% of reperfusion arrhythmias were initiated by oscillatory afterpotentials (OAP) and the remaining 25% were due to a reentrant-type mechanism. The following will focus on mechanisms of arrhythmias, especially OAP.

i. Oscillatory Afterpotentials

OAP's (also referred to as delayed afterdepolarizations) are an important cause of arrhythmias in a wide range of settings (for reviews see Ferrier, 1977; Wit and Rosen, 1992). OAP's are oscillations in membrane potential that occur after repolarization of an action potential and that are caused by that action potential (Cranefield, 1977). Much of the interest in OAP's as an arrhythmogenic

mechanism occurred when it was found that toxic levels of cardiac glycosides could cause these types arrhythmias (Ferrier, 1977; January and Fozzard, 1988). The mechanism by which cardiac glycosides cause OAP's is by inhibiting the Na^+-K^+-ATPase pump. At high levels of drug, inhibition of this pump leads to an increase in intracellular Na^+. It is believed that this increase in intracellular Na^+ results in a reduced driving force for Na^+ across the sarcolemma. This, in turn, diminishes Ca^{++} extrusion via the Na^+-Ca^{++} exchange leading to an increase in intracellular Ca^{++} (Ferrier, 1977; Wit and Rosen, 1992). High intracellular Ca^{++} causes an oscillatory release of Ca^{++} from the SR and appearance of OAP's (Lee et al., 1980). Other conditions which increase intracellular Ca^{++}, such as elevation of extracellular Ca^{++} or removal of Na^+ in the superfusing buffer, also induce OAP's (Cranefield and Aronson, 1974). Therefore, interventions which increase intracellular Ca^{++} cause induction of OAP's.

ii. Transient Inward Current

It has been determined that OAP's are the result of a membrane current that is quite different from other membrane currents which occur during the course of the action potential. This current was called the transient inward current (I_{TI}) by Lederer and Tsien (1976) and was subsequently characterized by Kass et al. (1978a,b). They used two-electrode voltage clamp techniques in Purkinje fibres exposed to toxic levels of digitalis to study this current. Their

studies showed that I_{Ti} occurred in tissues exhibiting OAP and that I_{Ti} was observed upon repolarization following a depolarizing pulse (Kass et al., 1978a,b).

I_{Ti} can also be induced in single cell preparations. Fedida et al. (1987) characterized I_{Ti} and its associated aftercontractions in guinea pig ventricular myocytes exposed to high levels of digitalis. They characterized the voltage dependence of I_{Ti} as well as associated aftercontractions and found that the characteristics of I_{Ti} in ventricular tissue differed from those in Purkinje tissue (Fedida et al., 1987). One major difference is that I_{Ti} in Purkinje tissue exhibits a reversal at depolarized membrane potentials (Kass et al., 1978b; Cannell and Lederer, 1986; Han and Ferrier, 1992). However, when I_{Ti} is induced in ventricular muscle by digitalis (Fedida et al., 1987), low extracellular K^+ (Berlin et al., 1989), oxygen-derived free radicals (Matsuura and Shattock, 1992) or simulated ischemia and reperfusion (Cordeiro et al., 1994), no reversal potential is observed. This suggests that the underlying ionic mechanism responsible for I_{Ti} differs depending on the tissue being studied. It is believed that the charge carriers responsible for I_{Ti} are either Na^+-Ca^{++} exchange or a Ca^{++}-activated non-specific cation channel (for a review see January and Fozzard, 1988).

4. ADENOSINE DURING ISCHEMIA AND REPERFUSION.

A. Sources of ADN.

Formation and release of the nucleoside ADN from myocardial tissue has

been shown to occur in response to a decrease in Po_2 (Bardenheuer and Schrader, 1986) as well as to adrenergic stimulation (Miller et al., 1979). Both conditions are known to occur during myocardial ischemia (Belardinelli et al., 1990). ADN is believed to be derived from 2 main sources: a) ADN from hydrolysis of S-adenosylhomocysteine, and b) ADN derived from its phosphorylated derivatives AMP, ADP, and ATP. In myocardial ischemia, ADN is primarily produced through degradation of ATP, ADP and AMP (Lloyd et al., 1988; Olsson and Pearson, 1990). Under normal conditions, ADN is rapidly phosphorylated (in a process requiring energy) and is stored in cellular compartments. However, in the ischemic heart, the energy supply is compromised and ADN levels build up within the cell. ADN is then able to cross cells by both simple diffusion and carrier-mediated diffusion via a bi-directional nucleoside transporter resulting in ADN being available in the extracellular space (Jarvis and Young, 1983). Once in the extracellular space, ADN works by binding to specific subtypes of receptors; these receptors will be discussed in the next section.

B. Sub-types of ADN Receptors.

Currently, there are 4 sub-types of ADN receptors: A_1, A_{2A}, A_{2B}, and A_3 (for a review see Tucker and Linden, 1993). The A_1 and A_2 receptors have been classified based on their ability to either inhibit or stimulate the adenylate cyclase system, respectively (Tucker and Linden, 1993). In addition, rank order

of potencies of various ADN analogues have been used to classify A_1 and A_2 receptors. The actions of ADN at A_1 and A_2 receptors can be antagonized by methylxanthines. Recently, an additional ADN receptor subtype has been identified and isolated in rat testes (Meyerhof et al., 1991). This novel ADN receptor, termed the A_3 receptor, is activated by ADN (for a review see Linden, 1994). Activation of the A3 receptor stimulates inositol trisphosphate production (Ramkumar et al., 1993). However, it exhibits a different rank order of potency to specific analogs of ADN. In addition, the actions of ADN at the A_3 receptor are more resistant to blockade by the methylxanthines.

Radioligand binding studies have shown that there is a low density of ADN receptors in the heart as compared to brain (Linden et al., 1985). Evidence suggests that the A_1 receptor seems to predominate in the heart (Tawfik-Schlieper et al., 1989; Visentin et al., 1990; Belardinelli et al., 1990).

C. Actions of ADN.

The cardiovascular actions of ADN have been extensively examined (for review see Olsson and Pearson, 1990; Pelleg and Belardinelli, 1993). Activation of A_1 receptors by ADN is thought to be protective in myocardial ischemia (Murry et al., 1991; Karmazyn and Cook, 1992) and has been implicated in reduction of infarct size (Liu et al., 1991). ADN is also believed to mediate the protective effects exerted by preconditioning (Shiki and Hearse, 1987). Preconditioning occurs when the heart is subjected to brief repetitive ischemic periods followed

by reperfusion; this has been found to protect the myocardium against a greater ischemic insult (Shiki and Hearse, 1987). It is believed that ADN released during the brief period of ischemia mediates the effects through A_1 receptors, since the effects of preconditioning can by blocked by A_1 selective antagonists. In addition, ADN also alters the electrical and contractile activity in the myocardium by affecting ion conductances. ADN inhibits SA node activity (West and Belardinelli, 1985a,b; Belardinelli et al., 1988), AV node conduction (Clemo and Belardinelli, 1986; Dennis et al., 1992), atrial contractility (Linden et al., 1985) and reverses the effects of β-adrenergic stimulation (Belardinelli and Isenberg, 1983; Isenberg and Belardinelli, 1984) by activating specific A_1 receptors. The A_1 receptor appears to be coupled to the L-type Ca^{++} channel via an inhibitory G_i protein (Belardinelli et al., 1990). In addition, A_1 receptors are coupled, probably via a cAMP-independent pathway, to a $K^+_{Ach,Ado}$ channel in the AV node, SA node, atrial muscle (Belardinelli et al., 1990).

i) Actions of ADN in Ventricular Tissue

Numerous studies have provided evidence for the existence of A_1 receptors in ventricular muscle (Belardinelli et al., 1990). It is generally believed that, under control conditions, application of ADN to guinea pig ventricular myocytes does not produce any electrophysiological changes (Belardinelli and Isenberg, 1983; Isenberg and Belardinelli, 1984). However, when the L-type Ca^{++} current (I_{Ca}) is augmented by β-adrenergic stimulation, ADN attenuates the

increase in I_{Ca} (Isenberg and Belardinelli, 1984). In contrast, Eckert et al. (1993) demonstrated that application of ADN to guinea pig ventricular myocytes in the absence of β-agonists resulted in a decrease in I_{Ca}. The decrease in I_{Ca} was antagonized by A_1 selective blockers, which demonstrated that these effects were mediated via A_1 receptors. The decrease in I_{Ca} by ADN was not a direct effect, but was produced indirectly by causing Ca^{++} release from IP_3-sensitive intracellular stores. Elevated intracellular Ca^{++} levels can reduce the magnitude of I_{Ca} (You et al., 1994). The reason for the disparate results is unclear. One possibility may be the different concentrations of ADN used in the two studies. Isenberg and Belardinelli (1984) found no effect of ADN on I_{Ca} when ADN concentrations between 50-200 μM were used. Eckert et al. (1993) found a decrease in I_{Ca} using ADN concentrations between 10-100 μM. In addition, the decrease in I_{Ca} produced by ADN could be antagonized by the selective A_1 receptor antagonist DPCPX (Eckert et al., 1993). These results suggest that at low ADN concentrations, only one ADN receptor subtype may be activated whereas at higher ADN concentrations multiple receptor subtypes are activated. However, it is unclear whether there is coexistence of multiple ADN receptor subtypes in ventricular tissue.

The existence of A_2 receptors in ventricular tissue is controversial. Shyrock et al. (1993) found that application of selective A_2 receptor agonists did not alter action potential duration, cell shortening or cAMP accumulation in rat, rabbit or guinea pig myocytes. In addition, Wilken et al. (1991) found no

evidence for the presence of A_2 receptors on guinea pig ventricular myocytes. However, Behnke et al. (1990) used a number of selective agonists and antagonists to demonstrate the coexistence of A_1 and A_2 receptors on ventricular myocytes. In another study, Stein et al. (1993) treated guinea pig ventricular myocytes with pertussis toxin to inactivate the inhibitory G-protein and unmasked the cAMP-stimulatory effects of A_2 ADN receptors. Stein et al. (1993) found that activation of the A_2 ADN receptor caused an increase in cAMP levels in ventricular myocytes. This increase in cAMP levels was not accompanied by an increase in I_{Ca} or contractility. This result was unexpected, since activation of the β-adrenergic pathway leads to augmentation of I_{Ca} and contraction via a G-protein cAMP-dependent and cAMP-independent pathway (Pelzer et al., 1990); therefore, it would be expected that A_2 ADN receptor activation would produce a similar effect on I_{Ca}. The exact function of the A_2 ADN receptor in ventricular myocytes remains to be elucidated. Furthermore, the physiological functions of cAMP-stimulatory A_2 receptors and cAMP-inhibitory A_1 receptors co-existing on ventricular myocytes remain to be determined.

There is little evidence for the existence of A_3 receptors in ventricular myocytes. However, it is possible that some of the effects of ADN analogs previously attributed to activation of either A_1 or A_2 receptors may be mediated by A_3 ADN receptors. For example, it was originally shown by Kirsch et al. (1990) that A_1 ADN receptors are coupled to ATP-sensitive K^+ channels via an inhibitory G_i-protein in rat ventricular myocytes. However, they found that the

selective A_1 receptor agonist N_6-cyclohexyladenosine potentiated ATP-sensitive K^+ channel activity in less than 50% of experiments. The low success rate of ATP-sensitive K^+ channel potentiation by A_1 receptor activation suggests that another ADN receptor subtype may mediate the effects of ADN on ATP-sensitive K^+ channels (for a review see Tucker and Linden, 1993).

D. ADN during Myocardial Ischemia.

As mentioned previously (page 37), ADN can be derived either from hydrolysis of S-adenosylhomocysteine or from dephosphorylation of its phosphorylated derivatives (AMP, ADP, and ATP). In conditions of reversible myocardial ischemia it has been shown that large amounts of ADN are released in ischemia and washed away on reperfusion (Berne, 1963). Much of the ADN produced in ischemia is produced through degradation of adenine nucleotides (Jennings and Steenbergen, 1985). Evidence suggests that the release of ADN is protective and that drugs which enhance its release, block its re-uptake or stimulate ADN's effects at the receptor level should increase its protective effects (for review see Boachie-Ansah et al., 1993). Conversely, blockade of ADN receptors should be detrimental (Boachie-Ansah et al., 1993). Janier et al. (1993) examined the role of ADN in isolated rabbit hearts subjected to low-flow ischemia followed by reperfusion. They found that the time of onset of ischemic contracture was delayed in the presence of 1 μM ADN. This protective effect was accompanied by an increase in cytosolic ATP levels, and enhanced

contractile function upon reperfusion. In addition, all the protective effects of ADN were blocked by the non-selective ADN antagonist 8-phenyltheophylline (8-PT), which indicates that the effects were receptor-mediated. In another study, Lasley and Mentzer (1993) demonstrated that the cardioprotective effects of ADN in ischemia were mediated via A_1 ADN receptors. Furthermore, they demonstrated that pretreatment with pertussis toxin abolished the protective effects of ADN. This suggests that these effects of ADN are mediated via A_1 ADN receptors coupled to a pertussis toxin-sensitive G-protein, probably G_i.

Increasing the ADN levels in the interstitial space during myocardial ischemia should increase the protective effects. The drug acadesine has been shown to augment the ADN concentration in the interstitial space during ischemia suggesting that acadesine either enhances release or prevents reuptake of ADN (Gruber et al., 1989). In a rat ventricular myocyte model of simulated ischemia and reperfusion, acadesine was shown to partially protect against ischemia and reperfusion injury (Gruver et al., 1994). Furthermore, the effects of acadesine were abolished by the non-selective ADN antagonist 8-sulfophenyltheophylline which shows that the effects are receptor mediated (Gruver et al., 1994).

Several studies, however, have suggested that ADN might not be protective in ischemia. In rat ventricular myocytes Ganote et al. (1993) demonstrated that ADN and A_1 selective agonists exhibit minor protective effects against ischemic injury. In another study which utilized isolated perfused rabbit

hearts subjected to ischemia and reperfusion, A_1 receptor stimulation facilitated ventricular fibrillation by decreasing the action potential duration (Chi et al., 1994). However, they found the arrhythmogenic actions of A_1 receptor stimulation could be modulated by simultaneous activation of ventricular A_2 ADN receptor activation.

In summary, large amounts of ADN are released from myocardial tissue during ischemia. Much of the evidence suggests that the release of ADN is protective and that the protective effect is mediated via the A_1 ADN receptor subtype.

E. ADN Antagonism of Oscillatory Afterpotentials

Addition of the β-adrenergic agonist isoproterenol has been shown to induce I_{Tl} in guinea pig ventricular myocytes (Belardinelli and Isenberg, 1983). The effect of β-adrenergic stimulation on ventricular tissue has been well documented (for a review see Trautwein and Hescheler, 1990). Activation of β_1 receptors on the sarcolemma leads to an increase in the L-type Ca^{++} current by both a cAMP-dependent and a cAMP-independent mechanism (Pelzer et al., 1990). The cAMP independent mechanism occurs via a membrane-delimited G_s-protein acting directly on the Ca^{++} channel. It is believed that this increase in I_{Ca} results in an elevation of cytosolic Ca^{++} levels. Indeed, studies using fluorescent dyes sensitive to Ca^{++} showed that addition of isoproterenol results in an elevation in the fluorescence signal and hence the Ca^{++} level (Fenton et

al., 1991). As previously mentioned (page 36), I_{Ti} is the underlying current responsible for OAP's and the appearance of both is closely linked to a rise in intracellular Ca^{++} (Kass et al., 1978a,b). ADN has anti-adrenergic effects on myocardial ventricular tissue (Belardinelli et al., 1990). Addition of ADN, in the continued presence of isoproterenol, results in a decrease in the Ca^{++} transient signal (Fenton et al., 1991). In addition, ADN is capable of reducing or abolishing I_{Ti} induced by isoproterenol (Belardinelli and Isenberg, 1983; Song et al., 1992). It has been shown that ADN is capable of abolishing I_{Ti} induced by β-adrenoceptor stimulation but not by Na^+-K^+-ATPase inhibition (Belardinelli and Isenberg, 1983; Song et al., 1992). This suggests that when I_{Ti} is induced by a mechanism that elevates cAMP, ADN can abolish I_{Ti}, most likely through a mechanism involving an inhibitory G_i-protein. However, ADN has no effect on I_{Ti} induced by inhibition of Na^+-K^+-ATPase.

5. MODELS OF ISCHEMIA AND REPERFUSION.

A. Multicellular Models of Ischemia and Reperfusion.

Previous investigations have shown that there are a number of changes that occur in the myocardium in response to ischemic conditions and reperfusion. Some of these changes include electrophysiological disturbances, changes in ionic gradients within myocardial cells, and profound reduction in the magnitude of contraction. However, there are a number of difficulties associated with studying myocardial ischemia and reperfusion in intact hearts. The ideal

system to study various factors that contribute to ischemic myocardial injury would be one in which the investigator could independently modulate the intensity and duration of ischemia or elements of ischemia such as hypoxia (Murphy et al., 1987). Therefore, a number of studies have utilized isolated segments of myocardial tissue exposed to simulated ischemia and reperfusion of a known duration.

Multicellular models of simulated ischemia and reperfusion have been developed in Purkinje fibre preparations (Ferrier et al., 1985) and isolated right ventricular free wall preparations (Ferrier and Guyette, 1991). These models of simulated ischemia and reperfusion utilized a modified Tyrode's solution to mimic specific conditions of ischemia. These conditions include hyperkalemia, hypercapnia, lactate accumulation, acidosis, substrate deprivation and hypoxia. The right ventricular free model of simulated ischemia and reperfusion produced abbreviation of APD and depolarization of MDP during ischemia. In addition, this model generates arrhythmias with characteristics of both reentry and OAP's both during exposure to this ischemic solution and upon reperfusion. This model has been used extensively to correlate cellular and electrophysiological actions of drugs with pro- and anti-arrhythmic drug effects (Lukas and Ferrier, 1987; Li and Ferrier, 1991, 1992; Pasnani and Ferrier, 1992). However, this multicellular preparation does not permit study of changes in ionic currents and contractile activity induced by exposure to simulated ischemia and reperfusion. Adaptation of this model to single cardiac myocytes would allow simultaneous measurement

of transmembrane voltage, ionic currents and contraction.

B. Previous Single Cell Models

In an attempt to study changes at the cellular level during myocardial ischemia and reperfusion, various methods have been employed. Studies at the level of single cardiac myocytes have focused on effects of metabolic inhibition (Lederer et al., 1989; Goldhaber et al., 1991), hypoxia/anoxia (Stern et al., 1985; Benndorf et al., 1991a,b) and systems which generate oxygen-derived free radicals (Matsuura and Shattock, 1991). However, the responses of cardiac tissue to metabolic inhibition and hypoxia are quite different from those seen in response to myocardial ischemia (Lee and Allen, 1991). Since true myocardial ischemia consists of numerous components, it is difficult to mimic the components of true ischemia at the cellular level. However, cellular models do offer advantages not seen in tissue models. With cellular models, the extracellular conditions can be controlled with consistency.

Many models of hypoxia in single myocytes have been developed. Some of these models have employed a layer of inert gas over the superfusion chamber containing the myocytes in an attempt to lower the oxygen tension (Stern et al., 1985, 1988; Benndorf et al., 1991a,b). Results of all these studies have demonstrated that when the myocytes are made adequately hypoxic, inhibition of contraction occurs prior to the onset of a semi-contracted state called rigor. Depending on the duration of hypoxia, reoxygenation results in

either partial recovery of the myocytes or hypercontracture (Stern et al., 1985). However, hypoxia only inhibits oxidative phosphorylation and does not inhibit glycolysis.

In an attempt to simulate other components of ischemia, some studies have used the metabolic poisons cyanide (to inhibit oxidative phosphorylation) and 2-deoxyglucose (to prevent glycolysis) (Lederer et al., 1989; Goldhaber et al., 1991). Results from these studies are similar to those obtained from studies of hypoxia with the exception that cytosolic ATP content is depleted more rapidly during metabolic inhibition. However, cellular models of hypoxia and metabolic inhibition do not mimic certain other aspects of tissue ischemia including extracellular K^+ accumulation, lactate accumulation and a reduction in pH.

Various cellular models of simulated ischemia and reperfusion have previously been developed (Koyama et al., 1991; Nishida et al., 1993). In both these studies ischemia was mimicked by superfusing the myocytes with a buffer simulating specific conditions of ischemia. However, in both these models, transmembrane voltages, ionic currents and cell shortening were not recorded.

6. OBJECTIVES

1) To develop a cellular model of simulated ischemia and reperfusion and to use this model to examine changes in transmembrane ionic currents (I_{Ca} and I_{Ti}), transmembrane voltages (MDP and APD) and cell shortening during ischemic conditions and reperfusion.

2) To examine the effect of simulated ischemia and reperfusion on contractions initiated by a voltage sensitive Ca^{++} release mechanism.

3) To determine whether pharmacological agents which alter intracellular Na^{+} and Ca^{++} loading affect signs of Ca^{++} overload in reperfusion.

4) To determine whether exogenous ADN affects changes in contraction, MDP, APD and I_{Ca} in ischemia and reperfusion. A further objective was to determine whether the effects of ADN are receptor mediated and to establish which receptor subtype is involved.

MATERIALS AND METHODS

1. ANIMALS

All experiments were conducted on isolated guinea pig ventricular myocytes. Male High Oak guinea pigs (350-450 g) were obtained from Montreal, Canada. Animals were housed in the Dalhousie Animal Care facility and were maintained according to the guidelines issued by the Canadian Council on Animal Care (2 Volumes, Ottawa, Ontario: CCAC, 1980-1984). Food and water were freely available.

2. MYOCYTE ISOLATION

A. Perfusion Protocol

Guinea pigs were given heparin (1000 units/ml stock solution) administered by the intraperitoneal route (0.4 units/g body weight). After 45 minutes, animals were sacrificed by stunning followed immediately by exsanguination via the carotid vessels. The heart was rapidly removed through a parasternal incision and placed in a beaker containing 50-100 ml of a nominally Ca^{++}-free buffer whose composition is described in Table 1 (page 64). The heart was quickly trimmed of excess tissue then mounted, by the aorta, onto a cannula for perfusion in a retrograde fashion with the nominally Ca^{++}-free buffer. Perfusion of the heart was gravity-fed and the fluid level of the buffer in the column was kept at a constant height, to allow a constant perfusion pressure. The nominally Ca^{++}-free buffer was maintained at 36 ± 0.5° C and bubbled with a 95% O_2, 5%

CO_2 gas mixture (Union Carbide, Canada Limited). Perfusion of the heart was continued for a period of about 10 minutes, until the heart was cleared of blood and contractions had ceased.

B. Enzyme Digestion.

The myocyte isolation procedure was originally described by Bustamante et al. (1981) with minor modifications (Cordeiro et al., 1992). Following the perfusion, the heart was subjected to enzymatic digestion with nominally Ca^{++}-free buffer supplemented with protease (Sigma Type XIV) and collagenase (Sigma Type V). The protease and collagenase were dissolved in 100 ml of nominally Ca^{++}-free buffer which gave final concentrations of 5.2 units/ml and 142.6 units/ml, respectively. In addition, $CaCl_2$ was added to the enzyme solution, such that the final concentration of Ca^{++} in the enzyme solution was 200 μM. The enzyme solution was added to the column, and the heart was perfused for a period of 8-10 minutes. The enzyme was continuously recirculated to maintain adequate perfusion pressure. The enzyme solution was maintained at 36 ± 0.5° C and bubbled with a 95% O_2, 5% CO_2 gas mixture. After a period of 8-10 minutes, the heart was visually inspected and, if judged to be flaccid, the enzyme digestion was terminated. The ventricles were then excised from the rest of the heart and placed in a high K^+ (KB) solution (You et al., 1994) with minor modifications (Table 2, page 64). The ventricles were minced and gently swirled in the KB solution. The myocyte suspension was then

decanted into a beaker and stored at room temperature until use.

C. Superfusion of Myocytes.

An aliquot of the myocyte suspension was placed in a petri dish within an open perfusion micro-incubator (Model PDMI-2, Medical Systems Corporation, Greenvale, N.Y., U.S.A.) mounted on the stage of an inverted microscope (Olympus Model IMT-2, Tokyo, Japan). The myocytes were allowed to adhere to the bottom of the petri dish for a period of 10 minutes and were then superfused with 0.5 mM Ca^{++} Tyrode's solution (Table 3) bubbled with 95% O_2, 5% CO_2. The solutions were delivered to the bath by a peristaltic pump (Piper Pump, Dungey Incorporated, Agincourt, Ont.) through plastic tubing at a flow rate of 3 ml/minute. The solution change time was measured by observing the change in membrane potential of a myocyte in response to a step change in K^+ concentration. The solution change over time was found to be 45 seconds. The superfusing buffer within the petri dish was removed by a suction needle placed at the opposite end from the inlet tubing. The placement of the suction needle ensured that all parts of the bath were adequately superfused with fresh buffer, as determined by visualization of a coloured dye flowing through the superfusion chamber. In addition, the level of the buffer within the superfusion chamber was kept constant. The temperature of the Tyrode's solution within the superfusion chamber was maintained at 36.5 ± 0.5° C by a bipolar temperature controller (Model TC-202, Medical Systems Corporation). Following the initial superfusion

of the myocytes with 0.5 mM Ca^{++} Tyrode's solution, the concentration of Ca^{++} in the Tyrode's solution was gradually increased until a final concentration of 2.5 mM Ca^{++} was achieved. With this isolation procedure, a 50-80% yield of Ca^{++}-tolerant rod shaped myocytes was obtained. Experiments were performed only on myocytes which were free of membrane blebs and had a resting membrane potential greater than -80 mV.

3. MICROMANIPULATORS AND ELECTRODES.

All transmembrane electrical activity from ventricular myocytes was recorded with high resistance glass microelectrodes filled with filtered 2.7 M KCl (pore size 0.2 μm) to avoid buffering intracellular Ca^{++}. Tip resistances of the microelectrodes used in these studies varied between 18-28 MΩ. Tip resistance was measured by placing the filled electrode into the perfusion chamber, applying a 1 nA current and calculating the microelectrode resistance from the voltage reading on the Axoclamp-2A (Axon Instruments, Burlingame, Calif., U.S.A.). The microelectrodes were fabricated from glass capillaries which contained an inner filament and had an outer diameter of 1.2 mm and an inner diameter of 0.78 mm (World Precision Instruments, Incorporated, New Haven, CT., U.S.A.). Microelectrodes were pulled using a programable micropipette puller (Flaming/Brown Micropipette Puller Model P-87, Sutter Instrument Company, Novato, Ca., U.S.A.). Microelectrodes were inserted into microelectrode holders (MEH-1S12, World Precision Instruments) which

contained a Ag/AgCl pellet and were filled with filtered 2.7 M KCl. Both the microelectrode and the microelectrode holder were held in a mechanical micromanipulator (Leitz Incorporated, Wetzlar, Germany). Once the ventricular myocyte was impaled with the microelectrode, both conventional and voltage clamp recordings could be made.

4. RECORDING SETUP

A. Conventional Recording Studies.

All transmembrane voltages were recorded from ventricular myocytes impaled with microelectrodes (described in the previous section). Microelectrodes were connected to a high impedance unity gain headstage (model HS-2, gain=X1, Axon Instruments) which in turn was connected to the Axoclamp-2A console (Axon Instruments). The reference electrode was fabricated from the same capillary tubing used to make the microelectrodes. The capillary tube was filled with 2.7 M KCl and the tip of the capillary tube was plugged with asbestos. The capillary tube was placed downstream from the myocytes near the efflux of the bath. When both the microelectrode and the reference electrode were placed into normal Tyrode's solution within the perfusion chamber, the offest potential was zeroed with the input offset on the Axoclamp-2A. Cells were impaled through the microelectrodes and the membrane potential measured from the myocyte was connected to the Axoclamp-2A. The output of the voltage signal was connected to an Acquisition

Logic board (16 channel analog to digital conversion board, Axon Instruments). The A/D board was connected to an IBM compatible microcomputer (80286 and 80287 coprocessors) which acquired and stored the data. The maximum acquisition rate was 125 kHz for A/D conversion. The output of the membrane potential from the Axoclamp-2A was also displayed on a four channel, dual time base oscilloscope (Tektronix model 5110, Beaverton, Oregon, U.S.A.). Both the Axotape (version 1.2) software program and pClamp (version 5.1) software program (Axon Instruments) were used to acquire and store the data. Action potentials were initiated by 3 msec current pulses (2-3 nA) delivered through the microelectrode in trains of 15 (separated by 3 sec pauses) at a basic cycle length of 500 msec. Current pulses were generated by triggering the Axoclamp-2A with a digital pulse generator (Pulsar 6i, Frederick Haer and Co.). The 3 sec pause was controlled by a Pulsar 4i which was TTL-paired to the Pulsar 6i.

B. Cell Shortening Measurements

The myocytes were viewed through a closed circuit video camera (Panasonic, Model WV-1410) and displayed on a video monitor (Hitashi-Denshi, Model VM-1220C). Cell shortening measurements were made with a video edge detector (Crescent Electronics, Sandy, Utah, U.S.A.). The sampling rate of the video edge detector was 120 kHz. The output of the video edge detector was connected to the oscilloscope. The output of the video edge detector was also connected to the Acquisition Logic board which in turn was connected to an IBM

compatible microcomputer (as described in the previous section). The corresponding cell shortening traces elicited by either voltage clamp or conventional recording, were displayed simultaneously on the microcomputer and on the oscilloscope.

C. Voltage Clamp Studies

All voltage clamp studies reported here were performed with single electrode voltage clamp using intracellular microelectrodes. In most of these studies, the discontinuous single electrode voltage clamp technique was used. With discontinuous single electrode voltage clamp, the Axoclamp-2A cycles between voltage recording and current passing modes. The microelectrode spends 70% of the cycle in voltage recording mode and 30% of the cycle in current passing mode. With discontinuous single electrode voltage clamp, there are no errors produced by the microelectrode resistance in series with the cell. This method of voltage clamping has been described in detail (Finkel and Redman, 1984).

With the Axoclamp-2A set in Bridge mode, the microelectrode was manipulated until it was just above the chosen myocyte. A 1 nA current was then injected through the microelectrode using the Axoclamp-2A and the microelectrode resistance was measured. The resistance of the microelectrode was then zeroed using the bridge balance dial on the Axoclamp-2A console. The Axoclamp-2A was then set in discontinuous current clamp (DCC) mode and

a square current command of 1 nA was applied to the electrode. The oscilloscope was set at a rapid time base so that the injected waveform could be observed. The injected waveform was optimally adjusted using the capacitance neutralization dial on the Axoclamp-2A so that the waveform decayed most rapidly but without any overshoot. In addition, the switching rate was set to allow the injected current to decay before the next current injection cycle. The switching rate was usually set between 12-16 kHz. When the equipment was adjusted for optimal discontinuous single electrode voltage clamp, the Axoclamp-2A was set in Bridge mode and the injected current was removed.

When a myocyte was successfully impaled, both maximum diastolic potential (MDP) and action potential duration (APD) were recorded. The Axoclamp-2A was set in discontinuous single electrode voltage clamp (SEVC) mode. The microelectrode was connected to a high impedance unity gain head stage which, in turn, was connected to the Axoclamp-2A console. The ionic currents elicited by voltage clamp were filtered at 3 kHz by the Axoclamp-2A console. The output of the filtered current was connected to an Acquisition Logic board which, in turn, was connected to an IBM compatible microcomputer which acquired and stored the data. The filtered currents were also displayed on the oscilloscope. The voltage clamp protocols were generated by the Clampex software program (Axon Instruments). The pClamp software program was used for data acquisition and storage as well as to generate voltage clamp protocols.

5. EXPERIMENTAL PROTOCOL

A. Experimental Methods.

In a cell successfully impaled with a microelectrode, both conventional and voltage clamp recordings were made. These initial measurements were the control recordings. After a period of 10 minutes, myocytes were exposed to simulated ischemia. Simulated ischemia was produced by superfusion of the myocytes with a modified "ischemic" Tyrode's solution (Ferrier et al., 1985) for a period of 20 minutes (Table 4, page 65). Optimization of the time period to 20 min was performed empirically. These results will be discussed in the Results section. The modified Tyrode's solution mimicked several conditions of ischemia, namely hypoxia, acidosis, hyperkalemia, lactate accumulation and substrate deprivation. The oxygen tension was measured with a blood-gas analyzer (Corning). When the ischemic Tyrode's solution was bubbled with 90% N_2, 10% CO_2 gas (Union Carbide) the resulting Po_2 was 26.9 ± 6.0 mm Hg (n=4). In addition, a 90% N_2, 10% CO_2 gas phase was layered over the superfusion chamber to exclude atmospheric oxygen. Ischemic conditions were not possible unless the 90% N_2, 10% CO_2 gas phase was present over the superfusion chamber. Reperfusion was simulated by superfusing the myocytes with normal Tyrode's solution. The reperfusion period was 30 minutes. In control experiments, no significant run down in transmembrane currents and contractions occurred, even after 60 minutes of recording with intracellular electrodes. After one ischemia and reperfusion experiment was performed, the

myocytes were removed from the superfusion chamber and another aliquot of cell suspension in KB solution was placed in the perfusion chamber. Thus, no myocyte was exposed to simulated ischemia more than once.

B. Conventional Recordings

In conventional recording mode, action potentials were initiated by 3 msec current pulses (2-3 nA) delivered through the microelectrode in trains of 15 (separated by 3 sec pauses) at a basic cycle length of 500 msec. Current pulses were generated by triggering the Axoclamp-2A with a digital pulse generator (Pulsar 6i). APD and MDP measurements were made at 5 min intervals from conventional recordings. Abnormal electrical activity such as OAP and early afterdepolarizations were recorded with conventional recordings. Abnormal electrical activity tended to occur in early reperfusion. Therefore, APD and MDP sampling was more frequent during this period.

C. Voltage Clamp Recordings

During voltage clamp the holding potential was -80 mV. For most experiments, the voltage clamp protocol consisted of the following: one pre-pulse to +20 mV for 200 msec followed by a return step to -80 mV for 500 msec; a 500 msec step to -40 mV to discharge and inactivate Na^+ channels; a 300 msec step to -10 mV to activate and allow measurement of I_{Ca}; a 300 msec step to +20 mV and repolarization to -60 mV for 1.8 sec to determine whether I_{Tl} could

be induced. The peak amplitude of I_{Ca} was recorded from 5 averaged traces and measured as the difference between the peak inward current and the net current after 300 msec. Contraction was recorded from 5 averaged traces and the magnitude was measured as the difference between the relaxed and the contracted state. For comparison purposes, contractions were expressed as a percentage of the pre-ischemic contraction.

Because of the rapid kinetics and large magnitude of I_{Na}, it was not possible to maintain voltage control when this current was activated. Therefore, no quantitative data on I_{Na} are reported in these studies. However, smaller currents with slower time courses (such as I_{Ca} and I_{Tl}) were recorded accurately. To confirm that the membrane potential measured by the single electrode during switch clamp was an accurate measure of the membrane potential, a second independent electrode was used to continuously monitor the membrane potential of the myocyte (Ferrier and Howlett, 1995). It was determined that the potential recorded by the current passing electrode was accurate reflection of the membrane potential. In addition, the average rise time for the voltage steps was between 1-2 ms (Ferrier and Howlett, 1995).

6. PHYSIOLOGICAL BUFFERS

During the myocyte dissociation procedure, preparations were superfused with the nominally Ca^{++}-free Tyrode's solution described in Table 1 (page 64). Storage of the isolated ventricular myocytes was in a high K^+ KB solution (You et

al., 1994) as described in Table 2 (page 64). Under control conditions and during reperfusion, myocytes were superfused with normal Tyrode's solution, the composition of which is shown in Table 3 (page 65). Normal Tyrode's solution was bubbled continuously with a 95% O_2, 5% CO_2 gas mixture to keep the pH at 7.35 ± 0.05. Ischemia was simulated by superfusing the myocytes with an "ischemic" Tyrode's solution (Ferrier et al., 1985) whose composition is described in Table 4 (page 65). Ischemic Tyrode's solution was bubbled continuously with a 90% N_2, 10% CO_2 to make the buffer hypoxic. Acidosis of the ischemic buffer was achieved by reducing the $NaHCO_3$ concentration from 20 to 6 mM (Table 4, page 65).

7. DRUGS AND REAGENTS

The drugs used in this study include: lidocaine hydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.), verapamil hydrochloride (Sigma), amiloride hydrochloride (Sigma), protease type XIV (Sigma), collagenase type IA (Sigma), adenosine (Sigma), 8-phenyltheophylline (Sigma), 8-cyclopentyltheophylline (Research Biochemicals Incorporated, Natick, Ma., U.S.A.), ryanodine (Calbiochem Corp.), manganous chloride (BDH), nickel chloride (BDH), HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma). Nifedipine was a gift from Miles Pharmaceutical (New Haven, Conn., U.S.A.). All drugs were added from an aqueous stock solution and all drugs were water soluble with the exception of 8-phenyltheophylline, 8-

cyclopentyltheophylline and nifedipine. Both 8-phenyltheophylline and 8-cyclopentyltheophylline were dissolved in a 0.1 N NaOH solution. Addition of either 8-PT or CPT did not alter the pH of either the normal or ischemic Tyrode's solution. Nifedipine was dissolved in 100% ethanol as a stock of 10^{-2} M. The final ethanol concentration superfusing the myocytes did not exceed 0.05%. This concentration did not affect electrical or contractile activity.

8. STATISTICAL ANALYSIS

All data are presented as mean ± S.E.M. unless otherwise indicated. Each replicate represents a cell from a different animal. Thus, only one cell from each heart was used in a given experimental condition. Statistical differences within an experimental group were analysed by a one way repeated measures ANOVA. Multiple comparisons versus control were performed with a Dunnett's test. Statistical differences between two experimental groups were analysed with a two way repeated measures ANOVA. Multiple comparisons versus control were performed with a Student-Newman-Keuls test. Changes in the incidence of I_{Tl} were performed with a Chi-squared test. All statistical analyses were performed by the computer program SigmaStat (Jandel Scientific).

Table 1. Composition of Nominally Ca^{++}-free Tyrode's Solution for Myocyte Isolation (mM)

NaCl	120
KCl	4
NaH_2PO_4	4
$NaHCO_3$	22
D-glucose	10
$MgCl_2$	1
pH	7.4 ± 0.05

Table 2. Composition of High K^+ KB Solution (mM) (You et al., 1994)

KCl	30
KOH	80
KH_2PO_4	30
$MgSO_4$	3
Glutamate	50
Taurine	20
EGTA	0.5
HEPES	10
D-glucose	10

Table 3. Composition of Normal Tyrode's Solution (mM)

NaCl	129
KCl	4
NaH_2PO_4	0.9
$NaHCO_3$	20
D-glucose	5.5
$MgSO_4$	0.5
$CaCl_2$	2.5
Bubbled with	95% O_2, 5% CO_2
pH	7.4
Po_2 (mm Hg)	811.7 ± 25.9

Table 4. Composition of Ischemic Tyrode's Solution (mM) (Ferrier et al., 1985)

NaCl	123
KCl	8
NaH_2PO_4	0.9
$NaHCO_3$	6
Na-lactate	20
$MgSO_4$	0.5
$CaCl_2$	2.5
Bubbled with	90% N_2, 10% CO_2
pH	6.8
Po_2 (mm Hg)	26.9 ± 6.0

RESULTS

1. CELLULAR MODEL OF ISCHEMIA AND REPERFUSION.

A. Development of Single Cell Model of Ischemia and Reperfusion

The single myocyte model of simulated ischemia and reperfusion reported in this thesis is an adaptation of a model originally developed for use in multicellular preparations (Ferrier et al., 1985; Ferrier and Guyette, 1991). In this model, cells are superfused with a solution which mimics certain conditions of ischemia, namely hyperkalemia, hypercapnia, lactate accumulation, acidosis, substrate deprivation and hypoxia. In preliminary experiments, exposure to "ischemic" Tyrode's solution (Table 4, page 65) resulted in depolarization of the membrane potential. However, none of the other myocardial changes reported previously (i.e., APD shortening and contractile failure) occurred upon prolonged exposure to ischemic Tyrode's solution. Moreover, reperfusion did not result in abnormal electrical activity. It was hypothesized that the absence of significant electrophysiological effects in ischemia and reperfusion might be due to atmospheric oxygen dissolved in the ischemic solution. Previous studies in other laboratories have used an argon gas phase over the superfusion chamber to achieve hypoxia (Stern et al., 1985, 1988); this gas phase occluded much of the atmospheric oxygen which may dissolve into the superfusing solution. Therefore, subsequent experiments involved exposing the myocytes to ischemic

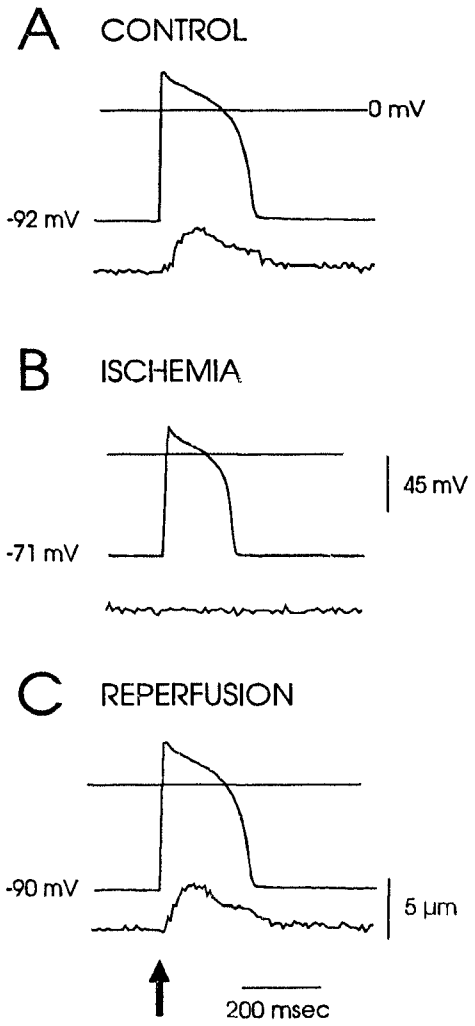

FIGURE 1: Representative examples of action potentials and contractions observed at various stages of an experiment. The horizontal line represents 0 mV. Cell shortening was measured in micrometers (µm). **Panel A)** Action potential and associated contraction recorded prior to exposure to simulated ischemia. **Panel B)** APD_{90} decreased and contraction was abolished after 20 min exposure to ischemic conditions. **Panel C)** APD_{90} and contraction recovered to control levels after 30 min of reperfusion. The time at which stimulation was delivered is indicated by the arrow.

Tyrode's solution in the presence of a 90% N_2/10% CO_2 gas phase layered over the superfusion chamber. This gas mixture was identical to the gas used to bubble the ischemic Tyrode's solution. Upon exposure to the ischemic Tyrode's solution in the presence of the gas phase, contractile failure was consistently observed. In addition, the APD gradually decreased as duration of exposure to ischemic conditions increased. If the duration of ischemia was of sufficient length (about 20 minutes), reperfusion resulted in the appearance of abnormal electrical activity within the first few minutes of reperfusion. In contrast, spontaneous electrical activity was never observed in ventricular myocytes under control conditions. Occasionally, myocytes rounded-up and hypercontracted during reperfusion however most cells recovered normal electrical activity. Under conditions developed with these preliminary experiments, transmembrane voltages, ionic currents, and contraction were measured from myocytes exposed to simulated ischemia and reperfusion.

B. Changes in Membrane Voltage and Associated Contraction during Simulated Ischemia and Reperfusion.

Figure 1A shows a representative action potential and corresponding contraction recorded from a guinea pig ventricular myocyte in normal Tyrode's solution. Action potentials were initiated by a 3 msec depolarizing current pulse delivered to the impaled myocyte via the microelectrode. These action potentials were measured in conventional recording mode. The arrow in figure 1

illustrates the time at which the stimulation was delivered. Under these control conditions, the mean MDP of the cells was -89±1 mV and the action potential duration at 90% repolarization (APD_{90}) was 257±14 msec (n=18). Thus, action potential configuration was similar to that reported previously for guinea pig ventricular myocytes (Isenberg and Klockner, 1982). Spontaneous activity was never observed in ventricular myocytes under control conditions.

Following the control period, cells were exposed to simulated ischemia. In preliminary experiments, the duration of the ischemic period was varied. A 45 min ischemic period resulted in marked depolarization (to approximately -30 mV) and eventual loss of both electrical and contractile activity. Upon reperfusion, electrical activity failed to recover and cells hypercontracted. A 10 min period of ischemia resulted in little or no abnormal electrical activity upon reperfusion. Based on these initial experiments we chose to utilize a 20 min ischemic period. A 20 minute exposure to ischemic Tyrode's solution resulted in contractile failure and APD abbreviation during ischemia. In addition, reperfusion resulted in consistent appearance of abnormal electrical activity; however, the 20 min ischemic period usually allowed the myocytes to completely recover electrical and contractile activity upon reperfusion. Figure 1B shows a representative action potential and contraction recorded from a cell after exposure to ischemic conditions for 20 min. The APD_{90} was reduced to 188±12 msec and contractions were abolished by this treatment. Cells never exhibited contracture throughout the 20 min ischemic period. In addition, MDP was -67±4 mV (n=18). Figure 1C,

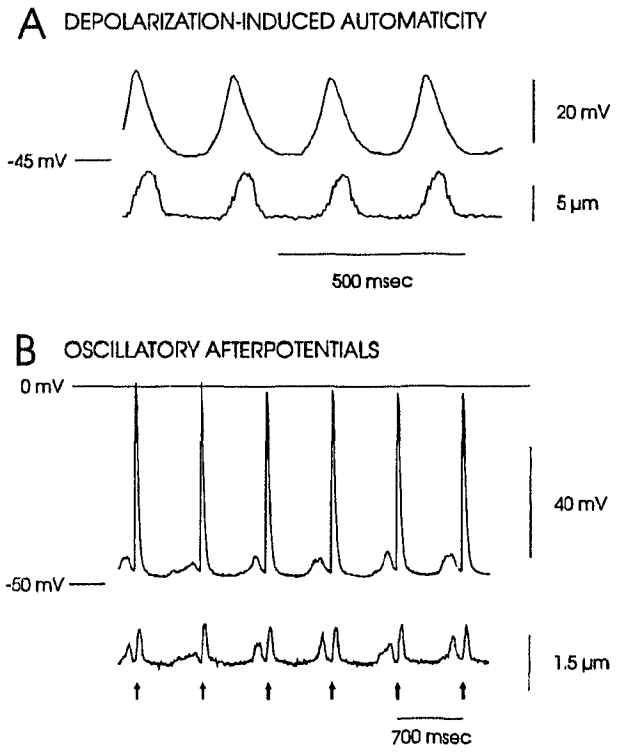

FIGURE 2: Representative examples of abnormal electrical activity and contractions recorded in early reperfusion (3-4 min). **Panel A)** Electrical activity resembling depolarization-induced automaticity. Spontaneous potentials (upper trace) and contractions (lower trace) appeared in this cell when the membrane potential decreased to -45 mV. **Panel B)** Action potentials and OAP's (upper trace) were accompanied by contractions and aftercontractions (lower trace), respectively. The horizontal line represents 0 mV. Stimulation is indicated by the arrows.

recorded following 25 min of reperfusion, shows that action potential configuration and contraction returned to control values in late reperfusion. Earlier stages of reperfusion were characterized by complex changes in electrical and contractile activity as described below.

Figure 2 shows examples of abnormal electrical and contractile activity recorded from single myocytes within the first 10 mins of reperfusion. Some cells exhibited automaticity associated with spontaneous depolarization to membrane potentials positive to -50 mV (Figure A). This activity most likely corresponds to that referred to as depolarization-induced automaticity by Katzung (1975). Each spontaneous action potential was accompanied by a contraction (Figure 2A). Depolarization-induced automaticity was recorded in 6 out of 23 cells. Other cells exhibited OAP's and aftercontractions, commonly associated with Ca^{++} overload (18/23 cells). The arrows in figure 2B illustrate the time at which the stimulating current pulse was given. OAP's and aftercontractions occurred late in diastole, just in advance of the next driven beat as shown in Figure 2B. A few cells (6/23) exhibited early afterdepolarizations (data not shown). Abnormal activity occurred in 16 of 23 experiments and some cells exhibited multiple effects. Occasionally, myocytes rounded-up and hypercontracted during reperfusion (3/23 cells) however most cells recovered normal electrical and contractile activity after 10-20 min of reperfusion (Figure 1C).

It has been reported that the underlying current responsible for

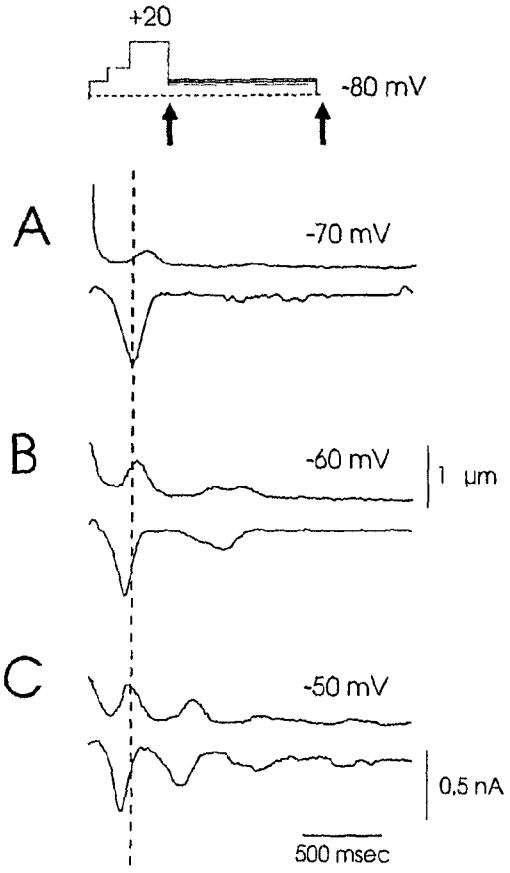

FIGURE 3: Examples of I_{Tl} and associated aftercontractions observed upon repolarization to various potentials during reperfusion. Voltage clamp protocol is illustrated at the top of the figure. Arrows indicate the portion of the voltage clamp protocol for which currents and contractions are illustrated. **Panel A)** A single I_{Tl} and aftercontraction were recorded at -70 mV. Multiple I_{Tl}'s and aftercontractions were recorded upon repolarization to -60 mV **(panel B)** and -50 mV **(panel C)**. I_{Tl} occurred earlier at more depolarized potentials (see dashed line for reference). Aftercontractions always occurred with a lag with respect to I_{Tl}

appearance of OAP is I_{TI}. I_{TI} always appears upon repolarization following a depolarizing activating step and appearance of I_{TI} is closely linked to a rise in intracellular Ca^{++}. To determine whether I_{TI} was present, cells also were voltage clamped in early reperfusion. Figure 3 shows representative examples of I_{TI} and aftercontractions recorded under voltage clamp conditions after 3 min of reperfusion. The voltage clamp protocol is illustrated at the top of figure 3. The holding potential was -80 mV. Prior to the test step, there was a 300 msec pre-pulse to +10 mV (pre-pulse not shown). The voltage clamp protocol consisted of a 300 msec step to -40 mV to discharge and inactivate Na^+ channels; a 300 msec step to -10 mV to activate and allow measurement of I_{Ca}; an activation step to +20 mV and repolarization to -60 mV to determine whether I_{TI} was inducible. I_{TI} was observed upon repolarization following a depolarizing activating step. Repolarization to -70 mV elicited a single I_{TI} and aftercontraction (panel A). Repolarization to more depolarized potentials induced multiple I_{TI} and aftercontractions (panels B and C). I_{TI} always preceded aftercontractions, however the number and timing of oscillations was dependent on membrane potential. Both I_{TI} and aftercontractions occurred earlier following the repolarization step as the repolarization potential was made less negative (figure 3). I_{TI} and aftercontractions were observed in 18/23 cells in early reperfusion and characteristically appeared during the first 10 min of reperfusion. I_{TI} declined in amplitude as the reperfusion time increased and gradually disappeared after 10-20 min of reperfusion. Corresponding aftercontractions

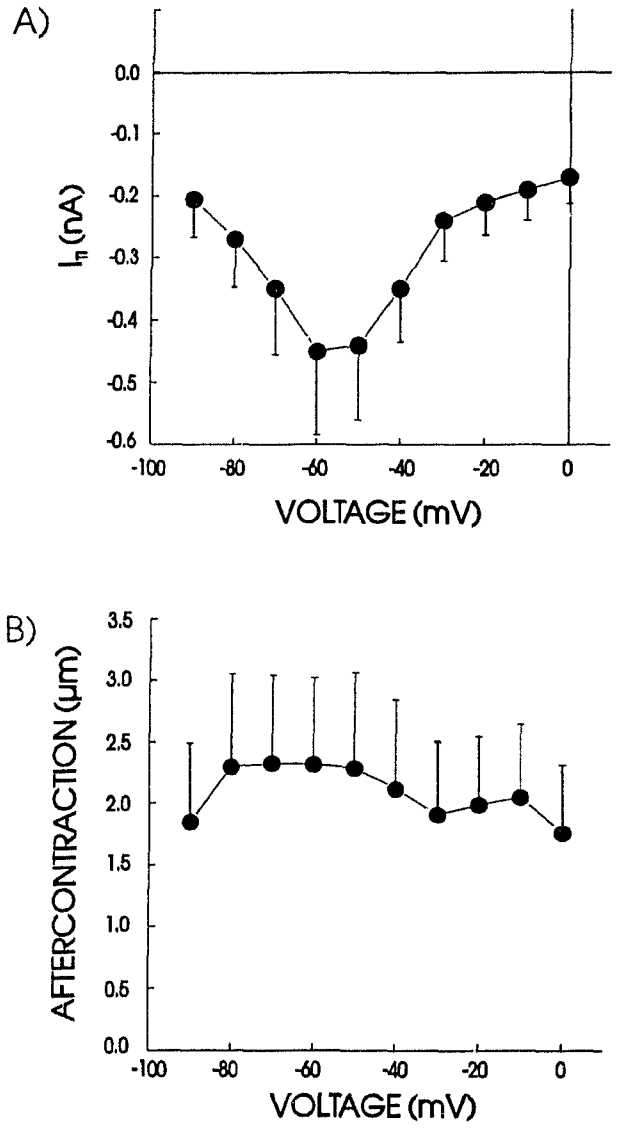

FIGURE 4: Mean ± SEM (n=8) I-V and contraction-voltage relationships for I_{Tl} and aftercontractions induced by reperfusion. These relationships were determined by varying the repolarization voltage following a 300 ms activation step to +20 mV. I_{Tl} increased to a peak at about -60 mV and then declined. However, the magnitude of aftercontractions was not voltage-dependant. No reversal of I_{Tl} was observed with repolarization steps up to 0 mV.

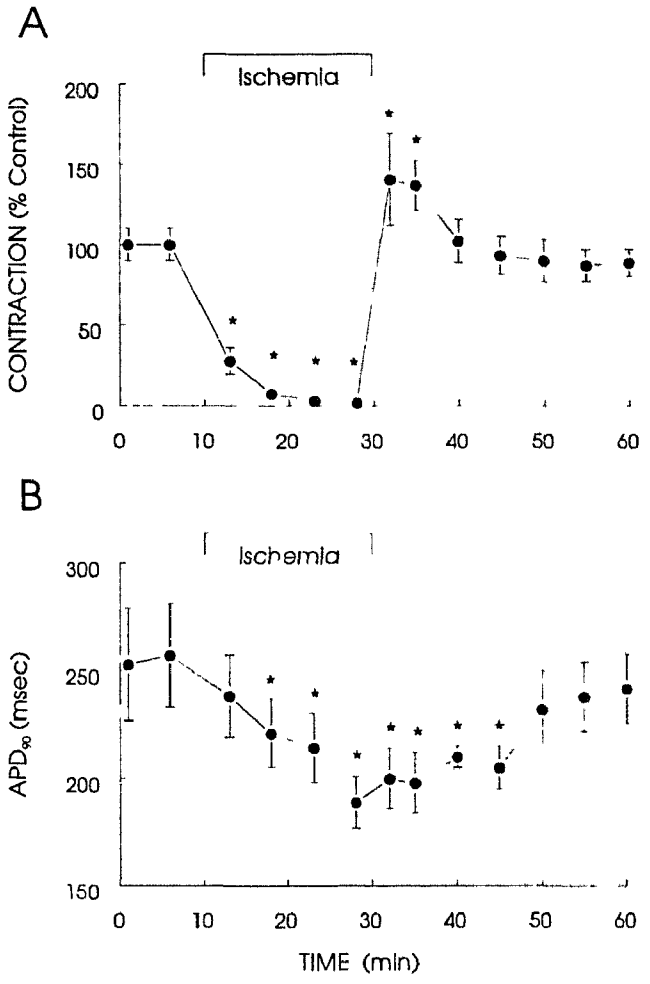

FIGURE 5: The time course of changes in APD_{90} and contractions in response to simulated ischemia and reperfusion. **A)** Exposure to ischemic conditions abolished contractions. Reperfusion resulted in a rapid recovery of contractions which temporarily exceeded control levels and then declined to control. **B)** APD_{90} progressively decreased during the ischemic period and then gradually returned to control upon reperfusion. Values are mean ± SEM (n=18). * = significantly different from control ($p<0.05$).

also declined as reperfusion time increased. This voltage clamp protocol did not elicit I_{Tl} under control conditions. Corresponding aftercontractions also declined as reperfusion time increased. When the voltage clamp protocol described above was run during simulated ischemia, I_{Tl} and aftercontractions were never observed.

Figure 4 shows the mean values for the current-voltage (I-V) relation for I_{Tl} and the contraction-voltage relation for aftercontractions recorded after 3 min of reperfusion (n=8). Both the current-voltage and contraction-voltage relations were generated by the voltage clamp protocol described in figure 3, however the repolarization step was varied from -90 mV to 0 mV. I_{Tl} was voltage-dependent; however aftercontractions did not change with membrane potential. The peak amplitude of I_{Tl} was reached upon repolarization to a membrane potential of -60 mV. I_{Tl} gradually decreased in amplitude with repolarization to more positive potentials while the amplitude of aftercontractions remained unchanged. No reversal of I_{Tl} was seen with repolarization steps up to 0 mV.

Next, the time course of changes in electrical and contractile activity during simulated ischemia and reperfusion was determined. Figure 5 compares the time courses of changes in APD_{90} and contractions associated with action potentials in response to simulated ischemia and reperfusion. APD_{90} and magnitude of cell shortening were measured in myocytes stimulated every 500 msec as described in the methods section. Magnitude of contractions was significantly decreased within 5 min of ischemia when compared to control

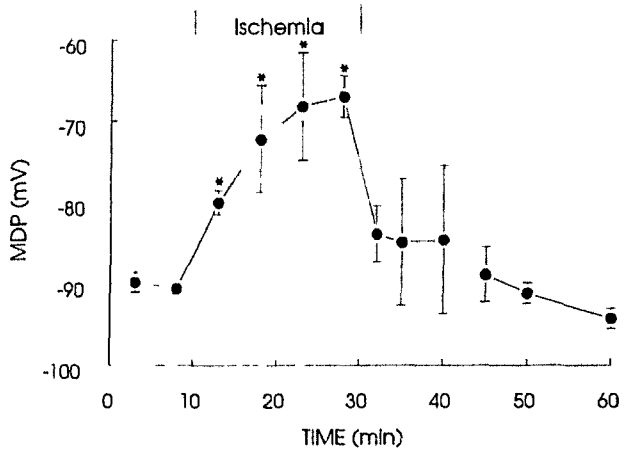

FIGURE 6: Time course of changes in MDP in response to simulated ischemia and reperfusion. Cells gradually depolarized upon exposure to simulated ischemia and MDP returned to control levels during reperfusion. Values are mean ± SEM (n=18); * = significantly different from control ($p<0.05$).

($p<0.05$). By the end of ischemia, contractions were abolished (figure 5A). Upon reperfusion, contractions returned rapidly and the magnitude of cell shortening transiently exceeded control ($p<0.05$). With continued reperfusion, contractions gradually returned to pre-ischemic levels (figure 5A). The time course of changes in APD_{90} was distinctly different (figure 5B). There was a progressive shortening of APD_{90} during ischemia, which was significantly different from control after 8 min of ischemia ($p<0.05$). The changes in APD elicited by simulated ischemia were not prevented by current clamping MDP back to control levels. In fact, this resulted in a further abbreviation of APD (data not shown). Upon reperfusion, APD_{90} recovered slowly and was not significantly different from control by the end of reperfusion (figure 5B).

MDP changes in response to simulated ischemia and reperfusion were also measured. Figure 6 shows that exposure to ischemic Tyrode's solution caused a progressive decrease in MDP which was statistically significant. After 20 min of ischemia, MDP had decreased from -89±1 to -67±4 mV (n=18). MDP recovered to pre-ischemic values after about 20 min of reperfusion.

C. Changes in Membrane Currents and Associated Contractions During Voltage Clamp.

Figure 7 shows representative recordings of I_{Ca} and corresponding cell shortening recorded during voltage clamp in myocytes exposed to simulated ischemia and reperfusion. The voltage clamp protocol is shown in Panel A. The

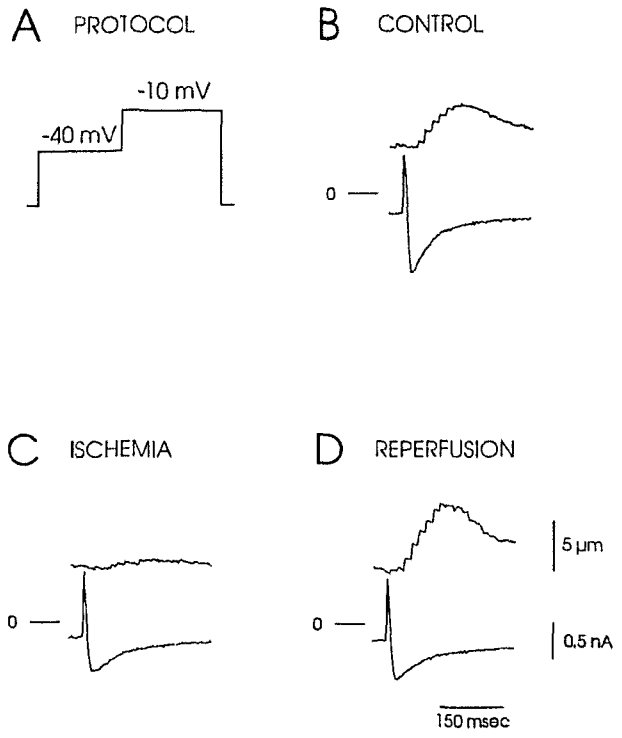

FIGURE 7: Representative experiment showing changes in I_{Ca} and contractions in response to simulated ischemia and reperfusion. **Panel A)** The voltage clamp protocol consisted of a step depolarization to -40 mV to discharge and inactivate I_{Na}. A second step depolarization to -10 mV activated I_{Ca}. The holding potential was -80 mV. **Panel B)** I_{Ca} and corresponding contraction prior to ischemic conditions. **Panel C)** After 20 min of simulated ischemia, contraction was abolished yet I_{Ca} only decreased by about 50%. **Panel D)** At 3 min reperfusion, I_{Ca} was still depressed yet the magnitude of contraction exceeded control levels. Horizontal line indicates zero current.

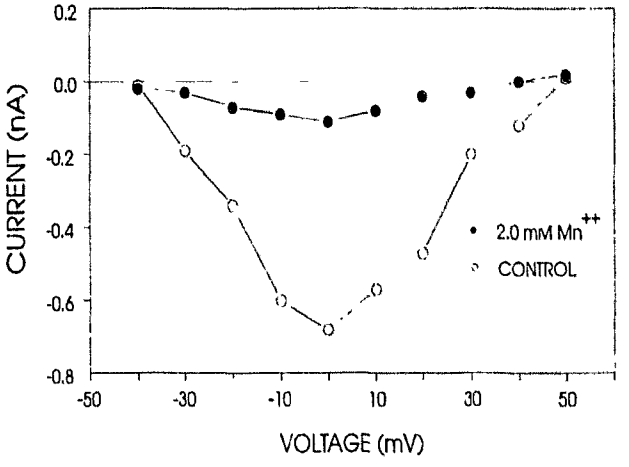

FIGURE 8: Representative example of I-V relationship for the L-type Ca^{++} current recorded from a ventricular myocyte. The threshold for activation was at -30 mV and the peak of the Ca^{++} current was 0 mV. Addition of 2 mM Mn^{++} reduced the Ca^{++} current at all potentials.

holding potential was -80 mV. The voltage clamp protocol consisted of a 300 msec pre-pulse to +10 mV (pre-pulse not shown), followed by a 300 msec depolarizing step to -40 mV to discharge and inactivate Na^+ channels. This was followed by a 300 msec step to -10 mV to activate and allow measurement of I_{Ca}. I_{Ca} was recorded from 5 averaged traces and measured as the difference between the peak inward current and the net current after 300 msec Panel B of figure 7 shows I_{Ca} and contraction recorded under control conditions. The average magnitude of I_{Ca} was -0.98±0.08 nA (n=11) and contraction always accompanied inward current. Figure 7C illustrates the effects of 20 min of ischemia on I_{Ca} and contractions. Exposure to simulated ischemia virtually abolished contractions, however I_{Ca} was reduced only by about 50% (-0.50±0.06 nA, n=11). Figure 7D shows I_{Ca} and contraction recorded after 3 min of reperfusion. Contractions recovered and exceeded pre-ischemic levels, however the magnitude of I_{Ca} remained depressed (-0.49±0.07 nA, n=11). The validity of this measure of I_{Ca} was tested in separate experiments by exposure of cells to the Ca^{++} channel blockers verapamil (5 μM), nifedipine (2 μM) or Mn^{++} (2 mM). Ca^{++} channel blockers substantially inhibited inward current identified as I_{Ca} under both control conditions and during simulated ischemia. Inhibition of I_{Ca} by 2 mM Mn^{++} under control conditions is shown in the example in figure 8. Similar results were obtained when either verapamil or nifedipine were added (data not shown).

The time courses of changes in cell shortening and I_{Ca} throughout

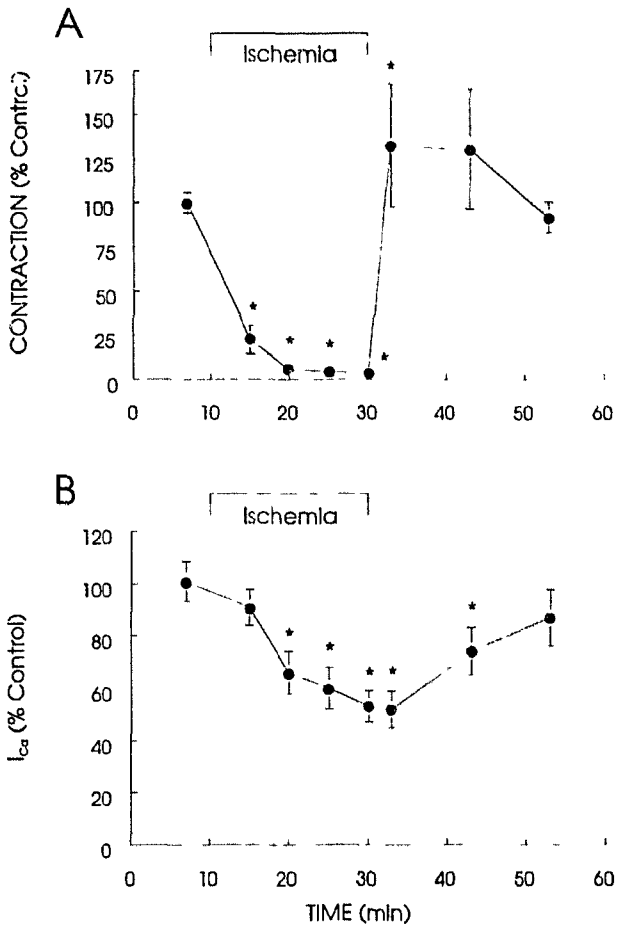

FIGURE 9: Changes in I_{Ca} and contractions in response to simulated ischemia and reperfusion. **A)** Contractions were abolished by exposure to ischemic conditions. Reperfusion resulted in a rapid recovery of contractions which initially exceeded control values. **B)** I_{Ca} decreased gradually during ischemia although about 50% of I_{Ca} was still present at the end of ischemia. Reperfusion resulted in a slow return of I_{Ca} to control values. Values are mean ± SEM (n=11). * = significantly different from control ($p<0.05$).

ischemia and reperfusion are shown in figure 9. Contractions were significantly reduced throughout ischemia when compared to control ($p<0.05$) and were abolished after 10 min of ischemia (figure 9A). Upon reperfusion, contractions recovered rapidly and temporarily exceeded control levels ($p<0.05$, figure 9A). These results also show that changes in contraction observed under voltage clamp conditions are virtually identical to those observed with conventional recording techniques (compare Figures 5A and 9A). The time course of changes in the magnitude of I_{Ca}, however, was markedly different from that of contraction. I_{Ca} declined gradually throughout ischemia and recovered very slowly upon reperfusion (Figure 9B). Thus, changes in I_{Ca} had a much slower time course than changes in contraction.

2. PHARMACOLOGICAL INTERVENTIONS DURING ISCHEMIA AND REPERFUSION

Early reperfusion was characterized by signs of Ca^{++} overload which included induction of I_{Ti} and overshoot of contractions in early reperfusion. Ca^{++} overload in reperfusion may occur as a result of Ca^{++} and/or Na^{+} loading which occur during ischemia and reperfusion (Tani, 1990). There are many mechanisms by which Ca^{++} overload is believed to occur during ischemia and reperfusion (described on pages 15 and 26 respectively) . This section of the thesis examines the effects of several pharmacological agents which affect cellular Ca^{++} and Na^{+} loading in myocytes exposed to simulated ischemia and

Nifedipine (2 μM)

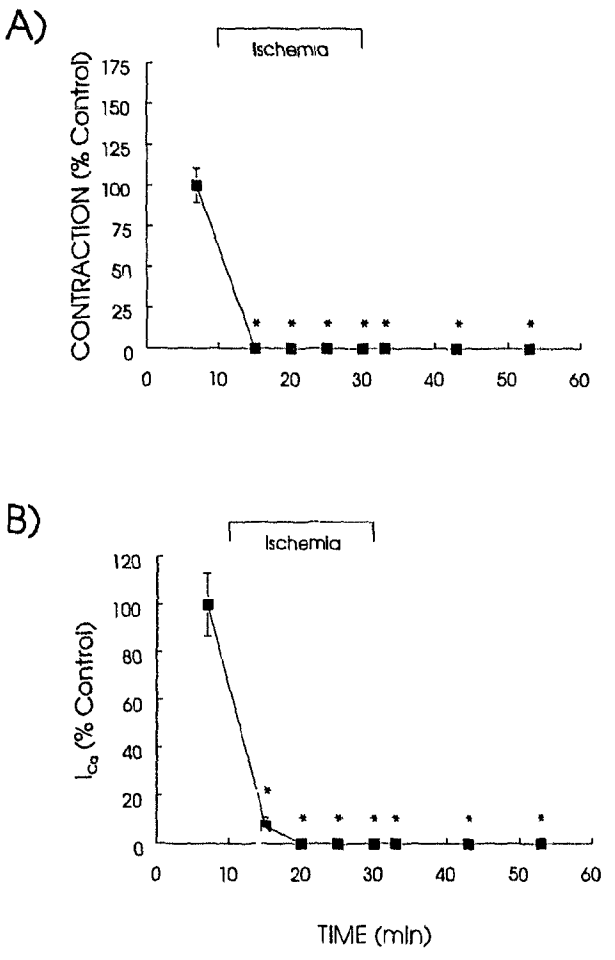

FIGURE 10: Changes in cell shortening (panel A) and I_{Ca} (panel B) in response to simulated ischemia and reperfusion in the presence of nifedipine (2 μM). The voltage clamp protocol consisted of a step depolarization to -40 mV to discharge and inactivate I_{Na}. A second step to -10 mV activated I_{Ca} and contraction. the holding potential was -80 mV. Both contractions (panel A) and I_{Ca} (panel B) were abolished throughout ischemia and reperfusion in the presence of nifedipine. * = significantly different from control ($p<0.05$).

reperfusion.

A. Effects of Pharmacological Agents on I_{Ca}, L-Channel Associated Contraction and Incidence of I_{TI}.

i. Effect of Ca^{++} Channel Blockade during Ischemia and Reperfusion.

Results presented thus far have demonstrated that, in ischemia, significant inhibition of contraction occurs within 10 min whereas by the end of 20 min of ischemia I_{Ca} was only inhibited by 50%. In addition, upon reperfusion a transient overshoot of contraction occurred early in reperfusion but the L-type Ca^{++} current was still 50% of pre-ischemic levels; with further reperfusion, L-type Ca^{++} current gradually recovered. These results demonstrate that there is a dissociation of the magnitude of L-type Ca^{++} current and the magnitude of contraction associated with the L-type Ca^{++} current during ischemia and reperfusion. This suggests that signs of Ca^{++} overload observed upon reperfusion (i.e. the appearance of I_{TI} and overshoot of contraction) are likely not due to enhanced Ca^{++} influx via L-type Ca^{++} channels. To confirm this hypothesis, ventricular myocytes were exposed to simulated ischemia and reperfusion in the presence of the L-type Ca^{++} channel blocker nifedipine (2 μM). Nifedipine was added during simulated ischemia and was present throughout reperfusion. Figure 10 illustrates the time course of changes in contraction (panel A) and I_{Ca} (panel B) during simulated ischemia and reperfusion. The voltage clamp protocol consisted of a step depolarization to -40 mV to discharge

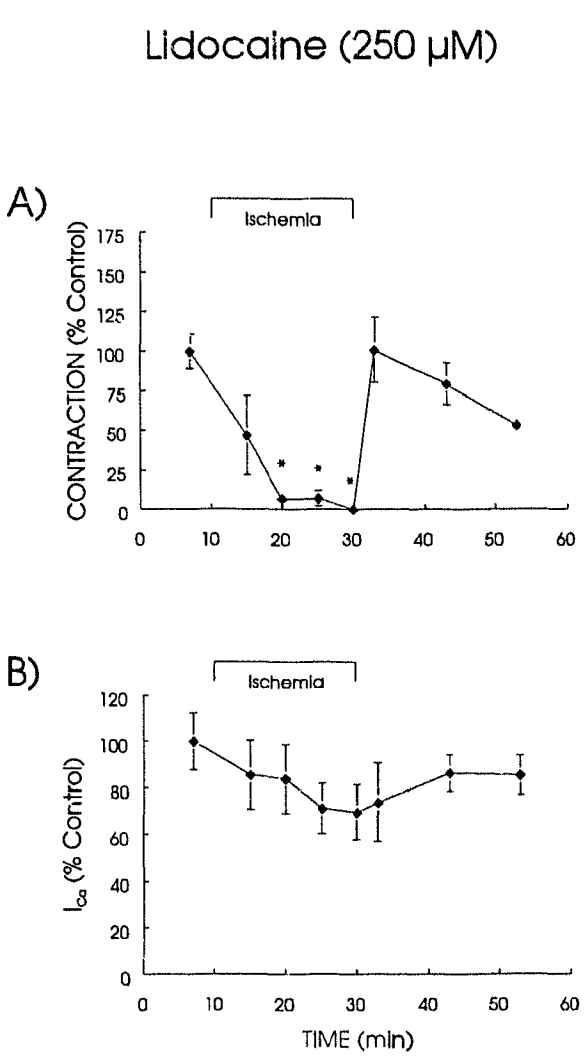

FIGURE 11: Time course of changes in cell shortening (panel A) and I_{Ca} (panel B) in response to simulated ischemia and reperfusion in the presence of lidocaine (250 µM). The voltage clamp protocol consisted of a step depolarization to -40 mV to discharge and inactivate I_{Na}. A second step to -10 mV activated I_{Ca} and contraction. The holding potential was -80 mV. The overshoot of contraction in early reperfusion was prevented by lidocaine. I_{Ca} declined slightly during ischemia in the presence of lidocaine, while reperfusion resulted in a slow return of I_{Ca} toward control levels. * = significantly different from control ($p<0.05$).

and inactivate I_{Na}. A second step depolarization to -10 mV activated I_{Ca}. The holding potential was -80 mV. Current and contraction were expressed as a percentage of control value observed prior to ischemia. In myocytes exposed to nifedipine, both I_{Ca} and contraction were immediately abolished early in ischemia and remained abolished throughout ischemia and reperfusion. The effect of nifedipine on the incidence of I_{Ti} in early in reperfusion was also determined. In the absence of drug, I_{Ti} was present in 78% of experiments (18/23 cells). Interestingly, nifedipine had no effect on the incidence of I_{Ti} in early reperfusion; I_{Ti} was observed in 75% of experiments (6/8 cells)(Table 5, page 101). These results demonstrate that L-channel blockade during ischemia and reperfusion did not diminish the incidence of I_{Ti} upon reperfusion.

ii. Effect of Na^+ Channel Blockade during Ischemia and Reperfusion.

If Ca^{++} overload in early reperfusion is the result of Na^+ loading in ischemia, which in turn, exchanges for Ca^{++} via the Na^+-Ca^{++} exchange, then preventing Na^+ loading should abolish signs of Ca^{++} overload. One mechanism by which Na^+ loading may occur is through prolonged activation of Na^+ channels. Therefore, the effects of the Na^+ channel inhibitor lidocaine (250 μM) on I_{Ca}, contraction and incidence of I_{Ti} were determined in myocytes exposed to simulated ischemia and reperfusion. Lidocaine was added during ischemia and was present throughout reperfusion. Figure 11 illustrates the time course of changes in I_{Ca} and contraction during ischemia and reperfusion in the presence

Amiloride (100 µM)

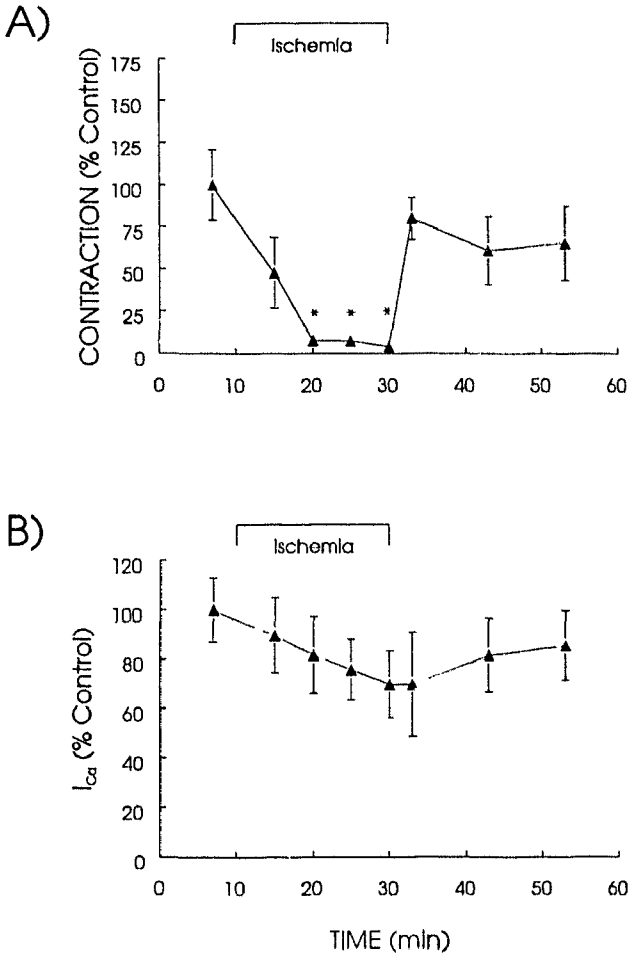

FIGURE 12: Changes in cell shortening (**panel A**) and I_{Ca} (**panel B**) in response to simulated ischemia and reperfusion in the presence of amiloride (100 µM). Contractile failure in ischemia was unaffected but the overshoot of contractions in early reperfusion was prevented by amiloride. I_{Ca} declined gradually during ischemia with amiloride present. Reperfusion resulted in a slow return of I_{ca} towards control levels. * = statistically significant from control ($p<0.05$).

of lidocaine. The Na^+ channel blocker had no effect on the time course of contractile failure in ischemia, however the statistically significant overshoot in the magnitude of contraction in early reperfusion was inhibited by lidocaine (figure 11A). In addition, the decline in the magnitude of I_{Ca} in ischemia was less than observed in control myocytes and was no longer statistically significant. Surprisingly, the incidence of I_{Tl} in early reperfusion was unaffected by lidocaine; I_{Tl} was observed in 71% of cells in reperfusion (5/7 experiments)(Table 5, page 101). These results show that Na^+ channel blockade reduced the overshoot in the magnitude of contractions in early reperfusion. However, it is unclear why the incidence of I_{Tl} was unaffected in early reperfusion.

iii. Effect of Na^+-H^+ Exchange Blockade during Ischemia and Reperfusion.

It has been demonstrated that intracellular pH decreases during ischemia (Lazdunski et al., 1985). The accumulation of protons within the myocyte may exchange for Na^+ via the Na^+-H^+ exchange, resulting in an accumulation of Na^+. Thus, the effects of the Na^+-H^+ exchange inhibitor amiloride (100 μM) on I_{Ca}, contraction and incidence of I_{Tl} during ischemia and reperfusion were also determined. Figure 12 shows the effect of amiloride on the time course of changes in contraction (panel A) and I_{Ca} (panel B). Amiloride had no effect on contractile failure in ischemia. However, amiloride prevented the statistically significant overshoot in contractions observed in early reperfusion (panel A). In addition, the reduction in I_{Ca} was less than observed in control myocytes and

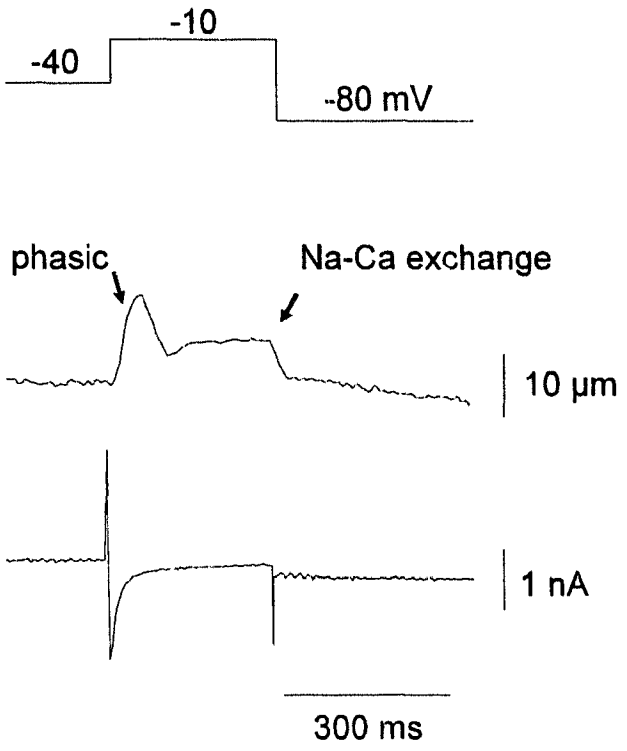

FIGURE 13: Representative traces of contraction (upper trace) and current (lower trace) in response to voltage clamp protocol. An activation step from -40 to -10 mV elicited I_{Ca} (lower trace). A phasic contraction followed by a slowly developing Na^{+}-Ca^{++} exchange contraction which disappeared upon repolarization is shown in the upper trace. For clarity, the Na^{+}-Ca^{++} exchange contraction illustrated in this figure is larger than average at this membrane potential.

was no longer statistically significant. The incidence of I_{Tl} observed upon early reperfusion in the presence of the Na^+-H^+ exchange inhibitor was significantly reduced; I_{Tl} was only observed in 28% of experiments (2 of 7 cells)(Table 5, page 101). These results suggest that blockade of the Na^+-H^+ exchange with amiloride prevented Na^+ loading in the myocyte which, in turn, prevented Ca^{++} overload via the Na^+-Ca^{++} exchange in early reperfusion. Alternatively, amiloride may inhibit the Na^+-Ca^{++} exchange which also would reduce Ca^{++} overload Na^+-Ca^{++} exchange . Thus, addition of the Na^+-H^+ exchange inhibitor amiloride significantly inhibited signs of Ca^{++} overload in ischemia and reperfusion.

B. Effect of Ischemic Conditions and Reperfusion on Na^+-Ca^{++} Exchange Contractions.

i. Measurement of the Na^+-Ca^{++} Exchange Contraction.

The results presented thus far have demonstrated that the contraction associated with the L-type Ca^{++} channel is inhibited during ischemic conditions. Furthermore, upon reperfusion contractions rapidly reappear and transiently exceed control levels. The contraction associated with the L-type Ca^{++} current is phasic. Under voltage clamp conditions, a slowly developing contraction which is believed to be due to Ca^{++} influx via the Na^+-Ca^{++} exchange (Terrar and White, 1990) was often noted in these experiments. Figure 13 illustrates current as well as both the phasic contraction and the slowly developing contraction from a

myocyte under normal conditions. The holding potential was -80 mV and there was a pre-pulse to +20 mV (pre-pulse not shown). The voltage clamp protocol (illustrated at top of figure) consisted of a step depolarization to -40 mV to discharge and inactivate Na^+ channels. A step depolarization from -40 to -10 mV elicited I_{Ca}. A phasic contraction corresponding to activation of the L-type Ca^{++} current is observed in response to the -10 mV step depolarization. Following the phasic contraction, a slowly developing contraction can be observed throughout the -10 mV step depolarization. The cell continues to shorten until the voltage is returned to -80 mV. Previous studies have suggested that this tonic contraction was attributable to Ca^{++} influx via the Na^+-Ca^{++} exchange (Terrar and White, 1989). Terrar and White (1989) have demonstrated that this slowly developing contraction gets larger as the voltage clamp steps become more positive. Furthermore, this tonic contraction persists until the myocyte is repolarized to more negative membrane potentials. The mechanism by which this phasic contraction occurs is via the Na^+-Ca^{++} exchange working in reverse mode. Under normal conditions, it is believed that the Na^+-Ca^{++} exchange works to remove 1 Ca^{++} ion out of the cell in exchange for 3 Na^+ ions being moved into the cell. However, at more positive voltages, the Na^+-Ca^{++} exchange may actually bring Ca^{++} into the cell and directly activate the myofilaments resulting in cell shortening (figure 13). Tonic contractions elicited by the Na^+-Ca^{++} exchange can be used to indirectly assess the degree of Na^+ loading and, therefore, the amount of Ca^{++} influx via the Na^+-Ca^{++} exchange.

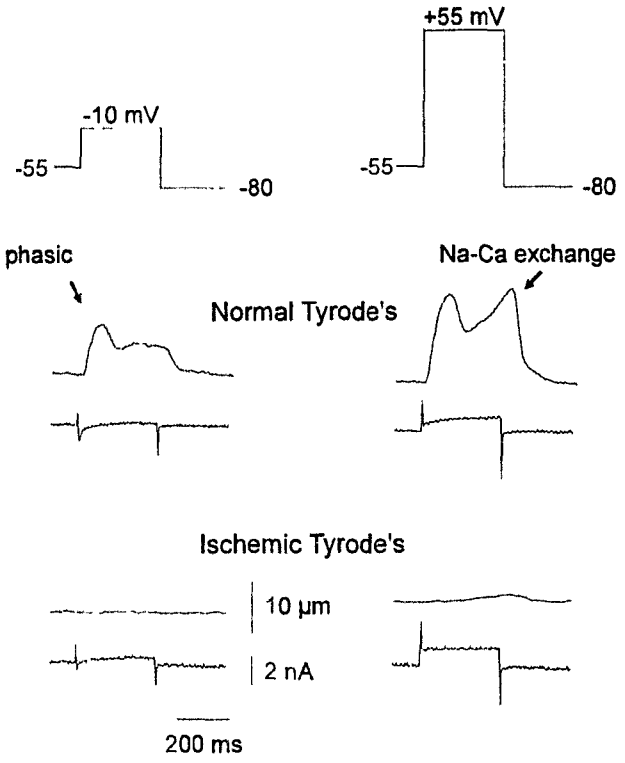

FIGURE 14: Representative traces of contraction (upper trace) and current (lower trace) in response to voltage clamp steps to either -10 mV (left panels) or +55 mV (right panels). The holding potential was -80 mV. Post-conditioning potential was -55 mV. In normal Tyrode's solution, the activation step to -10 mV elicited I_{Ca} (lower trace) and associated phasic contraction (upper trace) followed by a Na^{+}-Ca^{++} exchange contraction. The step to +55 mV elicited a larger phasic contraction and Na^{+}-Ca^{++} exchange contraction. Exposure to ischemic Tyrode's (18 min) resulted in abolition of the phasic contraction and a marked reduction in the Na^{+}-Ca^{++} exchange contractions.

ii) Effects of Simulated Ischemia and Reperfusion on Na^+-Ca^{++} Exchange Contractions.

Figure 14 illustrates representative examples of Na^+-Ca^{++} exchange contractions under normoxic conditions and following 20 min of simulated ischemia. The left side of the figure shows the contractions observed in response to a step depolarization to -10 mV and the right side of the figure illustrates the contractions observed at +55 mV. All recordings were made from the same myocyte. The voltage clamp protocol is displayed at the top of the figure. Prior to the voltage clamp protocol, there was a pre-pulse to +20 mV followed by a return step to -55 mV (pre-pulse not shown). Under normal conditions, a 300 msec step depolarization from -55 to -10 mV elicited a phasic contraction followed by a slowly developing Na^+-Ca^{++} exchange contraction (left side of figure). A voltage clamp step of identical duration to +55 mV elicited a larger phasic contraction and a Na^+-Ca^{++} exchange contraction (right side of figure). The Na^+-Ca^{++} exchange contraction is larger at more positive potentials and the rate at which the cell shortens is much greater. The bottom portion of figure 14 illustrates the contractions observed following 18 min of simulated ischemia. All contractions were abolished in response to the step depolarization to -10 mV. The Na^+-Ca^{++} exchange contraction observed in response to the step depolarization to +55 mV was greatly attenuated.

The Na^+-Ca^{++} exchange contraction increased in magnitude with more

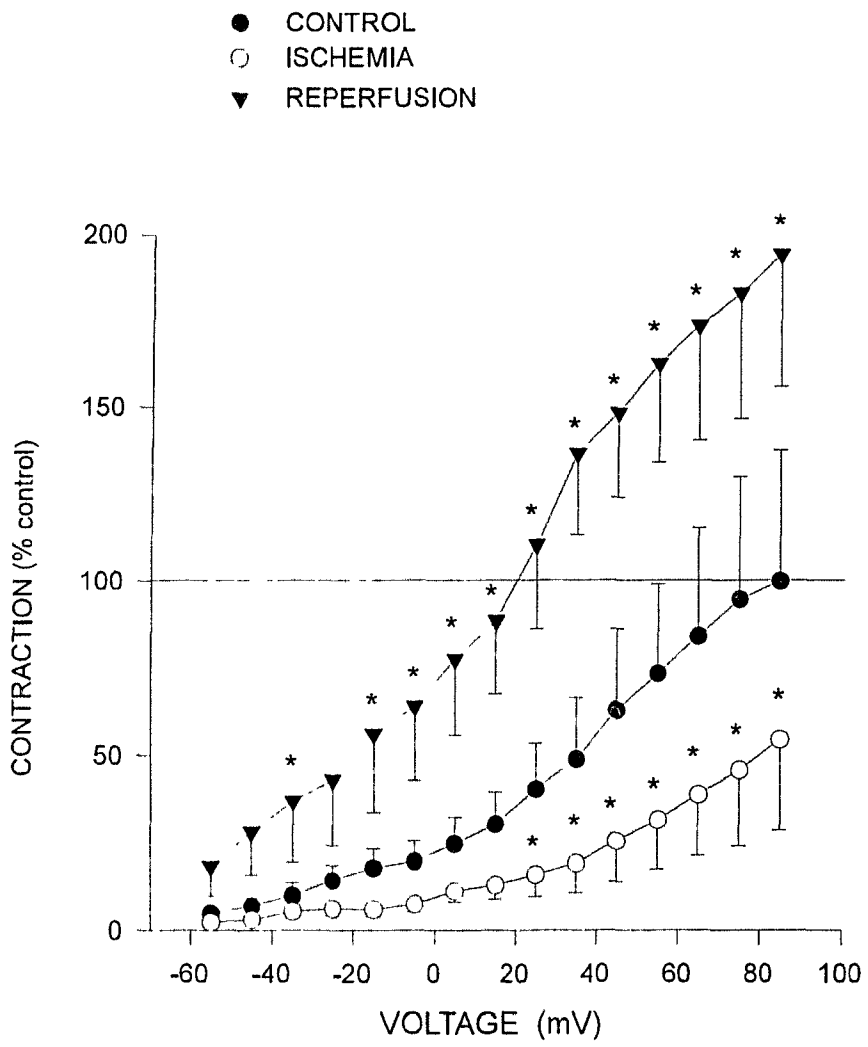

FIGURE 15: Contraction-voltage curves for the slowly developing Na^{+}-Ca^{++} exchange contraction. Contraction were measured at potentials from -55 to +85 mV following a pre-pulse to +20 mV in the absence of drug (n=8). Data are expressed as a percentage of the maximum value observed under control conditions. After 18 min of ischemia, there was a decrease in the magnitude of exchange contractions at all potentials when compared to control. At 4 min reperfusion, the magnitude of contraction significantly greatly exceeded control values. * = significantly different from control ($p<0.05$).

positive voltages. A contraction-voltage curve was generated using the same voltage clamp protocol illustrated in figure 14 except the activation voltage was varied between -55 and +85 mV. Figure 15 illustrates the contraction-voltage relationship for the Na^+-Ca^{++} exchange contraction under the different experimental conditions. All contractions were expressed as a percent of the maximum contraction observed under control conditions. Under control conditions, the Na^+-Ca^{++} exchange contraction developed slowly and had a curvilinear contraction-voltage relationship. Following 18 minutes of simulated ischemia, the magnitude of the Na^+-Ca^{++} exchange contraction was significantly reduced at the more positive membrane potentials when compared to control. Upon reperfusion, the magnitude of the Na^+-Ca^{++} exchange contraction was significantly greater than control. These contractions gradually decreased in magnitude with continued reperfusion and returned to control levels in late reperfusion.

iii) Effect of Amiloride on Na^+-Ca^{++} exchange Contractions During Simulated Ischemia and Reperfusion.

The results from figure 15 demonstrate that in early reperfusion, Na^+-Ca^{++} exchange contractions were potentiated with respect to control. These observations suggest either that the myofilaments became more sensitive to the cytosolic Ca^{++} concentration in reperfusion or that a greater amount of Ca^{++} entered the cell via Na^+-Ca^{++} exchange during early reperfusion.

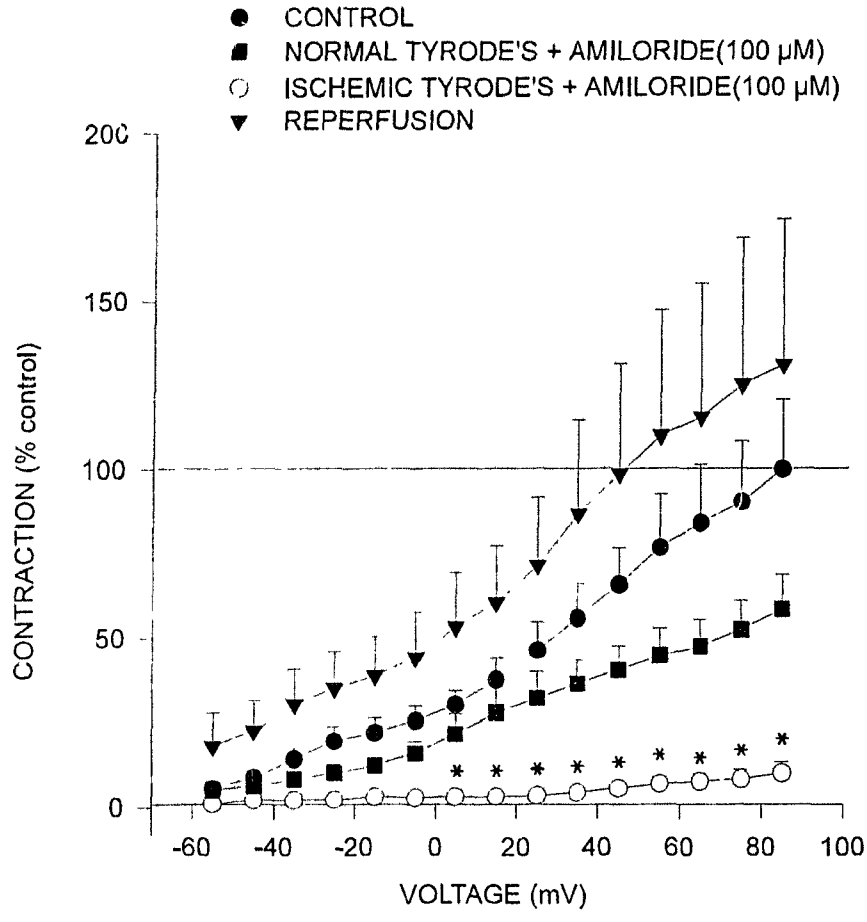

FIGURE 16: Contraction-voltage curves for the slowly developing Na^{+}-Ca^{++} exchange contraction in the presence of amiloride (100 µM, n=7). Data are expressed as a percentage of the maximum value observed under control conditions. Ischemic conditions (18 min) virtually abolished contractions at all potentials. Reperfusion (4 min) resulted in a slight overshoot in the magnitude of the exchange contraction which was much less than that observed in the absence of amiloride (compare with figure 15). * = statistically significant from control ($p<0.05$).

Since the Na^+-Ca^{++} exchange working in reverse mode removes Na^+ from the cell in exchange for Ca^{++}, ischemic conditions or early reperfusion may cause an accumulation of intracellular Na^+. Results previously described demonstrated that amiloride decreased both the overshoot of contractions and the incidence of I_{Ti} in early reperfusion. However, it was unclear whether the reduction in signs of Ca^{++} overload were due to inhibition of the Na^+-H^+ exchange or Na^+-Ca^{++} exchange.

Therefore, the effects of amiloride on contractions elicited by Na^+-Ca^{++} exchange were examined. Figure 16 illustrates the contraction-voltage relationship for Na^+-Ca^{++} exchange contractions in the presence of amiloride in ischemia. Contractions are expressed as a percentage of the maximum contraction observed under control conditions. Under control conditions, the contraction-voltage relationship gradually increased at more positive voltages and was linear. Exposure to ischemic conditions in the presence of amiloride resulted in a statistically significant decrease in the magnitude of Na^+-Ca^{++} exchange contractions at all potentials. Reperfusion (4 min) resulted in a reduction in the overshoot of the Na^+-Ca^{++} exchange contraction at all potentials. The results obtained with amiloride are not conclusive but demonstrate that addition of a Na^+-H^+ exchange inhibitor during ischemia prevented the overshoot of the Na^+-Ca^{++} exchange contraction. Taken together with the results which show that amiloride attenuated the overshoot of the L-current induced contraction and decreased the incidence of I_{Ti} upon reperfusion, these results

suggest that during ischemia, intracellular acidification occurs as previously demonstrated. A decrease in intracellular pH would increase cytosolic Na^+ loading via the Na^+-H^+ exchange during ischemia and/or early reperfusion which, in turn, would result in Ca^{++} overload via the Na^+-Ca^{++} exchange. Alternatively, amiloride may directly inhibit the Na^+-Ca^{++} exchanger, as previously suggested (Lazdunski et al., 1985). Direct inhibition of the Na^+-Ca^{++} exchanger would result in a reduction in magnitude of the Na^+-Ca^{++} exchange contraction. In addition, direct inhibition of Na^+-Ca^{++} exchange would reduce the incidence of I_{TI} since the Na^+-Ca^{++} exchange is believed to be a charge carrier of I_{TI} (January and Fozzard, 1988).

iv) Effect of Reperfusion with Acidic Buffer on Na^+-Ca^{++} Exchange Contractions.

Ca^{++} overload during early reperfusion may be related to changes in intracellular pH. It is believed that, during ischemia, both intracellular and extracellular pH decrease to low levels (i.e. 6.5-6.8). During the early stages of reperfusion the extracellular pH returns to 7.4. However, the intracellular pH remains low. Therefore, a pH gradient develops across the cell membrane which results in H^+ being extruded from the cell in exchange for Na^+. This massive Na^+ loading in early reperfusion would be expected to result in Ca^{++} overload, as Na^+ is removed from the cell in exchange for Ca^{++} via the Na^+-Ca^{++} exchange. If this mechanism is responsible for Ca^{++} overload in early

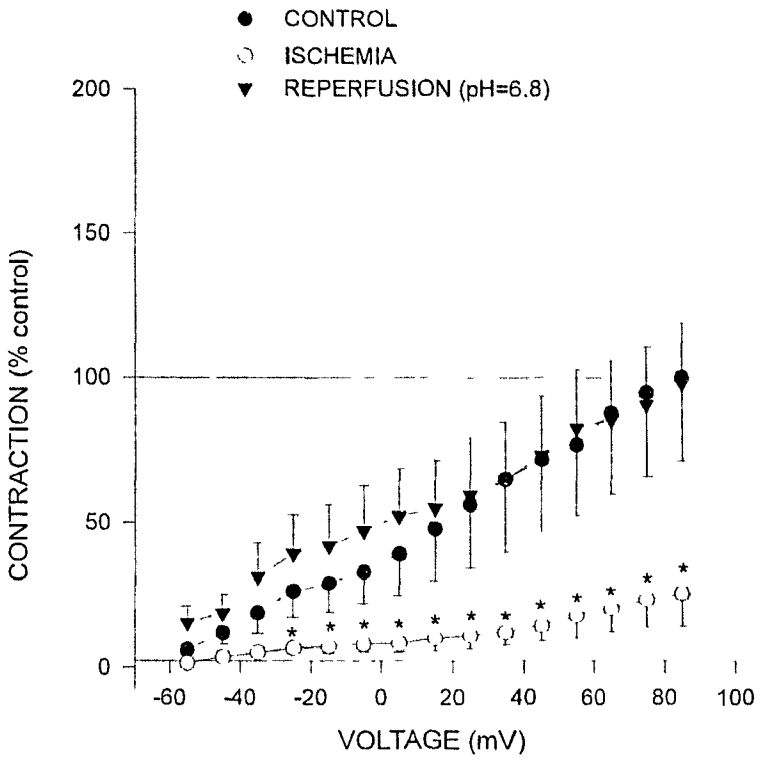

FIGURE 17: Contraction-voltage curves for Na^{+}-Ca^{++} exchange at various stages of the experiment. Exposure to simulated ischemia (18 min) virtually abolished contractions at all potentials. Reperfusion with normal Tyrode's solution at pH=6.8 prevented the overshoot in Na^{+}-Ca^{++} exchange contraction at all potentials.

TABLE 5: INCIDENCE OF I_{Ti} IN RESPONSE TO VARIOUS DRUG TREATMENTS.

TREATMENT	INCIDENCE
Control	18/23 (78%)
Nifedipine (2 μM)	6/8 (75%)
Lidocaine (250 μM)	5/7 (71%)
Amiloride (100 μM)	2/7 (28%)
Acidic reperfusion (pH=6.8)	1/6 (17%)

reperfusion then Ca^{++} overload should be prevented by preventing the pH gradient from developing in reperfusion. To test this hypothesis, cells were exposed to 20 min of simulated ischemia and then were reperfused with normal Tyrode's solution at pH=6.8. Figure 17 illustrates the contraction-voltage relationship for the Na^{+}-Ca^{++} exchange contraction when cells were reperfused with normal Tyrode's solution at pH=6.8. Contractions were expressed as a percentage of the maximum contraction observed under control conditions. Under control conditions the contraction-voltage curve gradually increases with more positive voltages and is linear. Following 18 min ischemia, the contraction-voltage curve is significantly reduced but remains linear. Reperfusion (4 min) with a Tyrode's solution at pH=6.8 prevented the overshoot of the Na^{+}-Ca^{++} exchange contractions when compared to control. Furthermore, reperfusion with an acidic buffer also prevented induction of I_{TI} upon reperfusion; I_{TI} was observed in only 1/6 experiments (17%)(Table 5, page 101). These observations demonstrate that reducing the Na^{+}-H^{+} exchange gradient across the myocyte membrane in reperfusion prevents signs of Ca^{++} overload in early reperfusion. This suggests that by preventing the Na^{+}-H^{+} exchange gradient, less Ca^{++} enters via the Na^{+}-Ca^{++} exchange.

3. EFFECTS OF SIMULATED ISCHEMIA AND REPERFUSION ON CONTRACTIONS ELICITED BY A VOLTAGE-SENSITIVE RELEASE MECHANISM.

A) Characteristics of Release Contraction.

Results presented thus far demonstrate that contractions associated with activation of L-type Ca^{++} current are abolished in ischemia and exhibit a transient overshoot in early reperfusion. However, previous studies have demonstrated that contractions also can be initiated at membrane potentials more negative than the threshold for activation of L-type Ca^{++} current (i.e -40 mV) (Ferrier and Howlett, 1995). These contractions are insensitive to blockade of L-type Ca^{++} current and Na^{+} current but are inhibited by ryanodine which suggests these contractions are initiated by Ca^{++} release from the SR. It was hypothesized that contractions initiated by activation of this voltage-sensitive release mechanism (release contraction) might also be affected by simulated ischemia and reperfusion.

i) Effect of L-Current Blockade on Release Contraction.

To separate the two components of contraction, a two-step voltage clamp protocol was used. Figure 18A shows the current and corresponding contraction from a myocyte prior to ischemia. The holding potential was -80 mV and there was a pre-pulse to +20 mV (pre-pulse not shown). A step depolarization to -40 mV from -80 mV initiated a large inward Na^{+} current and corresponding contraction. However, the mechanism responsible for initiation of the contraction associated with the step to -40 mV can not be determined under these conditions (150 mM external Na^{+} at 37°C) because of the loss of voltage

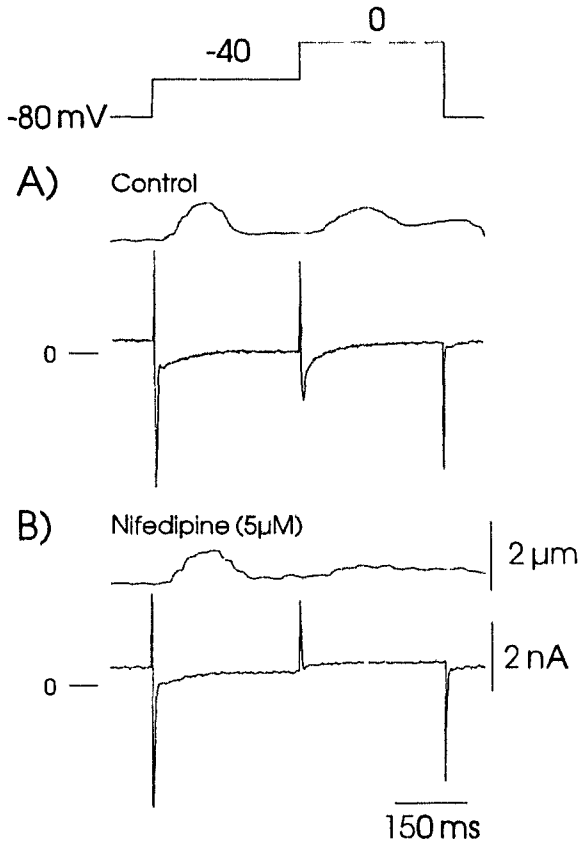

FIGURE 18: Separation of two components of contraction under voltage clamp conditions using a two-step voltage clamp protocol. The voltage clamp protocol is displayed at top of the figure. Prior to the voltage clamp protocol, there was a 200 msec pre-pulse to +20 mV (pre-pulse not shown). Panels show cell shortening (upper traces) and membrane current (lower traces) recorded in response to 300 msec depolarizing steps to -40 mV and 0 mV. **Panel A)** Current and contraction recorded under normal conditions (prior to exposure to ischemia). A step depolarization to -40 mV elicited a large Na^+ current and corresponding contraction. A further step to 0 mV elicited the L-type Ca^{++} current and corresponding contraction. **B)** Addition of 2 μM nifedipine abolished Ca^{++} current measured at 0 mV and the corresponding contraction but did not affect contraction initiated by the step to -40 mV. Horizontal line represents zero current.

control during the influx of Na^+. During the influx of Na^+, the membrane potential momentarily becomes more positive than -40 mV and likely results in activation of L-type Ca^{++} channels. This influx of Ca^{++} may initiate release of Ca^{++} from the SR and elicit a contraction. To determine whether activation of L-type Ca^{++} current in response to the voltage clamp step to -40 mV was responsible for the initiation of contraction, the effect of adding the L-type Ca^{++} channel blocker nifedipine was determined. Nifedipine at 5 μM should prevent the contraction observed at -40 mV due to loss of voltage control. In addition, nifedipine should also abolish contractions observed in response to the step depolarization to 0 mV. Panel B of figure 18 illustrates the effect of adding 5 μM nifedipine to the contraction. Both the current and contraction traces are from the same myocyte as in panel A. The step depolarization to -40 mV from a holding potential of -80 mV elicited a large Na^+ current and corresponding contraction. However, both I_{Ca} and the corresponding contraction elicited by the step to 0 mV were inhibited in the presence of nifedipine. These results suggest the contraction observed at -40 mV is not due to loss of voltage control during the Na^+ influx and subsequent activation of L-type Ca^{++} channels but must be due to another mechanism. Prolonged exposure to 5 μM nifedipine resulted in the abolition of all contractions. This may be due to the inability of the SR to load with Ca^{++} in the presence of nifedipine with the voltage clamp protocol employed (Ferrier and Howlett, 1995).

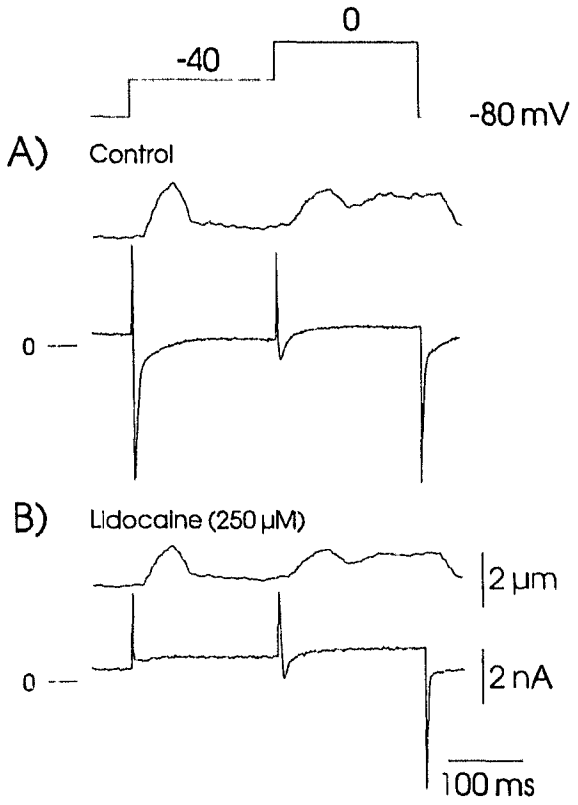

FIGURE 19: Separation of release and L-current associated contractions under voltage clamp using a two-step voltage clamp protocol. The protocol is shown at the top of the figure. Traces were recorded under normal conditions. **Panel A)** A large Na^+ current and contraction can be observed during a step depolarization to -40 mV. A further step to 0 mV elicited the L-current and corresponding contraction. **Panel B)** Addition of 250 µM lidocaine abolished the Na^+ current but not the contraction observed at -40 mV. Both the L-current and contraction are present in response to the step to 0 mV. Horizontal line represents zero current.

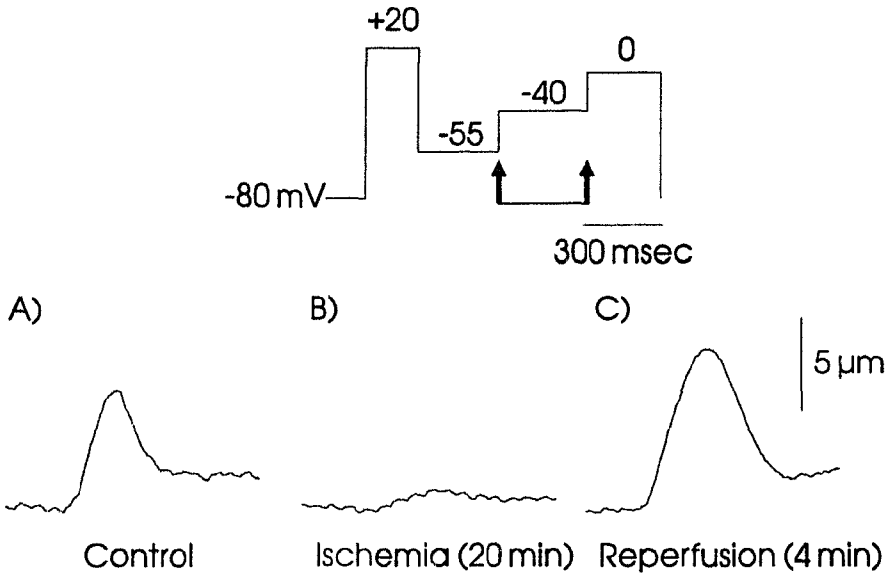

FIGURE 20: Representative example of release contractions recorded from a single myocyte in control, ischemia and reperfusion. 2 µM nifedipine was present throughout and release contractions were measured following a step depolarization to -40 mV from -55 mV. The holding potential was -80 mV and there was a 200 msec pre-pulse to +20 mV followed by a return step to -55 mV to discharge and inactivate I_{Na}. **Panel A)** The release contraction shown under normal conditions. Nifedipine (2 µM) was present for two minutes before the recording was made. **Panel B)** The release contraction was virtually abolished following 20 min ischemia in the presence of nifedipine. **Panel C)** Upon reperfusion and in the continued presence of nifedipine, there was a 130% overshoot in the magnitude of cell shortening relative to control levels. Arrows indicate the portion of the voltage clamp protocol for which contractions are illustrated.

ii) Effect of Na^+ Blockade on Release Contractions.

Activation of I_{Na} has been shown to activate phasic contractions by reverse mode Na^+ -Ca^{++} exchange (Leblanc and Hume, 1990). To eliminate contractions initiated by this mechanism, I_{Na} was inhibited with the Na^+ channel blocker lidocaine. Panel A of figure 19 illustrates both current and contraction in response to the two-step voltage clamp protocol shown at the top of the figure. The holding potential was -80 mV and there was one pre-pulse to +20 mV (pre-pulse not shown). As before, the step depolarization from -80 to -40 mV initiated a Na^+ current and corresponding contraction. The step to 0 mV elicited an L-type Ca^{++} current and corresponding contraction. Panel B illustrates both current and contraction from the same myocyte following addition of 250 μM lidocaine. Lidocaine blocked the Na^+ current in response to the step depolarization to -40 mV, however contraction observed at -40 mV was still present. A further step to 0 mV activated the L-type Ca^{++} current and corresponding contraction which also were unaffected by lidocaine.

B. Effects of Ischemic Conditions and Reperfusion on Release Contractions.

The effects of simulated ischemia and reperfusion on release contractions were determined next. Figure 20 (panel A) illustrates a representative example of the release contraction observed under normoxic conditions. Throughout the experiment, 2 μM nifedipine was present to eliminate the L-channel component and all traces in figure 20 are from the same myocyte. The holding potential was

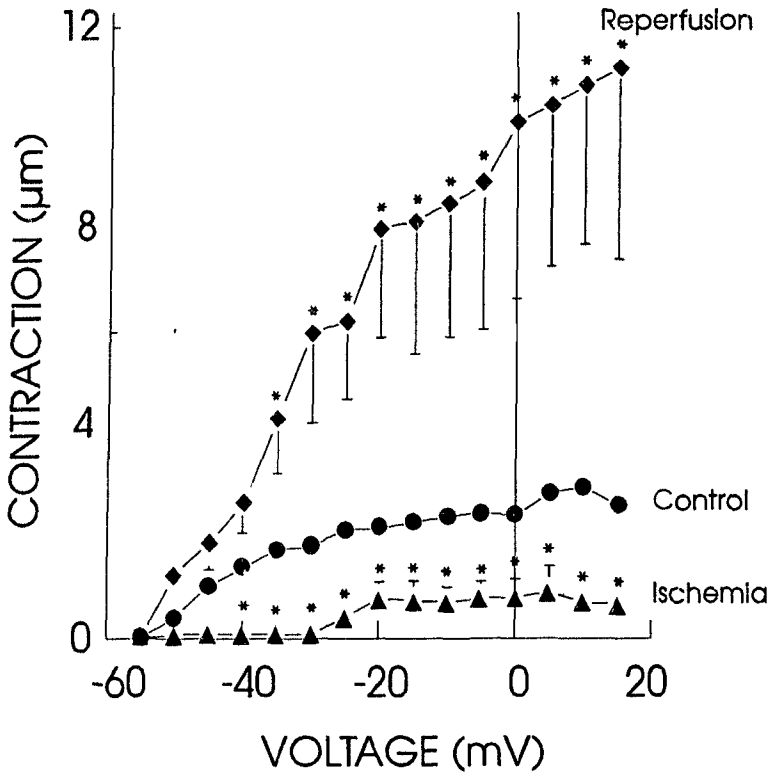

FIGURE 21: Mean ± SEM contraction-voltage relationships for release contractions measured in the presence of 2 µM nifedipine. Under control conditions, the threshold for contraction was -50 mV and contractions increased in magnitude with an increase in voltage. However, the contraction-voltage relationship seemed to plateau at potentials more positive than -20 mV. After 20 min ischemia, release contractions were virtually abolished ($p<0.05$, $n=5$). Upon reperfusion, contractions measured at all potentials were significantly greater than control ($p<0.05$, $n=5$). Experiments where I_{Ti} was observed upon reperfusion were not included in the pooled data.

-80 mV and there was a pre-pulse to +20 mV followed by a return step -55 mV to discharge and inactivate I_{Na}. A further step to -40 mV elicited the release contraction. In the presence of nifedipine, the L-current was not activated and contractions initiated by I_{Ca} were not observed (not shown). However, a large contraction was seen in response to the -40 mV step (panel A). Figure 20B illustrates that the release contraction is virtually abolished following a 20 min exposure to simulated ischemia in the continued presence of nifedipine. The magnitude of the release contraction following 4 min of reperfusion in the presence of nifedipine is shown in figure 20C. Release contraction in early reperfusion exhibited an overshoot when compared to control. The overshoot typically lasted only 5-10 min. With continued reperfusion, the release contraction gradually decreased and disappeared altogether after 30 min reperfusion.

Mean contraction-voltage relationships for the release contractions in control, ischemia and reperfusion are illustrated in figure 21. All experiments were done in the presence of L-channel blockade with nifedipine. Contractions were measured at various potentials from a post-conditioning potential of -55 mV (to discharge and inactivate Na^+ channels). Under control conditions, the threshold for the release contraction was -50 mV. As the voltage increased, the magnitude of the release contraction also increased, although it appeared to plateau in response to voltage clamp steps more positive than -20 mV. Following 20 min of simulated ischemia, the magnitude of contraction was

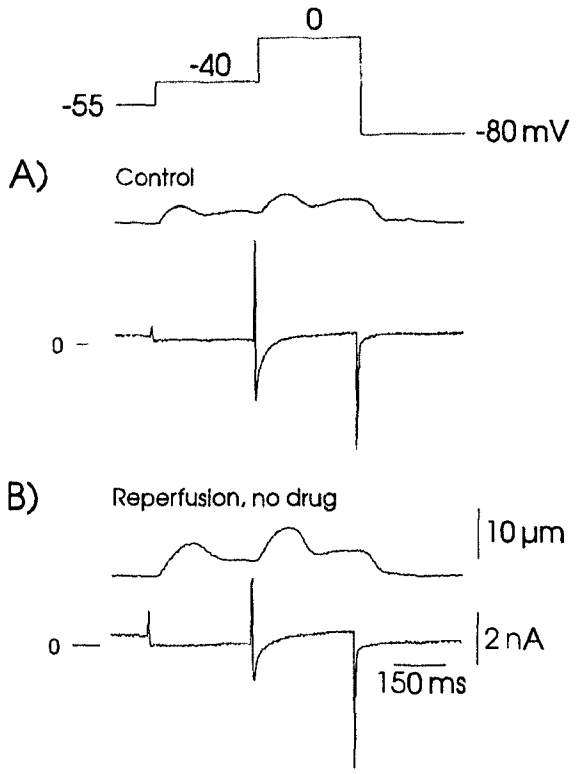

FIGURE 22: Representative examples of currents and contraction recorded from a single myocyte at different points in the protocol. Post-conditioning potential was -55 mV following a pre-pulse to +20 mV to discharge and inactivate I_{Na}. The voltage clamp protocol is displayed at the top. **Panel A)** Currents and contractions observed prior to 20 min ischemia. **Panel B)** Upon reperfusion and in the absence of drug, there was a rebound overshoot in the magnitude of the release contraction (at -40 mV) and the contraction associated with the L-type Ca^{++} current. Horizontal line represents zero current.

greatly reduced at all potentials ($p<0.05$, $n=5$). Reperfusion (4 min) resulted in a substantial overshoot in the magnitude of the release contraction measured at all potentials ($p<0.05$, $n=5$). In contrast to control conditions, the magnitude of the release contraction in early reperfusion did not plateau at more positive voltage clamp steps but continued to increase with increasing voltage.

C. Characteristics of Release Contractions during Reperfusion

The release contraction can be activated at potentials more negative than the threshold for activation of L-type Ca^{++} channels and does not show sensitivity to nifedipine. When nifedipine was present throughout ischemia and reperfusion, reperfusion following 20 minutes of simulated ischemia results in the reappearance and transient overshoot of the release contraction. It was determined whether the overshoot in the release contraction could be selectively inhibited by pharmacological interventions without affecting the L-channel associated contraction. As previously shown, the release contraction could not be abolished by either L-type Ca^{++} channel blockade or Na^{+} channel blockade. Panel A of figure 22 illustrates both current and contraction under normal conditions (ie. prior to ischemia). The voltage clamp protocol is illustrated at the top of figure 22. The holding potential was -80 mV. Prior to the two-step voltage clamp protocol, there was a pre-pulse to +20 mV followed by a return step to -55 mV to discharge and inactivate Na^{+} current. The step to -40 mV elicited a contraction; there was a small inward current associated with the contraction

(figure 22A). A further step to 0 mV activated the L-type Ca^{++} current and corresponding contraction. Panel B of figure 22 illustrates both current and contraction from the same myocyte as in panel A at 4 minutes reperfusion following 20 minutes ischemia. In this example, no drug was present during either simulated ischemia or reperfusion. During early reperfusion, there was a pronounced but transient overshoot in the magnitude of contraction observed from a step to -40 from -55 mV; however, there was still little inward current associated with this step. Also apparent in this figure is the overshoot of the L-current contraction in response to the step depolarization to 0 mV. The magnitude of the L-type Ca^{++} current was decreased about 50%, as described in a previous section of the thesis.

i) Effect of Ni^{++} on Overshoot of Release Contraction in Early Reperfusion.

Previous reports have suggested that T-type Ca^{++} channels may be able to initiate cardiac contraction (Zhou and January, 1994; Ferrier and Howlett, 1995). Therefore, it was determined whether low concentrations of Ni^{++} (100-200 μM), which inhibits T-channels (Tytgat et al., 1990), could prevent the transient overshoot of the release contraction upon reperfusion. In these experiments, 200 μM Ni^{+} was applied during both ischemic conditions and reperfusion to prevent the overshoot of the release contraction. A representative example of both contractions and currents observed under control conditions (panel A) and at 4 min reperfusion in the presence of Ni^{++} (panel B) is illustrated

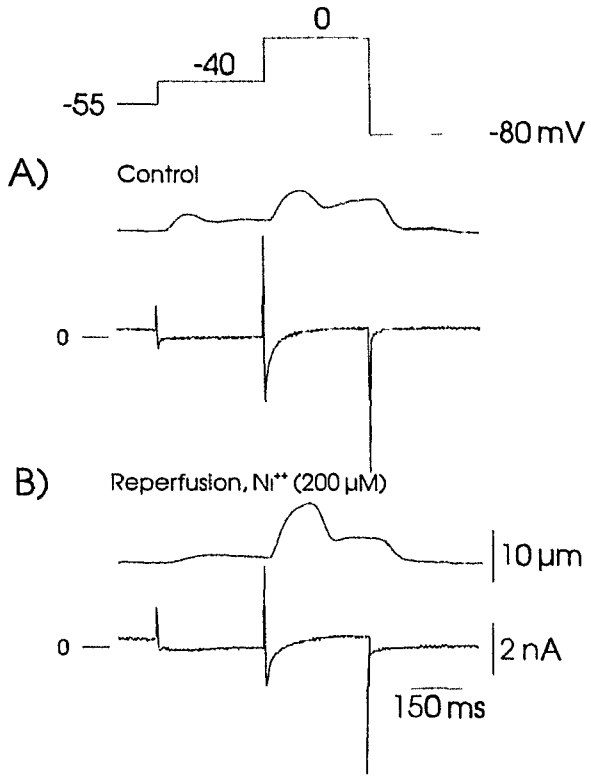

FIGURE 23: Representative examples of currents and contractions from the same myocyte under control conditions and upon reperfusion following pretreatment with Ni^{++}. The voltage clamp protocol is shown at the top of the figure and is identical to that in figure 22. **Panel A)** In this myocyte, the release contraction is present upon a step depolarization to -40 mV. A further step to 0 mV activated the L-current and contraction. **Panel B)** The release contraction was reduced by 200 µM Ni^{++} and the L-channel contraction exhibited a transient overshoot in early reperfusion. Horizontal line represents zero current.

in figure 23. The voltage clamp protocol is the same as in figure 22. Prior to ischemia and in the absence of drug, both the release contraction and the contraction associated with the L-type Ca^{++} current can be observed in response to voltage clamp steps to -55 and -40 mV respectively. Panel B of figure 23 shows current and contraction from the same myocyte at 4 minutes reperfusion. The release contraction observed at 4 min reperfusion was substantially reduced in the presence of Ni^{++}. The L-channel contraction exhibited a transient overshoot. Similar results were observed in 5 experiments. In another set of experiments, 200 μM Ni^{++} was applied only during reperfusion. In these experiments the transient overshoot was not prevented (n=5, data not shown).

ii) Effect of Ryanodine on Overshoot of Release Contraction in Early Reperfusion.

The low concentration of Ni^{++} used in the previous experiment has been shown to selectively inhibit T-channels (Tytgat et al., 1990). Millimolar concentrations of Ni^{++} have been shown to inhibit Na^{+}-Ca^{++} exchange thus a minor effect on Na^{+}-Ca^{++} exchange cannot be excluded. Since it has been reported that in early reperfusion, the Na^{+}-Ca^{++} exchange works in the reverse mode to bring Ca^{++} into the cell (Tani, 1990), Ni^{++} might decrease the amplitude of the release contractions by inhibition of the exchanger. Reverse mode of the Na^{+}-Ca^{++} exchange is believed to be a consequence of Na^{+} loading during ischemia (Haigney et al., 1992). The transient overshoot of the release

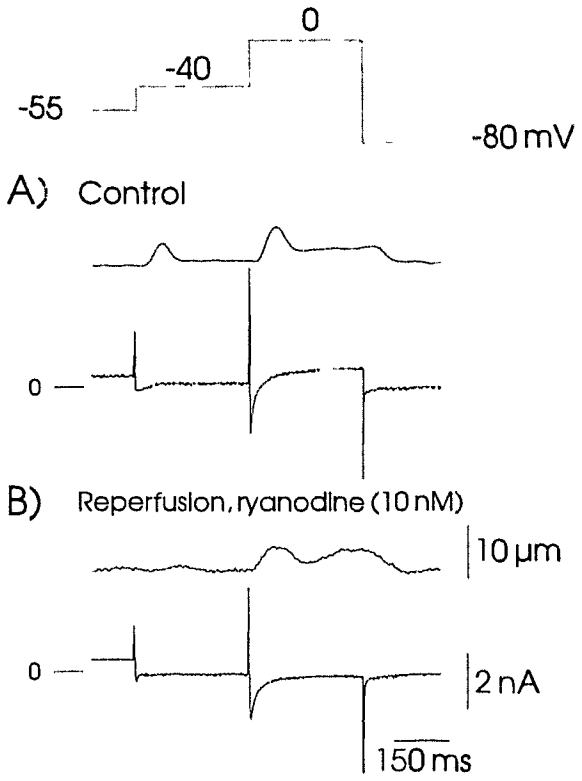

FIGURE 24: Representative examples of current and contraction from the same myocyte during control conditions (**panel A**) and upon reperfusion in the presence of ryanodine (10 nM)(**panel B**). The voltage clamp protocol is shown at the top of the figure. Under control conditions, both a release contraction and an L-current associated contraction were observed in response to step depolarizations to -40 and 0 mV respectively. **Panel B)** Ryanodine (10 nM) depressed the contraction observed at -40 mV and slightly reduced the L-current associated contraction. Horizontal line represents zero current.

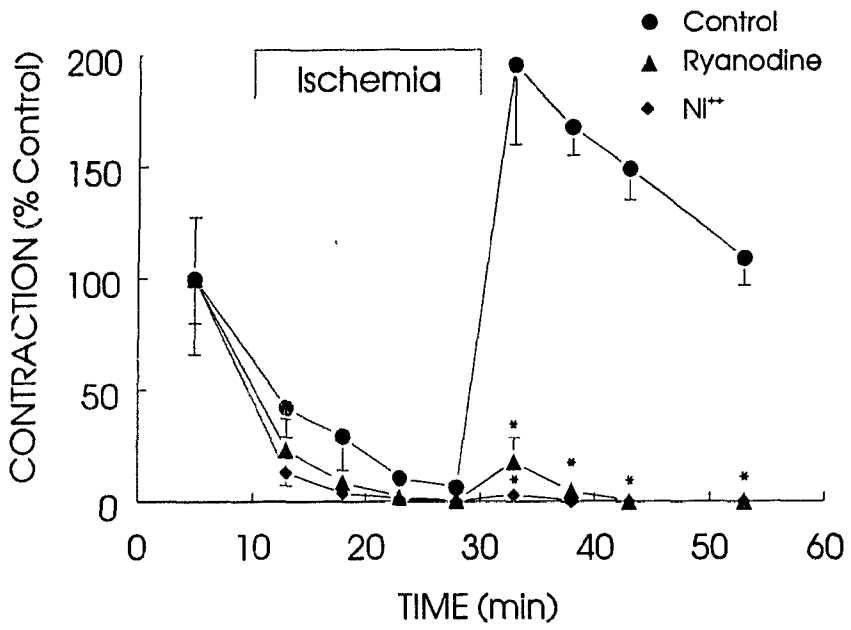

FIGURE 25: Time course of changes in release contractions in response to ischemia and reperfusion. Drugs were present during ischemia and reperfusion. In the absence of drug, exposure to ischemic conditions caused a rapid decrease in the magnitude of contractions. Reperfusion resulted in a transient overshoot in the magnitude of contractions. In the presence of either 200 μM Ni^{++} or 10 nM ryanodine, inhibition of contraction in ischemia was unaffected. However, the transient overshoot of contraction was prevented by both drugs.

* = significantly different from control.

contraction in early reperfusion may be due to SR Ca^{++} release or to direct activation of the myofilaments by Ca^{++} entering via the Na^{+}-Ca^{++} exchange. To test whether the release contraction was due to Ca^{++} release from the SR, the simulated ischemia and reperfusion experiments were repeated in the presence of 10 nM ryanodine. Ryanodine, at low concentrations, depletes SR Ca^{++} by locking the SR Ca^{++} release channel in an open subconducting state (Rousseau et al., 1987). Figure 24 is a representative example illustrating currents and contractions observed under control conditions and at 4 minutes reperfusion in the presence of ryanodine. The contraction in response to the -40 mV step was virtually abolished by ryanodine while the contraction elicited by the step to 0 mV was unaffected by ryanodine. Similar results were observed in 5 experiments.

Figure 25 shows the time course of release contractions in response to simulated ischemia and reperfusion. In the absence of drug, exposure to ischemic conditions caused a rapid decrease in the magnitude of contraction. Reperfusion resulted in a temporary overshoot in contraction to almost 200% when compared to levels seen prior to ischemia. With continued reperfusion, contractions gradually returned to control levels after 30 min. Contractile failure during ischemia was not affected by either ryanodine or Ni^{++}. However, the temporary overshoot observed upon reperfusion was greatly attenuated. These results suggest that release contractions are inhibited by ryanodine and Ni^{++}. Furthermore, the release contractions are inhibited during simulated ischemia and exhibit a marked overshoot upon reperfusion.

D. Induction of I_{Tl} in Early Reperfusion.

In some experiments, it was not possible to measure the release contraction upon reperfusion because of induction of I_{Tl}. It has been reported that I_{Tl} is seen upon repolarization following a depolarizing pulse and is due to an oscillatory release of Ca^{++} from the SR (January and Fozzard, 1988). In the voltage clamp protocol where the myocyte is held at -55 mV following a conditioning potential of +20 mV, I_{Tl} appeared in early reperfusion. Since appearance of I_{Tl} decreases the amount of Ca^{++} from the SR pool and thereby alters the amount of Ca^{++} available for subsequent contraction (Wolska and Lewartowski, 1993), experiments where I_{Tl} was induced upon reperfusion were not included in the pooled data.

The incidence of I_{Tl} in early reperfusion was also recorded in the presence of Ni^{++} and ryanodine. In the absence of drug, I_{Tl} was recorded in 18/23 (78%) of experiments. I_{Tl} was observed in only 1 out of 5 experiments when Ni^{++} (200 μM) was present during ischemia and reperfusion. In experiments where 10 nM ryanodine was present, I_{Tl} was observed in 2/5 experiments. When the concentration of ryanodine was increased to 100 nM, I_{Tl} was never observed during early reperfusion (0/3 experiments).

4. EFFECTS OF ADENOSINE DURING SIMULATED ISCHEMIA AND REPERFUSION.

A. Effects of Adenosine Under Normoxic Conditions.

The results presented thus far illustrate that significant electrophysiological and contractile changes occur in a cellular model of simulated ischemia and reperfusion. However, one component of true myocardial ischemia which was not included in the simulated ischemic Tyrode's solution was ADN. Formation and release of ADN from myocardial tissue has been shown to occur in response to a decrease in Po_2 (Bardenheuer and Schrader, 1986) as well as to adrenergic stimulation (Miller et al., 1979). However, little is known about the effects of ADN on the cellular electrophysiological and contractile responses of ventricular myocytes to ischemia and reperfusion.

In the following series of experiments the effects of ischemia and reperfusion in the presence and absence of ADN were determined. It is unclear whether ADN is released from ventricular myocytes during simulated ischemia in this model of ischemia and reperfusion. If ADN is released from ventricular myocytes, it may be washed away in the superfusion chamber. To determine whether endogenous ADN contributed to the effects of ischemia and reperfusion on the electrical and contractile responses, the non-selective ADN antagonist, 8-PT alone was applied during the ischemic period. The effects of exogenously applied ADN during simulated ischemia also were determined. ADN was

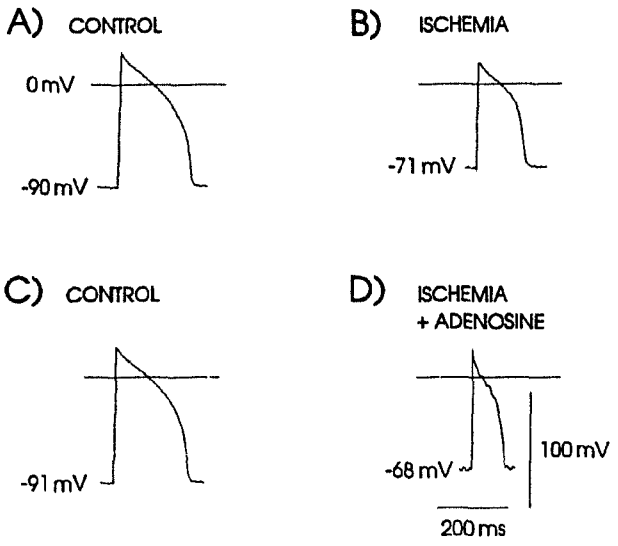

FIGURE 26: Representative examples of action potentials observed under control and ischemic conditions. **Panel A)** Action potential recorded prior to exposure to simulated ischemia. **Panel B)** Action potential from the same myocyte as in A. APD was abbreviated and the cell depolarized following a 20 min exposure to simulated ischemia. **Panel C)** Action potential from a different myocyte recorded prior to ischemia. **Panel D)** Action potential recorded from the same myocyte as in C in the presence of 50 μM ADN. ADN potentiated the abbreviation of APD induced by ischemia. Values of MDP are shown in each panel.

present during the ischemic period only.

Prior to determining the effects of ADN during ischemia and reperfusion, the effects of ADN were determined under normoxic conditions. Application of 1, 5, or 50 μM of ADN had no significant effects on contractile or cellular electrophysiological responses in ventricular myocytes (data not shown), which is in agreement with observations in previous studies (Isenberg and Belardinelli, 1987).

B. Effects of ADN on Membrane Voltage and Contraction during Simulated Ischemia and Reperfusion.

The effects of ADN were determined under conditions of simulated ischemia and reperfusion. Action potentials were recorded in conventional recording mode. Action potentials were elicited by applying a 3 msec depolarizing current pulse delivered through the microelectrode via the Axoclamp-2A console. Figure 26A shows a representative action potential recorded from a ventricular myocyte under control conditions. Figure 26B shows an action potential recorded from the same myocyte following 20 min of ischemia. Both MDP and APD decreased during ischemia. Figure 26C and D show action potentials recorded from another myocyte during control conditions (C) and after a 20 min exposure to simulated ischemia in the presence of 50 μM ADN (D). Abbreviation of APD in response to ischemia was greater in the presence of ADN than under control conditions.

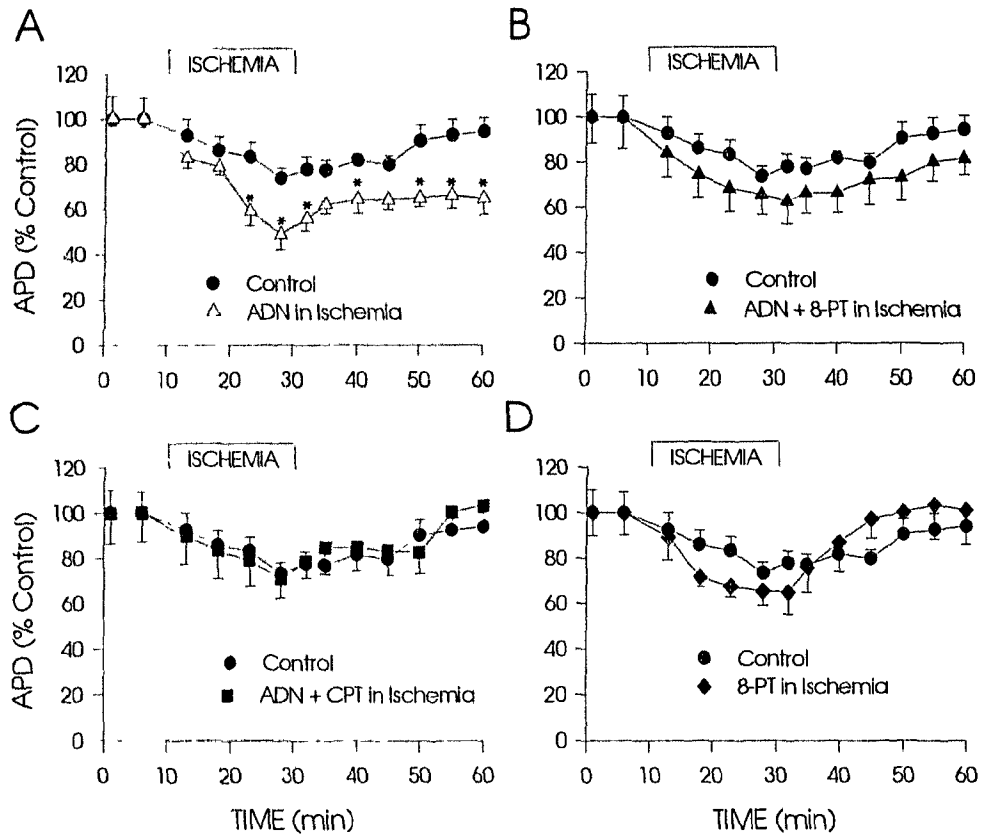

FIGURE 27: Panel A) Time course of changes in APD_{90} in response to ischemia and reperfusion. In absence of drug, there was a progressive decrease in APD in ischemia, followed by gradual return to pre-ischemic levels upon reperfusion. Addition of 50 μM ADN during simulated ischemia resulted in a greater abbreviation of APD during ischemia and delayed recovery in reperfusion. **Panel B)** The effect of ADN (50 μM) on changes in APD was prevented by 10 μM 8-PT. **Panel C)** The A_1 selective adenosine antagonist CPT (5 μM) also prevented the effect of ADN on APD. **Panel D)** 8-PT (10 μM) alone, present during ischemia, did not alter APD during simulated ischemia and reperfusion. * = significantly different from control ($p<0.05$).

Figure 27 illustrates the time course of changes in APD during simulated ischemia and reperfusion in the presence and absence of ADN and non-selective ADN antagonist 8-PT and selective A_1 adenosine receptor antagonist CPT. Panel A of figure 27 shows the time course of changes in APD during simulated ischemia and reperfusion in the presence and absence of 50 μM ADN. In myocytes not exposed to ADN, the mean APD_{90} was 253 ± 12 msec (n=12) during control conditions. APD gradually decreased to 188 ± 12 msec ($p<0.05$, n=12) at the end of 20 min of ischemia. Changes in APD elicited by simulated ischemia were not prevented by current clamping MDP back to control levels (not shown). In fact, this resulted in a further abbreviation of APD (data not shown). Reperfusion resulted in gradual recovery toward control values. The mean APD under control conditions for myocytes exposed to ADN in ischemia was 238 ± 9 msec. In the presence of ADN, APD decreased to 116 ± 16 msec ($p<0.05$) after 20 min of ischemia. In addition, although ADN was no longer present during reperfusion, APD remained significantly abbreviated even after 30 min reperfusion. Panel B of figure 27 illustrates the time course of changes in APD in the absence of drug, and when both 50 μM ADN and 8-PT (10 μM) were present during the ischemic period. The decrease in APD produced by ADN was reversed by 8-PT. Figure 27C shows the effects of 50 μM ADN plus CPT (5 μM) on APD compared to control. The effect of ADN on APD was completely reversed by CPT at all time points in the experiment. To determine whether endogenous ADN released from the myocytes during simulated

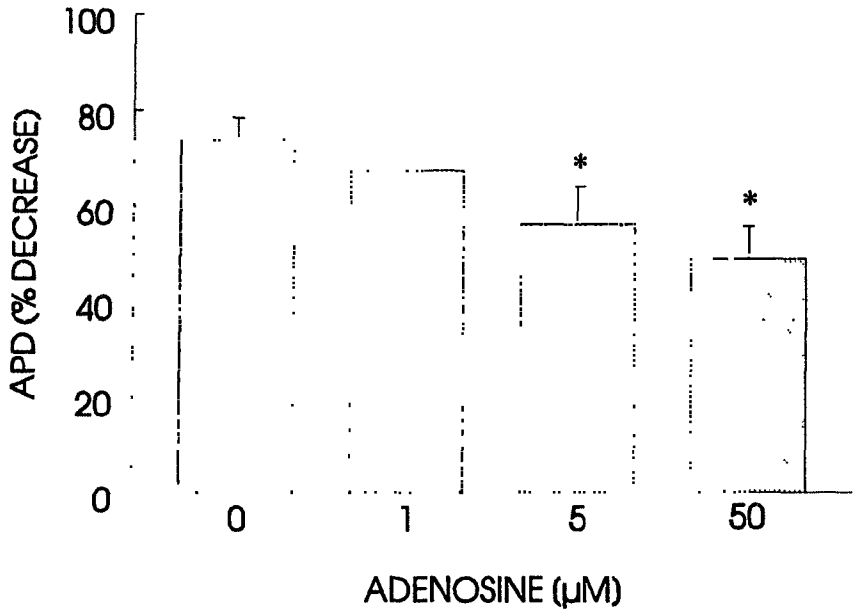

FIGURE 28: The concentration dependence of the effect of ADN on APD measured after 20 minutes of simulated ischemia. ADN produced a dose-dependent decrease in APD following 20 min of ischemia. The effects of both 5 and 50 μM ADN were statistically significant ($p<0.05$).

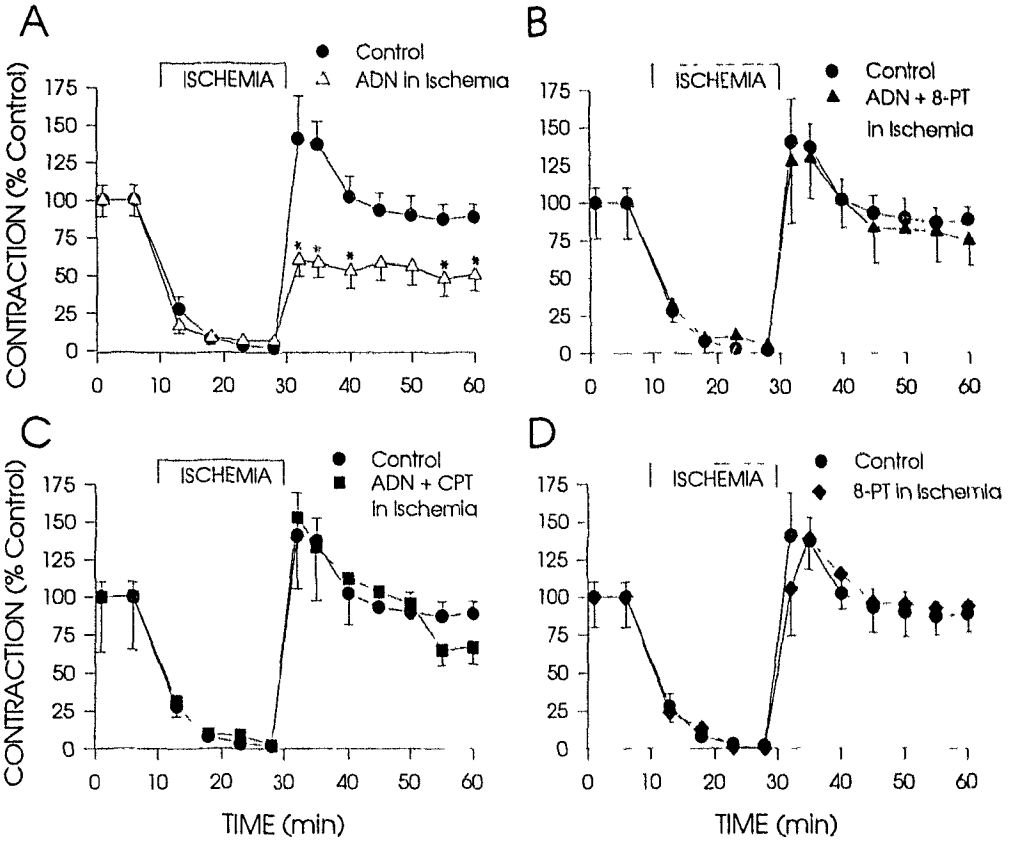

FIGURE 29: Time course of changes in contractions associated with action potentials. **Panel A)** Exposure to simulated ischemia resulted in a rapid and complete inhibition of contractions. Return to control conditions resulted in a temporary overshoot of contractions after about 5 min of reperfusion. ADN did not alter contractile failure during ischemia but prevented the transient overshoot in contractions upon reperfusion. The effects of ADN on contractions in early reperfusion were prevented by either 10 μM 8-PT **(Panel C)** or 5 μM CPT **(Panel C)**. **Panel D)** Application of 10 μM 8-PT alone during ischemia did not alter contractions during either ischemia or reperfusion. * = significantly different from control ($p<0.05$).

ischemia might have contributed to our results, 8-PT (10 μM) alone was applied during the ischemic period. Panel D of figure 27 illustrates that application of 8-PT during ischemia did not alter APD. This result suggests that there is little contribution of endogenous ADN to the observed effects on APD.

The concentration dependence of the effect of ADN on APD measured after 20 minutes of simulated ischemia is illustrated in figure 28. Addition of 1 μM ADN to the ischemic Tyrode's solution caused only a slight reduction in APD, whereas 5 and 50 μM ADN decreased APD to 56 ± 8% and 48 ± 7% of control, respectively by 20 min of ischemia. The effects of both 5 and 50 μM ADN were statistically significant ($p<0.05$). The concentration-dependence of the effects of ADN on I_{Ca}, contractions and MDP were also investigated. The weak effects of ADN on I_{Ca} and MDP were observed only at 50 μM.

Action potentials were accompanied by cell shortening. Figure 29 shows the time course of changes in the magnitude of contraction associated with action potentials. In the absence of drug, exposure to simulated ischemia caused the magnitude of contractions to decrease until they were abolished (panel A). Upon reperfusion, contractions returned rapidly and the magnitude of cell shortening temporarily exceeded control levels. Contractions gradually returned to control levels with continued reperfusion. With 50 μM ADN present during ischemia, contractile failure during ischemia was not altered (figure 29A). However, in early reperfusion the transient overshoot in magnitude of

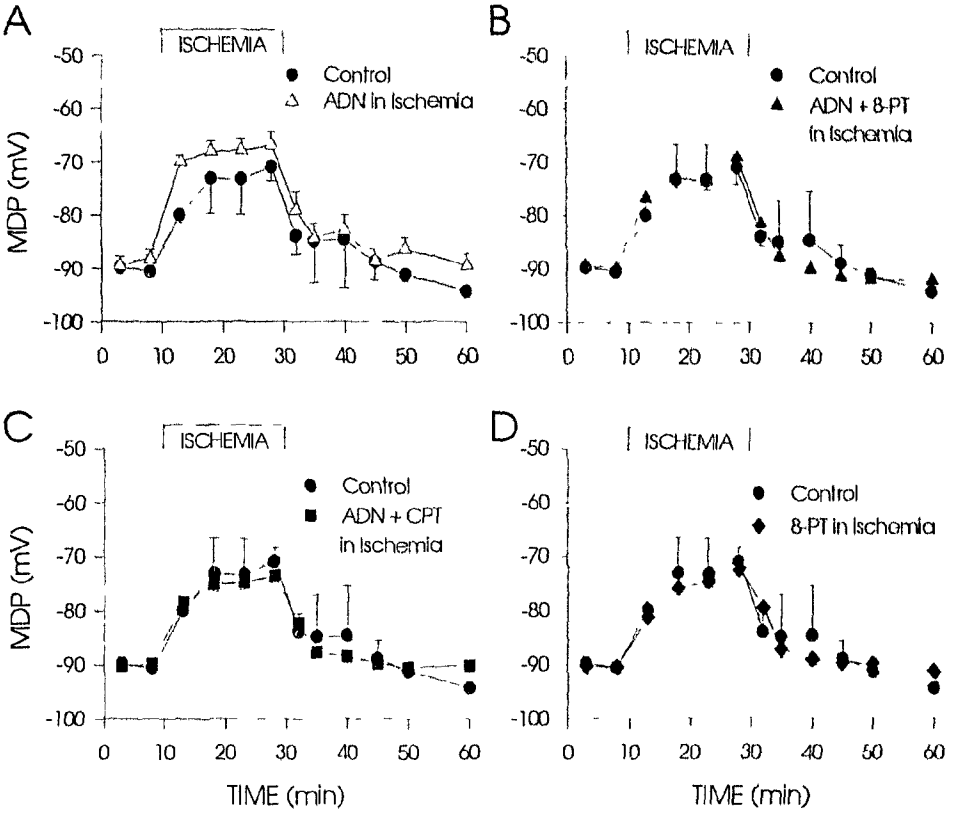

FIGURE 30: Time course of changes in MDP in response to simulated ischemia and reperfusion. **Panel A)** Upon exposure to ischemia, the cells gradually depolarized; return to normoxic conditions caused MDP to return to pre-ischemic values. MDP tended to be more depolarized during ischemia in the presence of ADN. Time course of changes in MDP was unaffected by application of either 8-PT **(Panel B)** or CPT **(Panel C)**. **Panel D)** Application of 8-PT alone during ischemia did not alter MDP during simulated ischemia or reperfusion.

contractions was abolished ($p<0.05$). In addition, the magnitude of contractions did not return to control levels and remained significantly depressed up to 30 min of reperfusion. Panel B of figure 29 illustrates the effects of ADN (50 μM) plus 8-PT (10 μM) on contractions elicited by action potentials during simulated ischemia and reperfusion. The non-selective antagonist 8-PT abolished the effects of ADN on contraction. Panel C of figure 29 shows the effect of ADN (50 μM) plus CPT (5 μM) on contractions elicited by action potentials. The selective A_1 receptor antagonist prevented the effects of ADN on contractions initiated by action potentials. When 8-PT (10 μM) alone was present during ischemia, there was no change in the magnitude of contractions observed during ischemia or reperfusion when compared to control conditions (panel D of figure 29).

Figure 30 illustrates changes in MDP in response to simulated ischemia and reperfusion. In the absence of ADN, myocytes gradually depolarized from -90 ± 1 mV to -73 ± 7 mV ($p<0.05$) after 20 min of simulated ischemia (figure 30A). MDP returned to pre-ischemic values after about 30 minutes of reperfusion. ADN resulted in a tendency toward greater depolarization in ischemia than observed in control myocytes, although these results were not statistically significant (figure 30A). In the presence of 50 μM ADN, myocytes depolarized from -87 ± 2 mV to -63 ± 5 mV after 20 min of ischemia. Addition of either ADN plus 8-PT (figure 30B) or ADN plus CPT (figure 30C) resulted in no significant change in MDP when compared to control. Application of 8-PT alone during ischemia did not alter the time course of changes in MDP when compared

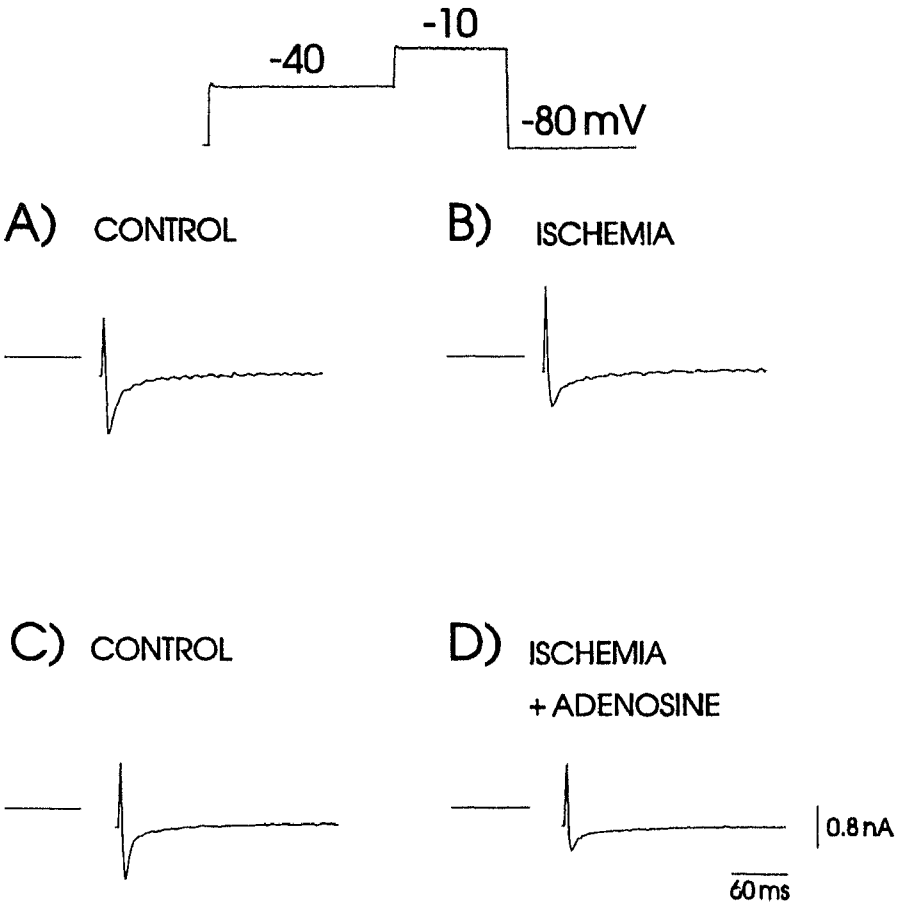

FIGURE 31: Representative examples of I_{Ca} recorded under control and ischemic conditions in the presence and absence of ADN. **Panel A)** I_{Ca} recorded prior to exposure to simulated ischemia **Panel B)** I_{Ca} from same myocyte as in A was decreased in magnitude after a 20 min exposure to simulated ischemia. **Panel C)** I_{Ca} from a different myocyte recorded prior to ischemia. **Panel D)** Exposure of the same myocyte as in C to simulated ischemia in the presence of 50 μM ADN resulted in no significant change in the magnitude of I_{Ca}. Horizontal line represents 0 current. The voltage clamp protocol is illustrated at the top of the figure.

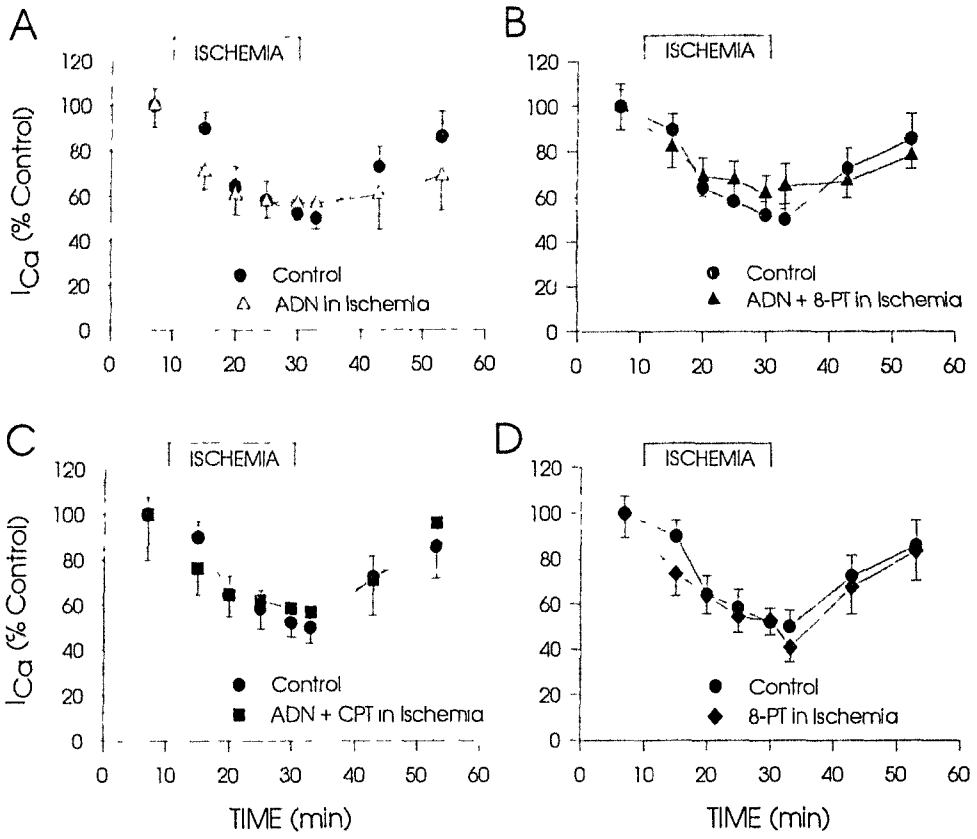

FIGURE 32: Time course of changes in I_{Ca} in response to simulated ischemia and reperfusion. **Panel A)** Normalized changes in I_{Ca} in response to ischemia and reperfusion in the presence and absence of ADN. ADN did not affect the magnitude of I_{Ca} in ischemia and reperfusion. **Panel B), C)** The time course of changes in I_{Ca} in response to ischemia and reperfusion was unaffected by either 8-PT or CPT. **Panel D)** 8-PT alone during ischemia did not affect I_{Ca} during simulated ischemia or reperfusion.

to control (panel D of figure 30).

C. Effects of ADN on Membrane Currents and Contraction during Simulated Ischemia and Reperfusion.

To determine effects of ADN on I_{Ca} in ischemia and reperfusion, cells were voltage clamped at regular intervals. The voltage clamp protocol is illustrated at the top of figure 31. The holding potential was -80 mV and there was a 200 msec pre-pulse to +20 mV (pre-pulse not shown). The voltage clamp protocol consisted of a step depolarization to -40 mV for 500 msec to discharge and inactivate Na^+ channels. I_{Ca} was measured during a 300 msec step depolarization from -40 to -10 mV. Figure 31 (panels A and B) shows examples of I_{Ca} measured in one representative myocyte. Prior to exposure to simulated ischemia, the magnitude of I_{Ca} in this cell was approximately -1.0 nA (panel A). Panel B illustrates I_{Ca} from the same myocyte following a 20 min exposure to simulated ischemia. In this example, the magnitude of I_{Ca} decreased by about 48% when compared to control (panel A). Panels C and D show I_{Ca} recorded from another myocyte. With ADN present in ischemia (panel D), I_{Ca} decreased by about 51% when compared to control (panel C).

The time course of changes in peak I_{Ca} normalized to each respective control in the presence and absence of drug are shown in figure 32. Peak I_{Ca} was measured at -10 mV. In the absence of ADN, exposure to ischemic conditions resulted in a decrease in peak I_{Ca} from -0.98 ± 0.08 nA to -0.50

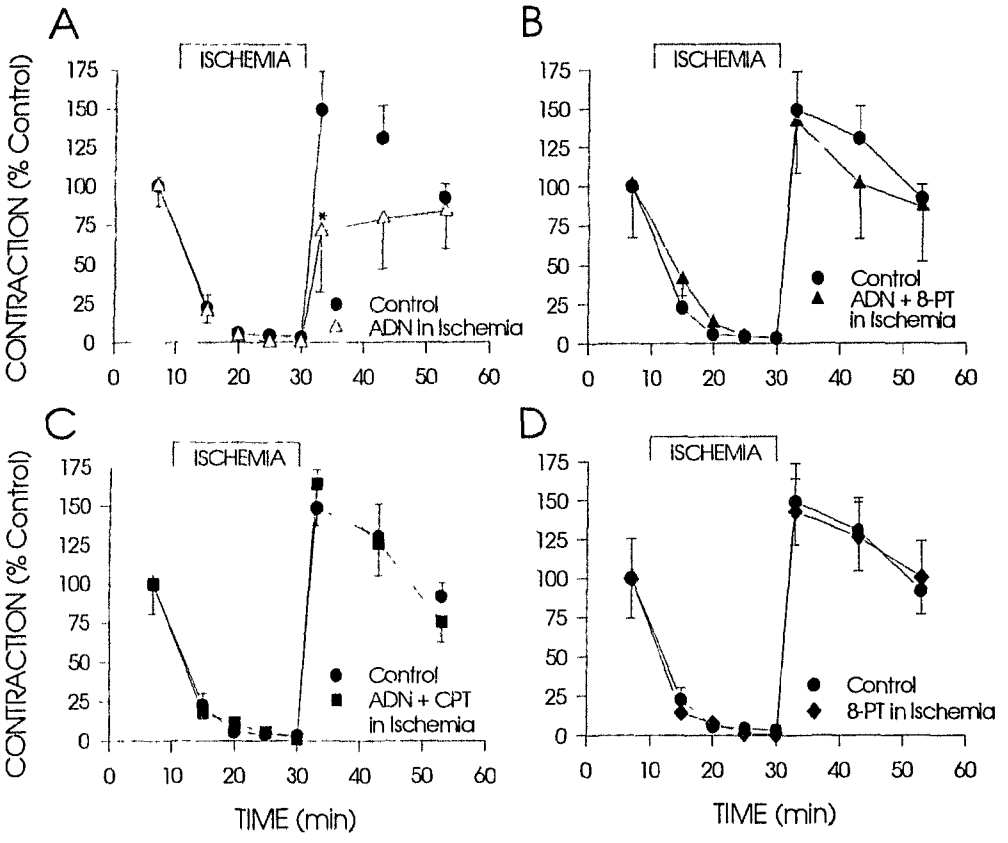

FIGURE 33: Contractions elicited by step depolarizations to -10 mV during voltage clamp experiments. **Panel A)** Upon exposure to ischemic conditions, there was a rapid and complete inhibition of contractions. Reperfusion resulted in a temporary overshoot of contractions after about 3 min. ADN did not alter contractile failure during ischemia but prevented the transient overshoot in contractions upon reperfusion. The blunting of contractions upon early reperfusion produced by ADN was prevented by both 8-PT **(Panel B)** and CPT **(Panel C)**. Application of 8-PT alone **(Panel D)** did not alter the magnitude of contraction during either ischemia or reperfusion. * = significantly different from control ($p<0.05$).

± 0.06 nA at the end of ischemia (panel A). Reperfusion was accompanied by a slow return of I_{Ca} toward pre-ischemic values. Addition of 50 μM ADN during ischemia did not significantly affect the decrease in peak I_{Ca} in ischemia, and there was no significant difference in the recovery of I_{Ca} during reperfusion (figure 32A). Panels B and C of figure 32 illustrate normalized data for both ADN plus 8-PT and ADN plus CPT, respectively. In the presence of either 8-PT or CPT, changes in the amplitude of I_{Ca} during simulated ischemia and reperfusion were not significantly different from control. The ischemia-induced changes in I_{Ca} were unaffected when 8-PT (10 μM) alone was applied during ischemia (figure 32B).

Voltage clamp steps from -40 to -10 mV which activated I_{Ca} also induced contractions. The time courses of changes in unloaded cell shortening during simulated ischemia and reperfusion under voltage clamp conditions is shown in Figure 33. Cell shortening is normalized as percent of pre-ischemic values for control and ADN treated myocytes (panel A). In the absence of ADN, exposure to simulated ischemia caused a rapid decline in cell shortening. Contractions were not measurable after 10 minutes of ischemia. Upon reperfusion, the magnitude of cell shortening in control myocytes rapidly returned and exceeded pre-ischemic levels (152% of control). Following this, contractions gradually decreased in magnitude and reached pre-ischemic levels after about 30 min reperfusion. When 50 μM ADN was present during ischemia, the time course of inhibition of cell shortening in ischemia was virtually identical to that seen in

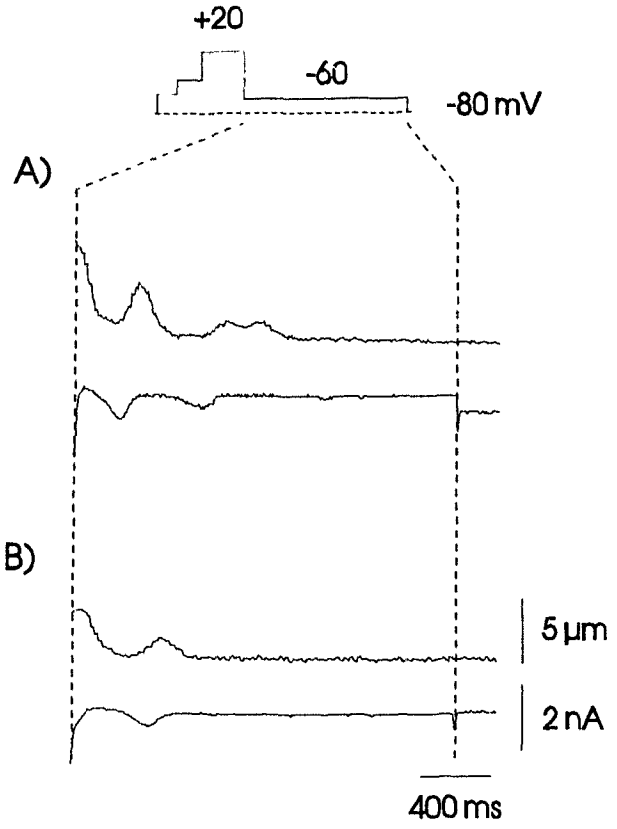

FIGURE 34: Examples of I_{Tl} (bottom trace of each pair) and associated aftercontractions (top trace in each pair) observed upon repolarization to -60 mV at 3 min reperfusion. **Panel A)** I_{Tl} was observed transiently during early reperfusion but dissipated after 15-20 min reperfusion. Multiple I_{Tl}'s and aftercontractions frequently appeared during reperfusion. **Panel B)** I_{Tl} recorded in a cell exposed to 50 µM ADN in ischemia. ADN reduced the incidence and magnitudes of reperfusion-induced I_{Tl} and aftercontractions. The voltage clamp protocol is shown schematically at the top of the figure. Illustrations depict current and contraction recorded during the period indicated by the dashed lines in the schematic.

control myocytes (figure 33A). However, cell shortening did not exceed pre-ischemic levels upon reperfusion. Recovery of contraction proceeded rapidly during the first 2 min of reperfusion and more slowly later in reperfusion. Contractile responses were similar to control when either ADN plus 8-PT (panel B of figure 33) or ADN plus CPT (panel C of figure 33) were applied during ischemia. Application of 8-PT alone (panel D) also had no effect on contractile responses in ischemia and reperfusion. The effects of 1 and 5 μM ADN on contractions initiated by voltage clamp steps which activate I_{Ca} also were determined. In the absence of ADN, the magnitude of cell shortening increased to 149 ± 24% of control at 3 min of reperfusion. There was no change in the magnitude of this response in 1 μM ADN (151 ± 22%). Addition of 5 μM ADN decreased contractions to 101 ± 28% of control whereas 50 μM ADN significantly reduced contractions to 70 ± 37% of control ($p<0.05$). A similar concentration dependence was demonstrated for the effects of ADN on contractions elicited by action potentials.

D. Effects of Adenosine on Incidence of I_{Tl} in Early Reperfusion.

In the absence of ADN, reperfusion induced I_{Tl} in 18 of 23 experiments (78%). I_{Tl} typically appeared during the first 10 min of reperfusion and gradually diminished in amplitude with time. I_{Tl} was accompanied by aftercontractions. Figure 34A shows representative traces of I_{Tl} and corresponding aftercontractions observed at 5 min of reperfusion under voltage clamp

TABLE 6: INCIDENCE OF I_{Tl} IN RESPONSE TO VARIOUS DRUG TREATMENTS.

TREATMENT	INCIDENCE
Control	18/23 (78%)
ADN (1 μM)	6/8 (75%)
ADN (5 μM)	4/8 (50%)
ADN (50 μM)	3/8 (38%)
ADN (50 μM) + 8-PT (10 μM)	5/8 (63%)
ADN (50 μM) + CPT (5 μM)	5/8 (63%)
8-PT (10 μM)	8/8 (100%)

conditions. The voltage clamp protocol is illustrated at the top of figure 34. The holding potential was -80 mV and there was a 200 msec pre-pulse to +20 mV (pre-pulse not illustrated). The voltage clamp protocol consisted of a 300 msec step to -40 mV to discharge and inactivate Na^+ channels; a step depolarization to -10 mV to activate and allow measurement of I_{Ca}; an activation step to +20 mV and repolarization to -60 mV to determine if I_{TI} was inducible. In some myocytes, only one I_{TI} and corresponding aftercontraction occurred, whereas multiple sequential I_{TI}'s and aftercontractions were observed in others. With 1 μM ADN present during ischemia, I_{TI} was observed upon reperfusion in 6 out of 8 experiments (75%)(Table 6, page 137). With 5 or 50 μM ADN present during ischemia, the incidence of I_{TI} observed during reperfusion was reduced to 4 of 8 experiments (50%) and 3 of 8 experiments (37.5%), respectively (Table 6, page 137). In addition, multiple I_{TI}'s and aftercontractions were no longer observed with the higher concentration of ADN. Original traces of I_{TI} and corresponding aftercontraction observed at 5 min of reperfusion in an experiment in which 50 μM ADN was present during ischemia are shown in figure 34B. The magnitudes of I_{TI} and aftercontractions were less following ADN pretreatment than observed in control myocytes. Both the selective and the non-selective ADN antagonists appeared to inhibit the effects of ADN on incidence of I_{TI}. When 50 μM ADN and 8-PT were present during ischemia, reperfusion resulted in induction of I_{TI} in 5 of 8 experiments (63%)(Table 6, page 137). Similarly, when ADN and CPT were present during ischemia, reperfusion induced I_{TI} in 5 of

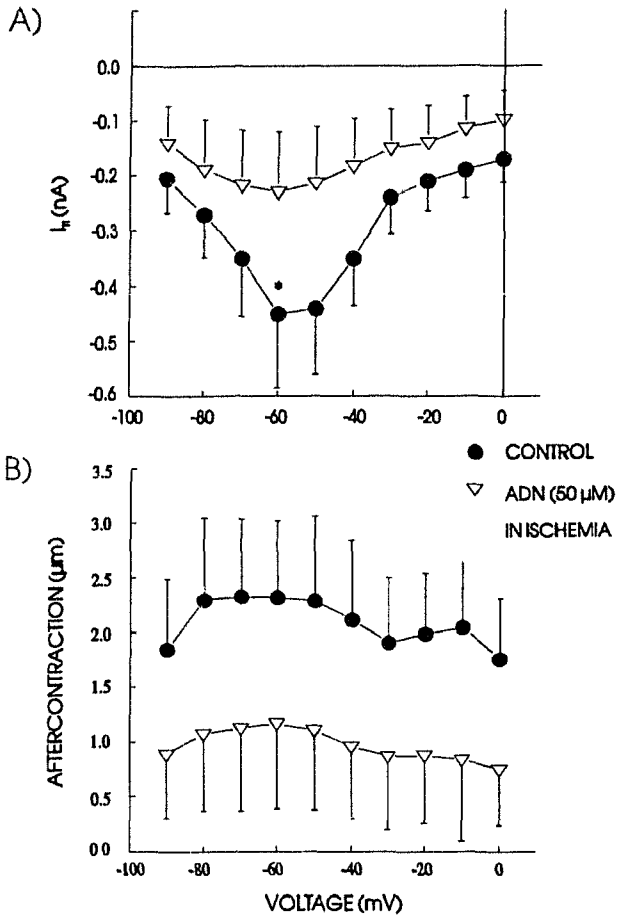

FIGURE 35: Panel A) I-V relationship for I_{Ti} induced by reperfusion (values are the means ± SEM, for 9 control experiments and 8 ADN experiments). These relationships were determined by varying the repolarization voltage following a 300 ms activation step to +20 mV. I_{Ti} increased to a peak at about -60 mV and then declined. No reversal potential was observed. Addition of 50 μM ADN during simulated ischemia resulted in a decrease in the magnitude of I_{Ti}. Statistical significance was observed at -60 mV ($p<0.05$). **Panel B)** Contraction-voltage relationship for aftercontractions associated with I_{Ti}. In the presence of ADN, the magnitude of aftercontractions was reduced at all potentials, although this was not statistically significant.

8 experiments (63%)(Table 6, page 137). Addition of 8-PT alone during ischemia resulted in an increase in the incidence of I_{TI} upon reperfusion. In these experiments I_{TI} was induced in 8/8 experiments (100%), which suggests that endogenous ADN may reduce the incidence of I_{TI} in early reperfusion (Table 6, page 137).

I-V and contraction-voltage relations for I_{TI} induced by reperfusion are shown in Figures 35A and 35B. The I-V and C-V relations were generated using the voltage clamp protocol illustrated in figure 34 except that the repolarization voltages were varied between -90 and 0 mV. I_{TI} induced by reperfusion was always inward at repolarization voltages between -90 and 0 mV. As the membrane potential became less negative, I_{TI} increased to a maximum between -60 mV and -50 mV and then declined in control myocytes. In myocytes pretreated with ADN, the magnitude of I_{TI} was reduced, especially near the peak of the I-V curve where there was a statistically significant reduction at -60 mV. The I-V relationship was not shifted with respect to voltage in the presence of ADN. Aftercontractions that were associated with I_{TI} are illustrated in figure 35B. Aftercontractions also were decreased in cells pretreated with ADN. In contrast to I_{TI}, magnitude of aftercontractions was decreased approximately equally at all membrane potentials tested, although none of the ADN values were statistically significant with respect to control (figure 35B).

DISCUSSION

1. DEVELOPMENT OF A CELLULAR MODEL OF SIMULATED ISCHEMIA AND REPERFUSION.

A. Comparison of Multicellular and Single Cells Model.

The present study describes and characterizes a cellular model of simulated ischemia and reperfusion which allows measurement of changes in action potentials, ionic currents and contractions. This model exhibits changes in electrical and contractile activity similar to those reported in studies of Langendorff perfused hearts subjected to low or no flow ischemia and reperfusion.

Various multicellular and whole heart models have been used to study different aspects of acute coronary occlusion and infarction. Recent studies have attempted to utilize isolated myocytes to determine relations between electrical and contractile activity. However, these studies mainly have examined the effects of profound anoxia, or combinations of metabolic poisons such as cyanide and 2-deoxyglucose rather than conditions observed during ischemia. Both anoxia and metabolic poisons cause a rapid fall in intracellular ATP and activation of ATP-sensitive K^+ channels (Stern et al., 1988; Lederer et al., 1989). Abbreviation of the action potential by $I_{K,ATP}$ may play an important role in rapid contractile failure in anoxia or in response to metabolic poisons (Stern et al., 1988; Nichols et al., 1991). In addition, contractile failure in these models also appears to involve inhibition of release of Ca^{++} from the SR. Goldhaber et al.

(1991) has shown that Ca^{++} transients detected by fura-2 are markedly reduced by metabolic inhibition in myocytes.

Anoxia likely occurs in tissues in the core of infarcts remote from collateral circulation. However, border zones adjacent to developing infarcts, or central tissues with some collateral circulation may experience milder conditions of ischemia. Tissues in these regions would be exposed to moderate levels of ischemia characterized by hypoxia rather than anoxia. Results from this thesis showed that simulated ischemia produced hypoxic conditions (Po_2 = 26.9 $\pm$ 6.0 mm Hg) rather than profound anoxia (Stern et al., 1985). Indeed, studies have demonstrated that the responses of cardiac tissues to ischemia are different from those to anoxia or to metabolic poisons (Lee and Allen, 1991). ATP levels fall much more slowly in ischemia. Contractile failure appears to precede marked reduction in ATP and is less well correlated with abbreviation of APD (Koretsune et al., 1991). Another major difference is that contractile failure occurs at a time when Ca^{++} transients indicate that SR release of Ca^{++} is still intact (Lee et al., 1988). This latter observation has led to the conclusion that contractile failure in ischemia is largely due to marked reduction of myofilament sensitivity to Ca^{++} (Lee et al., 1988).

Previous studies have developed multicellular models of simulated ischemia and reperfusion which utilized modified Tyrode's solution to mimic specific conditions of ischemia (Ferrier et al., 1985; Ferrier et al., 1991). These included hyperkalemia, hypercapnia, lactate accumulation, acidosis, substrate

deprivation and hypoxia. When ventricular tissues were exposed to this solution for 15 min, both ischemic conditions and reperfusion resulted in cardiac arrhythmias which were reversible in late reperfusion (Ferrier and Guyette, 1991; Li and Ferrier, 1991; Pasnani and Ferrier, 1992). In this thesis, exposure to ischemic conditions resulted in changes in electrical activity which also were reversible following 30 min of reperfusion. In a similar study, Koyama et al. (1991) exposed myocytes loaded with fura-2 to this solution. Contractile, but not electrical, activity was recorded while the myocytes were activated by field stimulation. Koyama et al. (1991) demonstrated that contractile failure occurred even though Ca^{++} transients persisted, as reported previously in Langendorff perfused hearts subjected to ischemia (Lee et al., 1988). Thus, this approach to modelling ischemia mimics both the electrical and contractile events seen in true ischemia. Therefore, we utilized this approach to develop a new, isolated myocyte model of simulated ischemia and reperfusion which allows intracellular recording of electrical activity, voltage clamp analysis of ionic currents, and measurement of contractile activity.

B. Myocardial Alterations in Ischemia.

i) Changes in Transmembrane Voltage.

Myocytes exposed to simulated ischemia and reperfusion exhibited changes in electrical activity similar to those observed in earlier studies of right ventricular multicellular preparations exposed to the same solution (Ferrier and

Guyette, 1991, Li and Ferrier, 1991, Pasnani and Ferrier, 1992) In this study, both MDP and APD_{90} decreased during ischemia while early reperfusion was accompanied by abnormal electrical activity including OAP and depolarization-induced automaticity In this thesis, it was also found that unloaded cell shortening which accompanied action potentials was abolished in ischemia This is in agreement with results of previous studies in other preparations which have reported that contractions are abolished during myocardial ischemia (Allen and Orchard, 1987)

ii) Relationship between Shortening of the APD and Contractile Failure.

Results of experiments which utilized metabolic inhibitors or anoxia have led to the suggestion that contractile failure under those conditions occurs in response to marked reduction in APD, which indirectly leads to contractile failure by limiting Ca^{++} influx (Stern et al , 1988, Lederer et al, 1989, Nichols et al , 1991, Cole et al , 1991) However, studies of APD in whole hearts or ventricular segments exposed to interruption of coronary flow showed more gradual and less pronounced abbreviation of APD (Culling et al , 1984) or little change in APD (Koretsune et al , 1991, Elliott et al , 1992) In this model of simulated ischemia and reperfusion in isolated myocytes, the relationship between ADP_{90} and contraction closely resembles that reported for ischemia, rather than for anoxia or metabolic inhibition In the present study in which cell shortening and APD_{90} are recorded from the same cell, we found that APD_{90} declined only

gradually in ischemia and that the time course did not closely match the time course of changes in magnitude of contraction. In addition, we found that when changes in APD_{90} were eliminated by voltage clamp, contractile failure persisted and exhibited a similar time course. This shows that the decrease in contraction in our model of ischemia was not linked to a reduction in APD_{90}. Thus, unlike cellular models which utilize metabolic inhibition or anoxia, our model exhibited a dissociation of electrical and contractile events in ischemia as reported in perfused heart models of ischemia (Koretsune et al., 1991; Elliott et al., 1992).

iii) Relationship between I_{Ca} and Contractile Failure.

The relationship between I_{Ca} and contractile failure has been examined in cellular models of metabolic inhibition with cyanide and 2-deoxyglucose (Lederer et al., 1989; Goldhaber et al., 1991). Conflicting results were reported. Lederer et al. (1989) found that failure of contractions preceded significant inhibition of I_{Ca} in rat myocytes. Goldhaber et al. (1991) reported that inhibition of contractions and Ca^{++} transients was primarily due to inhibition of I_{Ca} in guinea pig ventricular myocytes. In this model of simulated ischemia, it was found that the magnitude of I_{Ca} decreased slowly, to approximately 50% of control values by 20 min of ischemia. In contrast, contractions decreased more rapidly and were completely inhibited by the end of ischemia. The decrease in I_{Ca} reported in this thesis are consistent with Lederer et al. (1989) however, in this study, the myocytes never exhibited rigor following 20 min of simulated ischemia.

Furthermore, contractile failure during metabolic inhibition closely paralleled shortening of the APD (Lederer et al., 1989). Thus, our observation is consistent with the study of Koyama et al. (1991) which indicated that contractile failure represents a decrease in myofilament sensitivity to Ca^{++}.

C. Myocardial Alterations in Reperfusion.

i) Changes in I_{Ca} upon Reperfusion.

Changes in the magnitude of I_{Ca} in response to reoxygenation have been investigated by others in a cellular model of anoxia and reoxygenation (Benndorf et al., 1991a). That study showed that I_{Ca} decreased by between 20 and 100% upon reoxygenation (Benndorf et al., 1991a). The magnitude of I_{Ca} during anoxia however, was not measured in that study (Benndorf et al., 1991a). In this model of simulated ischemia and reperfusion, we found that I_{Ca} remained inhibited in early reperfusion, and recovered slowly to reach control levels after about 30 minutes. We also found that contractions recovered rapidly in early reperfusion and exhibited a rebound increase with respect to control levels. Thus, the rebound overshoot in amplitude of contraction also was not related to parallel changes in the magnitude of I_{Ca}.

ii) Signs of Ca^{++} Overload in Early Reperfusion.

When myocytes were exposed to the same solution used in this study, free intracellular Ca^{++} levels were reported to increase throughout ischemia

Koyama et al., 1991). Studies have shown that elevated free intracellular Ca^{++} affects the electrical properties of cardiac myocytes and is important in the initiation of cardiac arrhythmias (Manning and Hearse, 1984). In this study, abnormal electrical activity characteristic of Ca^{++} overload was observed in early reperfusion. In particular, OAP and I_{Ti} were observed in 78% of cells examined. Both OAP and I_{Ti} were accompanied by aftercontractions. These observations agree with results of previous studies which showed that cardiac tissues exposed to ischemic Tyrode's solution exhibit arrhythmic activity characteristic of Ca^{++} overload upon return to nonischemic conditions (Ferrier et al., 1985; Ferrier et al., 1991). The transient overshoot in the amplitude of contraction with respect to control levels was further evidence of Ca^{++} overload during early reperfusion in the present study.

In summary, a model of simulated ischemia and reperfusion in ventricular myocytes has been developed which clearly mimics the effects of ischemia, rather than metabolic inhibition. This model exhibits many of the features of multicellular preparations such as membrane depolarization and abbreviation of APD during ischemia, as well as appearance of OAP activity upon reperfusion. In this study, inhibition of contraction during ischemia cannot be attributed to abbreviation of APD_{90} or inhibition of I_{Ca}. Similarly, rapid recovery of contraction during reperfusion is independent of changes in I_{Ca}. This study also demonstrates that induction of aftercontractions, OAP's and I_{Ti} in reperfusion is associated with a reduction in I_{Ca}.

2. EFFECT OF PHARMACOLOGICAL AGENTS ON SIGNS OF Ca^{++} OVERLOAD IN EARLY REPERFUSION.

A) Effect of Ca^{++} Channel Blockade.

A number of studies have attempted to identify pharmacological interventions which might prevent Ca^{++} overload in ischemia and reperfusion. Excessive Ca^{++} influx through L-type Ca^{++} channels has been suggested as one pathway by which Ca^{++} overload occurs (Tani, 1990). Many studies have examined the effectiveness of Ca^{++} channel blockade during various stages of ischemia and reperfusion. Nayler et al. (1988) determined that acute Ca^{++} channel blockade delayed, but did not prevent, Ca^{++}-mediated damage upon reperfusion. Only, long-term pretreatment with Ca^{++} channel blockers decreased reperfusion-associated damage (Nayler et al., 1980). Results from the present study demonstrate that Ca^{++} channel blockade during both simulated ischemia and reperfusion did not prevent appearance of signs of Ca^{++} overload (OAP's, I_{TI} and aftercontractions) during reperfusion. This observation contrasts with many studies which have demonstrated that I_{TI} and OAP's induced by other conditions, such as digitalis intoxication, are suppressed by agents which inhibit Ca^{++} current (Ferrier, 1991). In addition, in the present study enhancement of I_{Ca} was never observed during either ischemia or reperfusion in the absence of drug. Thus, it would seem unlikely that influx of Ca^{++} through I_{Ca} is a primary cause of Ca^{++} overload in ischemia and reperfusion.

B) Effect of Na^+ Channel Blockade.

Another mechanism for Ca^{++} gain during reperfusion is thought to be via Na^+-Ca^{++} exchange secondary to an increase in intracellular Na^+ concentration (Tani, 1990). Na^+ influx through prolonged activation of voltage sensitive Na^+ channels is thought to play a role in Na^+ loading in ischemia and reperfusion, although this is controversial. Mejia-Alvarez and Marban (1992) suggested that intracellular Na^+ gain during metabolic inhibition is not mediated by voltage-sensitive Na^+ channels. In contrast, a study utilizing hypoxia rather than metabolic inhibition found that the rise in intracellular Na^+ during hypoxia could be prevented by R 56865, an agent capable of blocking the noninactivating component of the Na^+ current (Haigney et al., 1992). In our study, lidocaine prevented the overshoot of contraction in early reperfusion. This reduction in overshoot of contraction with lidocaine suggests that Na^+ loading through Na^+ channels may contribute to Ca^{++} overload during ischemia and reperfusion. Interestingly, alt[illegible]h lidocaine prevented the overshoot in contraction, it did not reduce the in[illegible]nce of I_{Ti} although it was unclear why this occurred. As noted with reference to Ca^{++} channel blockers, the latter observation was unexpected since many studies have demonstrated that I_{Ti} and OAP induced by other conditions are inhibited by a wide variety of Na^+ channel blockers (Ferrier, 1991). These results demonstrate that Na^+ or Ca^{++} channel antagonists are unable to abolish I_{Ti} induced by ischemia and reperfusion and suggest that events leading to induction of I_{Ti} may differ during ischemic conditions and

reperfusion.

C) Effect of Na^+-H^+ Exchange Blockade.

It has been demonstrated that amiloride delays the rise in cytosolic free Ca^{++} in perfused rat hearts exposed to global ischemia (Murphy et al., 1991). We also found that the Na^+-H^+ exchange inhibitor amiloride reduced the incidence of I_{TI} and prevented the overshoot of contraction in early reperfusion. This suggests that, in our model of ischemia and reperfusion, Na^+-H^+ exchange also may play a major role in Na^+ gain in ischemia and in subsequent Ca^{++} overload via Na^+-Ca^{++} exchange in reperfusion. It is important to note however, that amiloride also has been reported to inhibit Na^+-Ca^{++} exchange (Ladzunski et al., 1985). Thus, the attenuation of signs of Ca^{++} overload observed upon reperfusion in the presence of amiloride also may be due to other effects such as inhibition of Na^+-Ca^{++} exchange. Indeed, Na^+-Ca^{++} exchange is believed to be one of the charge carriers for I_{TI} in ventricular muscle (January and Fozzard, 1988). Thus, amiloride's marked efficacy in inhibiting I_{TI} and OAP may be mediated by two synergistic actions: 1) reduction of Ca^{++} overload through inhibition of Na^+-H^+ exchange; 2) inhibition of I_{TI} through inhibition of Na^+-Ca^{++} exchange.

D. Role of the Na^+-Ca^{++} Exchange.

i) Assessment of Na^+-Ca^{++} Exchange Activity.

Previous studies under voltage clamp conditions have demonstrated that Ca^{++} influx via the Na^{+}-Ca^{++} exchange can elicit a slowly developing contraction (Terrar and White, 1990) In experiments reported in this thesis, a slowly developing contraction (Na^{+}-Ca^{++} exchange contraction) was also noted in voltage clamp recordings (figure 13) The Na^{+}-Ca^{++} exchange contraction became greater with more depolarized voltage clamp steps, suggesting that more Ca^{++} was entering via the Na^{+}-Ca^{++} exchange

ii) Na^{+}-Ca^{++} Exchange in Ischemia.

Exposure to ischemic conditions resulted in a marked reduction in Na^{+}-Ca^{++} exchange contractions One explanation may be that the Na^{+}-Ca^{++} exchange is inhibited in ischemia with respect to control Caroni and Carafoli (1983) reported that phosphorylation of the Na^{+}-Ca^{++} exchange increased its activity In conditions of ischemia, low cytosolic ATP levels would prevent the Na^{+}-Ca^{++} exchange from being phosphorylated Furthermore, Daly et al (1984) showed that Ca^{++} transport via Na^{+}-Ca^{++} exchange was reduced in hearts made ischemic Another explanation may be inhibition of the Na^{+}-Ca^{++} exchange by protons Philipson et al (1982) demonstrated that a decrease in pH markedly reduced Na^{+}-Ca^{++} exchange activity Low pH also reduces myofilament sensitivity (Orchard and Kentish, 1990) Therefore, the decrease in the Na^{+}-Ca^{++} exchange contraction in ischemia may be mediated by two synergistic actions 1) inhibition of Na^{+}-Ca^{++} exchange by protons and 2) reduction in

myofilament sensitivity by protons.

iii) Na^+-Ca^{++} Exchange in Reperfusion.

In reperfusion, when normal conditions were restored, there was a marked overshoot in the magnitude of Na^+-Ca^{++} exchange contractions at all potentials when compared to control (figure 15). As previously suggested, Na^+ loading during ischemia leads to Ca^{++} overload via the Na^+-Ca^{++} exchange in reperfusion. Amiloride, the Na^+-H^+ exchange blocker, prevented the statistically significant overshoot of the Na^+-Ca^{++} exchange contraction in early reperfusion (compare figures 15 and 16). However, amiloride also reduced Na^+-Ca^{++} exchange contractions under control conditions. It has been reported that amiloride may inhibit the Na^+-Ca^{++} exchange (Lazdunski et al., 1985). Therefore, it is unclear whether the decrease in Ca^{++} influx via the Na^+-Ca^{++} exchange by amiloride is due to: 1) decreased Na^+ loading via Na^+-H^+ exchange which, in turn, reduced Ca^{++} loading via the Na^+-Ca^{++} exchange or 2) direct inhibition of Na^+-Ca^{++} exchange. Alternatively, amiloride derivatives have been shown to slow the recovery of intracellular pH following global ischemia (Vandenberg et al., 1993) which would result in decreased myofilament sensitivity to Ca^{++}.

3. EFFECT OF SIMULATED ISCHEMIA AND REPERFUSION ON RELEASE CONTRACTIONS.

Previous studies have identified two components of cardiac contraction corresponding to sarcolemmal Ca^{++} entry and SR Ca^{++} release. The results presented thus far demonstrate that both the L-current associated contraction and the Na^{+}-Ca^{++} exchange contraction are inhibited during ischemia and exhibit a marked overshoot in early reperfusion. This section of the thesis examined the effects of simulated ischemia and reperfusion on the release contraction and showed the release contraction also was inhibited in ischemia. In addition, there was a marked overshoot in the magnitude of the release contraction in early reperfusion. Even in the presence of L-channel blockade, results demonstrated that the release contraction showed a large transient overshoot (almost 200% compare to levels observed prior to ischemia) in the early stages of reperfusion. With continued reperfusion, the magnitude of contraction gradually returned to normal levels. Furthermore, the overshoot of the release contraction was not affected by nifedipine, but was abolished by Ni^{++} and ryanodine.

A. Identification of Release Contraction.

It is not entirely clear how contractions are initiated at negative potentials. Since we demonstrated that contractions were activated at potentials more negative than -35 mV, it is unlikely that L-current was involved. In addition, nifedipine inhibited L-current and L-contractions but not release contractions, particularly upon reperfusion. Thus, the contractions must be the result of a mechanism different from the CICR proposed by Fabiato (1985a,b,c). In isolated

myocytes, a number of studies have demonstrated that either Ca^{++} transients (Leblanc and Hume, 1990; Lipp and Niggli, 1994) or contractions (Howlett and Ferrier, 1995) can be elicited by a mechanism independent of L-type current. In this study, no L-type Ca^{++} current was observed at potentials less negative than -35 mV. In addition, L-type Ca^{++} current only begins to activate at potentials more positive than -40 mV (Hess, 1988). This suggests that L-current is not responsible for the initiation of contraction. It has previously been shown that Ca^{++} transients could be initiated by reverse Na^{+}-Ca^{++} exchange subsequent to activation of I_{Na} (Leblanc and Hume, 1990). In the present study, contractions elicited by I_{Na} were eliminated by a voltage clamp step to -40 mV from a post-conditioning potential of -55 mV which discharged and inactivated I_{Na} (Tytgat et al., 1990). Therefore, Na^{+} influx does not account for contractions since I_{Na} was eliminated either by 1) Na^{+} channel blockade with lidocaine or 2) voltage inactivation.

B. Release Contraction during Ischemia and Reperfusion.

Under normoxic conditions, 2 μM nifedipine inhibited both L-current and contraction immediately. With continuous superfusion with nifedipine, there was a gradual reduction in the magnitude of the release contraction. This decrease in the release contraction may be due to the inability of the SR to load with Ca^{++} during L-channel blockade (Ferrier and Howlett, 1995). Exposure of the myocytes to ischemic conditions with 2 μM nifedipine present did not alter

contractile failure (figure 10). However, upon reperfusion and in the presence of nifedipine, the release contraction returned and even exceeded pre-ischemic levels temporarily. These observations once again suggest that potentiation of the release contraction is not due to I_{Ca}. The recovery of the release contraction upon reperfusion suggests that the cytosol and/or the SR accumulates Ca^{++} during early reperfusion. It is well established that Ca^{++} overload occurs upon reperfusion (reviewed by Tani, 1990). In addition, the deleterious effects of reperfusion can be lessened by lowering the extracellular Ca^{++} concentration in the buffer (Shine and Douglas, 1983), suggesting that much of Ca^{++} overload is due to Ca^{++} influx from the extracellular space. Because signs of Ca^{++} overload (ie. potentiation of release contraction and induction of I_{TI}) occurred in the presence of nifedipine, I_{Ca} does not appear to play a role in Ca^{++} overload. Therefore, Ca^{++} influx by some other mechanism must be responsible for Ca^{++} overload in reperfusion. Results presented in this thesis have demonstrated that amiloride (100 μM), but not nifedipine or lidocaine, decreased signs of Ca^{++} overload, such as induction of I_{TI}, upon reperfusion. Amiloride, at a concentration of 100 μM, is believed to inhibit Na^+-H^+ exchange with some inhibition of the Na^+-Ca^{++} exchange (Lazdunski et al., 1985). This suggests that much of the Ca^{++} overload occurring upon reperfusion is through Na^+-Ca^{++} exchange secondary to Na^+ loading through Na^+-H^+ exchange. Potentiation of the release contraction upon reperfusion suggests that Ca^{++} which enters the cytosol via the Na^+-Ca^{++} exchange is immediately taken up by the SR and

becomes available for subsequent release. From this study, the potentiation of the release contraction most likely resulted from increased SR Ca^{++} release. With continued reperfusion, the release contraction gradually disappeared indicating that the SR was no longer loading with Ca^{++}. As reperfusion progressed, cytosolic Ca^{++} and Na^{+} recovered to normal levels.

The brief induction of I_{TI}, as well as potentiation of the release contraction during the early stages of reperfusion suggests that the cells were Ca^{++} overloaded. However, the underlying mechanism responsible for I_{TI} is unclear although it is believed to be the result of either A) a Ca^{++} activated non-selective cation channel or B) the Na^{+}-Ca^{++} exchanger (January and Fozzard, 1988). Previous studies have shown that high concentrations of ryanodine can abolish OAP by binding to the SR Ca^{++} release channel and blocking the oscillatory release of Ca^{++} from the SR (Hayashi et al., 1987). In our study, 10 nM ryanodine decreased but did not abolish induction of I_{TI} (2/5 experiments); however, the higher concentration of ryanodine (100 nM) prevented appearance of I_{TI} (0/3 experiments), which agrees with previous observations. Interestingly, 200 μM Ni^{++} present during both ischemic conditions and reperfusion resulted in appearance of I_{TI} in only 1/5 experiments. High concentrations of Ni^{++} (ie. concentrations in the mM range) have been reported to block the Na^{+}-Ca^{++} exchange (Kimura et al., 1987). If the low concentration of Ni^{++} used in this study did block Na^{+}-Ca^{++} exchange, this would explain the decreased incidence of I_{TI} when Ni^{++} was present. Alternatively, Ni^{++} may prevent I_{TI} by some other

mechanism, perhaps by directly interacting with the Ca^{++} binding site on the SR Ca^{++} release channel, thus preventing the oscillatory release of Ca^{++}.

In summary, changes in release contractions were investigated when guinea pig ventricular myocytes were exposed to simulated ischemia and reperfusion. The release contraction exhibits contractile failure during ischemia and a transient potentiation of contraction upon reperfusion. In addition, potentiation of the release mechanism occurred by a mechanism independent of L-current activation since these effects occurred in the presence of nifedipine. Continuous superfusion with nifedipine eventually abolished the release contraction under normoxic conditions which suggested that SR stores did not load with Ca^{++}; however, reperfusion following 20 min of ischemia with nifedipine results in overshoot of the magnitude of the release contraction. The overshoot of the release contraction could be selectively inhibited by ryanodine and Ni^{++}. These results suggest that upon reperfusion, the SR is able to accumulate Ca^{++} which then becomes available for release. Since the accumulation of Ca^{++} occurred in the presence of L-current blockade, Ca^{++} loading occurred via the Na^{+}-Ca^{++} exchange operating in the reverse mode due to Na^{+} loading via the Na^{+}-H^{+} exchange. The implications of the potentiation of the release mechanism during early reperfusion may be that arrhythmias, particularly those initiated by triggered activity, are closely related to this transient potentiation.

4. EFFECTS OF ADENOSINE DURING SIMULATED ISCHEMIA AND

REPERFUSION.

The nucleoside ADN has been shown to exert electrophysiological effects in sinoatrial node, atrioventricular node, and atrial muscle under normal conditions, even in the absence of autonomic agonists (Pelleg and Belardinelli, 1993). ADN can also affect the electrophysiological activity of ventricular tissues, but only after they have been stimulated by β-adrenergic agonists (Pelleg and Belardinelli, 1993). The results of this thesis study confirm the absence of measurable effects of ADN under normal conditions in isolated guinea pig ventricular myocytes. However, this study demonstrates that ADN does have marked effects on electrical and contractile activity of ventricular myocytes during simulated ischemia and reperfusion. ADN potentiated the effects of ischemia on APD, and delayed recovery of this parameter during reperfusion. In addition, ADN blunted the temporary overshoot of contraction in early reperfusion The actions of exogenous ADN appear to be mediated via specific ADN receptors, since the effects of ADN were blocked by both the non-specific antagonist 8-PT and the A_1 selective antagonist CPT. Application of 8-PT during ischemia in the absence of ADN did not result in augmentation of any of these parameters. This suggests that endogenous ADN release did not influence these results. This study also demonstrated that ADN treatment during ischemia decreased signs of cytosolic Ca^{++} overload upon reperfusion (OAP, I_{Ti} and aftercontractions). These effects were reversed by the ADN receptor antagonists 8-PT and CPT. Interestingly, 8-PT alone during ischemia resulted in

an increase in the incidence of I_{Tl} upon reperfusion, which suggests that endogenous ADN might decrease I_{Tl} in early reperfusion in this cellular model of ischemia and reperfusion.

Upon early reperfusion, ADN reduced the transient overshoot in the magnitude of contraction elicited either by action potentials or by voltage clamp activation of I_{Ca}. The mechanisms by which ADN might mediate this effect are unclear. It is believed that ADN does not affect contractile filaments directly (Neumann et al., 1989). Therefore, one might speculate that ADN may act by inhibiting I_{Ca} in ischemia and reperfusion. However, this is unlikely. As previously reported, changes in I_{Ca} do not parallel contractile responses to simulated ischemia and reperfusion. Also, results of the present study show that the rebound in cell shortening was not accompanied by a rebound in peak I_{Ca}. In addition, ADN did not alter the effects of ischemia and reperfusion on I_{Ca} up to the time at which the rebound in contraction occurred. Since the effect of ADN on overshoot of contraction was identical under both conventional conditions and voltage clamp, this also cannot be attributed to effects of ADN on APD or MDP.

A. ADN and Signs of Ca^{++} Overload in Reperfusion.

The rebound in contractile activity upon reperfusion most likely is related to Ca^{++} overload. Evidence for the occurrence of Ca^{++} overload on reperfusion in these experiments comes from the observation that reperfusion is

accompanied by the appearance of OAP, I_{TI}, and aftercontractions. These phenomena have been associated with Ca^{++} overload in earlier studies (Kaas et al., 1978a). Thus, attenuation of contractile overshoot and inhibition of these other signs of Ca^{++} overload by ADN suggests that ADN may decrease Ca^{++} overload during ischemia and/or reperfusion. Experimental evidence suggests that Ca^{++} overload during reperfusion results mainly through enhanced influx of Ca^{++} via the Na^{+}-Ca^{++} exchanger (Nayler et al., 1988). Therefore, it might be possible that ADN alters Ca^{++} handling by inhibiting Na^{+}-Ca^{++} exchange. Since there is evidence for phosphorylation-dependent regulation of Na^{+}-Ca^{++} exchange (Caroni and Carafoli, 1983; Collins et al., 1992), it is possible that this could be mediated by an effect on adenylate cyclase. In other conditions leading to Ca^{++} overload and the appearance of OAP and I_{TI}, a phosphorylation step appears to be necessary for ADN to exert an inhibitory effect. For example, ADN was shown to be capable of abolishing I_{TI} induced by β-adrenergic stimulation (Isenberg and Belardinelli, 1984; Song et al., 1992) but did not affect I_{TI} induced by Na^{+}-K^{+} pump inhibition (Song et al., 1992). Presently it is not known whether ischemia and/or reperfusion promote phosphorylation of the Na^{+}-Ca^{++} exchanger, therefore this hypothesis is speculative. Alternatively, direct inhibition of the Na^{+}-Ca^{++} exchanger would also decrease Ca^{++} overload. In addition, this action could lead to a lower incidence of I_{TI} since the Na^{+}-Ca^{++} exchange is thought to be a major charge carrier for I_{TI} in ventricular muscle.

Although the effects of ADN on contractile overshoot were the same

under conventional conditions or voltage clamp, recovery during later reperfusion clearly was different. By the end of reperfusion, contractions elicited by action potentials remained depressed in ADN treated myocytes, whereas I_{Ca} elicited contractions recovered to control magnitudes. This observation suggests that the actions of ADN may not be expressed equally under the two different experimental conditions. In the later stages of reperfusion, when Ca^{++} homeostasis is restored, the reduced level of contraction elicited by action potentials may be the result of the abbreviated APD. This effect might lead to earlier inactivation of I_{Ca} and thereby decrease Ca^{++} influx. Indeed, the depression of contractile activity in late reperfusion was not observed when the changes in APD and MDP were eliminated under voltage clamp conditions.

One of the most prominent effects of ADN in ischemia and reperfusion is enhanced abbreviation of APD. This effect is clearly not mediated by effects on I_{Ca}. However, it is possible that enhanced abbreviation of APD represents an effect on an ADN-sensitive K^+ channel. Two possible candidates are the ADN/acetylcholine-sensitive K^+ channel ($K^+_{Ach,Adn}$) and the ATP-sensitive K^+ channel ($I_{K,ATP}$). The $K^+_{Ach,Adn}$ channel has been reported in atrial tissue, sinoatrial node and atrioventricular node (Belardinelli et al., 1980; Belardinelli et al., 1988) but not in ventricular tissue (Jochem and Nawrath, 1983). Thus, it is unlikely that the $K^+_{Ach,Adn}$ channel is important here. The $I_{K,ATP}$ channel however, has been reported in mammalian ventricle (Noma, 1983). ADN binding to A_1 receptors has been shown to open $I_{K,ATP}$ channels through a mechanism

involving an inhibitory G protein (Kirsch et al., 1990). In addition, the $I_{K,ATP}$ channel is activated in guinea pig ventricular tissue by the same conditions used in these experiments (Pasnani and Ferrier, 1992).

In summary, we have demonstrated that ADN: (1) potentiates the effects of ischemia on APD and delays recovery of APD during reperfusion, and (2) inhibits signs of Ca^{++} overload, such as rebound increases in cell shortening, and induction of OAP, I_{TI} and aftercontractions, upon reperfusion. These effects of ADN could be reversed by both 8-PT and CPT suggesting that ADN receptors, specifically A_1 receptors, mediate these effects. Since OAP are an important mechanism in the development of cardiac arrhythmias, the effects described in this study may represent a protective effect of ADN in ischemia and reperfusion that is independent of β-adrenoceptor antagonism.

5. CONCLUSIONS.

This study demonstrates that a model of simulated ischemia and reperfusion in ventricular myocytes has been developed which clearly mimics the effects of ischemia, rather than hypoxia or metabolic inhibition. This model exhibits many of the features of multicellular preparations such as membrane depolarization and abbreviation of APD during ischemia, as well as appearance of OAP activity upon reperfusion. The effect of simulated ischemia and reperfusion on some of the ion channels and ion exchangers discussed in this thesis are illustrated in figure 36. In this study, inhibition of contraction during

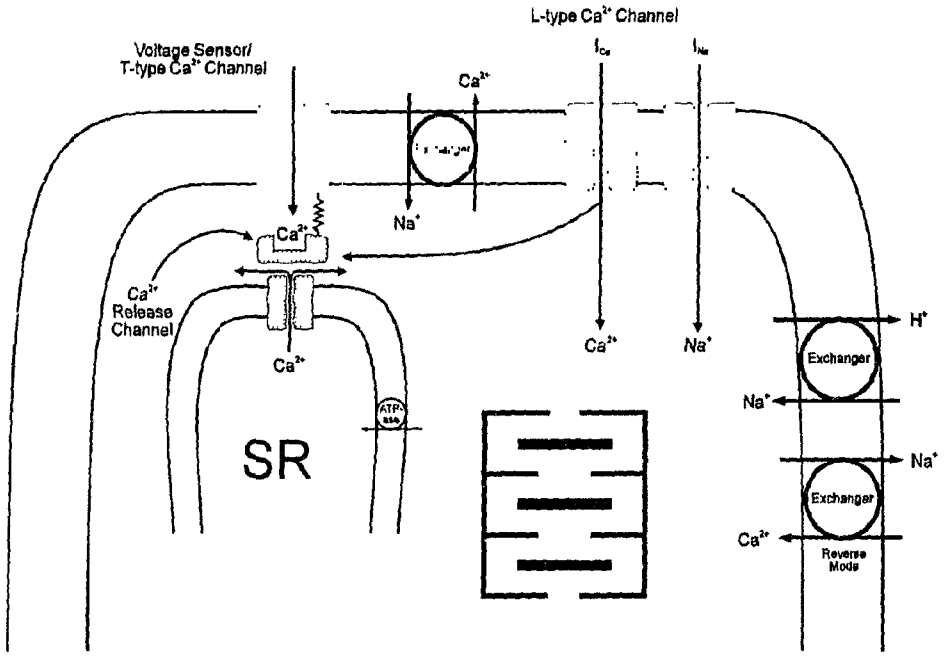

FIGURE 36: Schematic of a ventricular myocyte illustrating some of the ion channels and ion exchange processes that are affected by ischemic conditions and reperfusion

ischemia cannot be attributed to abbreviation of APD_{90} or inhibition of I_{Ca}. Similarly, rapid recovery of contraction during reperfusion is independent of changes in I_{Ca}. This study also demonstrates that induction of aftercontractions, OAP's and I_{Tl} in reperfusion is associated with a reduction in I_{Ca}. The development of this cellular model of ischemia and reperfusion allows screening of potential pharmacologic agents which may protect against ischemic injury. In this thesis, the effects of ischemia and reperfusion on signs of Ca^{++} overload in the presence agents which alter cellular Na^+ and Ca^{++} loading were examined. The results showed that neither inhibition of I_{Na} nor I_{Ca} could prevent induction of OAP's or I_{Tl} upon reperfusion. Ca^{++} overload may be due to Na^+ loading via Na^+/H^+ exchange and/or Na^+ channels which, in turn, leads to Ca^{++} overload via Na^+/Ca^{++} exchange. This model of simulated ischemia and reperfusion, in contrast to multicellular preparations, can be used effectively to simultaneously determine and compare effects of drugs on membrane currents and contractions. Since this is a model of simulated ischemia, it is not possible to incorporate all the components of true myocardial ischemia into the ischemic Tyrode's solution. In this thesis, addition of exogenous ADN, one component of true ischemia, was shown to produce profound changes in contractile and electrical activity. ADN potentiated the effects of ischemia on APD and delayed recovery of APD during reperfusion. ADN also inhibited signs of Ca^{++} overload, such as induction of OAP, I_{Tl} and aftercontractions upon reperfusion. These effects of ADN could be reversed by both 8-PT and CPT, suggesting that A_1

ADN receptors mediate these effects. The effects described in this thesis may represent a protective effect of ADN in ischemia and reperfusion that is independent of β-receptor antagonism.

6. FUTURE STUDIES.

The work presented in this thesis represents the development of a new cellular model of simulated ischemia and reperfusion. Furthermore, this model was used to examine some of the mechanisms which lead to myocardial injury due to ischemia and reperfusion. However, there are many questions left to be answered. An investigation of the effects of the individual components of the ischemic Tyrode's solution is necessary. This would allow identification of the components that are important in the electrophysiological changes seen during ischemia and reperfusion. Since this is a model of simulated ischemia, not all the components of true myocardial ischemia are incorporated in this model. As we have seen, exogenous ADN (one component of true myocardial ischemia) produced profound electrical and contractile changes. It would be interesting to determine the effects of ADN on contractions elicited by the voltage-sensitive release mechanism. Other components of myocardial ischemia may also be investigated using this model. Components which have been implicated in myocardial injury during ischemia are lysophosphatidylcholine and long-chain acylcarnitines. The effect of addition of these substances can be examined using this model. In this thesis, the effects of agents which alter cellular Ca^{++}

and Na^+ loading were investigated under ischemic conditions and reperfusion. The effects of new anti-arrhythmic drugs also can be investigated in this cellular model of ischemia and reperfusion.

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