Modulation of Cardiac Inotropy:
Contributions of Diastolic $[\text{Ca}^{2+}]$, Peak Systolic $[\text{Ca}^{2+}]$ and $\text{Ca}^{2+}$ Transient Amplitudes to Amplitudes of Contraction in Guinea Pig Ventricular Myocytes

by

Robin Helena Shutt

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
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DEDICATION

For

Greg: Dedication defined.

&

Peter Türcke, who is at the heart of it all.

"... on ne voit bien qu'avec le coeur.
L'essentiel est invisible pour les yeux."

- de St-Exupéry (2001)
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ABSTRACT

Positive inotropy following rest, in elevated extracellular calcium (Ca$^{2+}$), during hypothermia or with increasing stimulation frequency has been attributed to increased sarcoplasmic reticulum (SR) Ca$^{2+}$ content and/or increased trigger Ca$^{2+}$. However, each of these manipulations also may elevate diastolic [Ca$^{2+}$]. The objective of this study was to determine whether diastolic [Ca$^{2+}$] could contribute to positive inotropy in isolated guinea pig ventricular myocytes at physiological temperatures (37°C). Intracellular [Ca$^{2+}$] was measured with fura-2. Cell shortening was measured with an edge detector. SR Ca$^{2+}$ stores were assessed by rapid application of 10 mM caffeine. Under voltage clamp conditions, when extracellular [Ca$^{2+}$] was elevated contraction amplitude increased with increasing extracellular [Ca$^{2+}$], in parallel with increasing diastolic and peak systolic Ca$^{2+}$. Interestingly, Ca$^{2+}$ transient amplitude did not increase. Furthermore, SR Ca$^{2+}$ stores did not increase when extracellular [Ca$^{2+}$] was elevated. Thus, positive inotropy occurred in the absence of changes in Ca$^{2+}$ transient amplitude, indicating that diastolic Ca$^{2+}$ may be an important contributor to positive inotropy. When voltage clamped myocytes were stimulated from rest similar results were found; diastolic Ca$^{2+}$ and contraction amplitude increased while Ca$^{2+}$ transient amplitude did not. Thus, these data suggest that diastolic Ca$^{2+}$ is an important determinant of contraction amplitude. In contrast, both Ca$^{2+}$ transient amplitude and diastolic Ca$^{2+}$ increased in parallel with increasing contraction amplitude when rates of field stimulation were increased. Thus, when Ca$^{2+}$ transient amplitude and diastolic Ca$^{2+}$ both increased, positive inotropy occurred. In hypothermic myocytes (24°C) amplitudes of contractions, SR Ca$^{2+}$ stores
and Ca\textsuperscript{2+} transients were increased with respect to 37\textdegree C, while diastolic Ca\textsuperscript{2+} did not change. Therefore positive inotropy also occurred when Ca\textsuperscript{2+} transient amplitude increased, in the absence of increasing diastolic Ca\textsuperscript{2+}. Thus, the data presented in this thesis demonstrate that a change in either diastolic Ca\textsuperscript{2+} or Ca\textsuperscript{2+} transient amplitude was sufficient for positive inotropy to occur. Interestingly, when either diastolic Ca\textsuperscript{2+} or Ca\textsuperscript{2+} transient amplitude increased, the peak systolic [Ca\textsuperscript{2+}] achieved also was increased. Therefore, by influencing peak systolic Ca\textsuperscript{2+} both Ca\textsuperscript{2+} transient amplitude and diastolic Ca\textsuperscript{2+} could influence contraction amplitude.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A/D</td>
<td>analogue to digital</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxyethyl ester</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>enzyme that harnesses energy released from the hydrolysis of ATP to perform its function</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium-induced calcium release</td>
</tr>
<tr>
<td>CP</td>
<td>conditioning pulse</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>e340/e380</td>
<td>ratio of emissions evoked by excitation with 340 and 380 nm light</td>
</tr>
<tr>
<td>EC coupling</td>
<td>excitation-contraction coupling</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
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<tr>
<td>KH₂PO₄</td>
<td>potassium dihydrogen phosphate</td>
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<tr>
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<td>kilohertz</td>
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<tr>
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<td>potassium hydroxide</td>
</tr>
<tr>
<td>Lₜᵣₓ-L</td>
<td>L-type calcium current</td>
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<td>molar</td>
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<td>nanomolar</td>
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<tr>
<td>Symbol</td>
<td>Definition</td>
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<td>----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
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<tr>
<td>Na⁺ pump</td>
<td>Na⁺/K⁺ ATPase pump</td>
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</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchange(r)</td>
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<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>pH</td>
<td>negative log (base 10) of the molar concentration of hydrogen ions</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PTI</td>
<td>Photon Technologies International</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic or endoplasmic reticulum calcium adenosine triphosphatase</td>
</tr>
<tr>
<td>SL</td>
<td>sarcolemma; plasma membrane of a muscle cell</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum; endoplasmic reticulum of a muscle cell</td>
</tr>
<tr>
<td>SV</td>
<td>stroke volume</td>
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<tr>
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<td>V</td>
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<tr>
<td>Vₘ</td>
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ACKNOWLEDGEMENTS

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Everyone, for everything, thank you.
CHAPTER 1: INTRODUCTION

Never morning wore
To evening, but some heart did break.

Tennyson (1850)

1. History and Overview

In 1628, William Harvey published his observations and conclusions about the motion of the heart and circulation of the blood in *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* (Harvey, 1970). He determined that the contraction of the atria forces blood, returning to the heart via the venous system, into the ventricles. The filled ventricles then contract. Blood is ejected from the ventricles into the arteries that lead to the lungs and the body from the right and left ventricles respectively (Harvey, 1970). The heartbeat heard in the chest and the pulses that can be felt in the extremities are evidence of the pumping action of the heart, moving blood through the arteries to the extremities. Harvey (1970) determined experimentally that sheep and cattle move almost the entire volume of blood in their bodies in the space of thirty minutes (Harvey, 1970). Therefore, Harvey (1970) theorized, blood must move continuously in a circular manner, returning always to the heart, rather than being generated anew in the liver as was previously thought. He hypothesized that the blood returns to the heart via the venous system to be replenished with “nutritive factors” and “vital heat” (Harvey, 1970). Harvey also showed experimentally that valves within the veins ensured that venous
blood flowed only in one direction, towards the heart (Harvey, 1970). Finally, Harvey observed that the movement of blood in an individual could vary depending on age, physical activity, mood and other factors, both internal and external (Harvey, 1970). Harvey's conclusions about the circular movement of blood in the body flew in the face of conventional thought at the time (Manger, 1992), yet his observations and experiments form the basis for what is now known about cardiovascular physiology.

The basic components of the circulatory system and their functions described by Harvey in the seventeenth century (Harvey, 1970) remain the foundation of our current understanding of the function of the cardiovascular system. It is understood that the heart pumps blood efficiently against pressure gradients to the pulmonary and systemic circulatory systems (Mohrman & Heller, 1997). The volume of blood pumped by the heart per unit time is referred to as the cardiac output. Cardiac output is the product of heart rate and stroke volume (SV), where SV is the volume of blood moved during a single beat of the heart (Mohrman & Heller, 1997). SV is measured as the difference between the volume of blood in the heart at the end of diastole, the relaxation phase of cardiac contraction, and the volume of blood in the heart at the end of systole, the active force-generating phase of contraction (Mohrman & Heller, 1997).

In situations of increased metabolic demand on the body, such as exercise, cardiac output must be increased to maintain delivery of nutrients and oxygen to working tissues (Mohrman & Heller, 1997). Cardiac output is increased either by increasing heart rate, referred to as positive chronotropy, or by increasing SV. SV can be influenced by venous return, mean arterial pressure, arterial compliance and by the strength of cardiac contraction, or contractility (Mohrman & Heller, 1997). While in young populations
positive chronotropy is the primary mediator of elevated cardiac output, increased SV is the primary mediator of increased cardiac output in the elderly (Lakatta, 1993; Rodeheffer et al., 1984). Increased cardiac contractility, or positive inotropy, is an important determinant of SV when arterial compliance is decreased, or mean arterial pressure is elevated (Safar & London, 1987). Thus, positive inotropy, as a determinant of SV, determines the amount of blood moved through the body on a beat to beat basis. In the absence of changes in heart rate, or when maximum heart rate is reached, increased cardiac contractility is responsible for responding to increases in metabolic demand and to postural changes (Berne & Levy, 2001; Mohrman & Heller, 1997).

Studies of cardiac muscle following those of Harvey determined that certain factors were necessary for cardiac contraction to occur (Ringer, 1883a & b). Ringer (1883a) showed that calcium (Ca^{2+}) was required for the heart to contract. Ca^{2+} has been further shown to play a role in determining the strength of contraction (Niedergerke, 1956), and more recently transient increases in intracellular Ca^{2+} concentrations have been found to be the initiator of cardiac contraction in single cardiac muscle cells (Bers, 2001; Langer, 1997; Fabiato, 1985a, b & c; Fabiato & Fabiato, 1978 & 1975). Increases in the amplitude of this transient increase in intracellular Ca^{2+}, or Ca^{2+} transient, have been shown to contribute to positive inotropy at the cellular level (Bers, 2001; Langer, 1997; Fabiato, 1985a, b & c; Fabiato & Fabiato, 1978 & 1975). Many of the factors that influence Ca^{2+} transient amplitude, and thereby cardiac contractility, have been identified. These factors include: the amplitude of the inward Ca^{2+} current which is triggered by the cardiac action potential; the quantity of Ca^{2+} stored within the sarcoplasmic reticulum (SR), which is the primary Ca^{2+} store of the cell; and the
efficiency with which the SR Ca\(^{2+}\) stores are released, referred to as the gain of SR Ca\(^{2+}\) release. Each of these factors can produce positive inotropy alone, or in concert with the others. However, the exact contributions of each individual factor to positively inotropic stimuli such as changes in rate, changes in rhythm and changes in Ca\(^{2+}\) fluxes, have not been clearly defined.

The overall objective of this thesis is to examine factors that influence inotropy in isolated cardiac ventricular myocytes. In addition to conventional factors that influence inotropy, such as Ca\(^{2+}\) transient amplitude, inward Ca\(^{2+}\) current amplitude, SR Ca\(^{2+}\) stores, and the gain of SR Ca\(^{2+}\) release, the contribution of the diastolic Ca\(^{2+}\) concentration will be examined. Diastolic Ca\(^{2+}\) may contribute to positive inotropy by shifting the peak systolic intracellular [Ca\(^{2+}\)]. In the first part of this thesis, the responses of myocytes to classical initiators of positive inotropic responses, such as heart rate and quiescence, will be examined to determine if diastolic Ca\(^{2+}\) concentration plays a role in producing positive inotropy in response to these stimuli. In the second part of this thesis, the roles of Ca\(^{2+}\) current amplitude, SR Ca\(^{2+}\) content and the gain of SR Ca\(^{2+}\) release in the determination of Ca\(^{2+}\) transient amplitude in response to changes in extracellular Ca\(^{2+}\) will be examined in isolated cardiac myocytes. The contribution of Ca\(^{2+}\) transient amplitude and diastolic Ca\(^{2+}\) to inotropy associated with changes in extracellular Ca\(^{2+}\) also will be examined.

The chapters of the introduction, which follow, will establish the mechanisms of cardiac contraction at the whole heart and cellular levels, introduce the mechanisms by which the strength of cardiac contraction is increased in the whole heart, and finally will examine what is known about the factors that influence contraction in the isolated
myocyte. The mechanisms governing coordinated contraction of the heart, and the
initiation of contraction in single heart cells will first be reviewed.

2. Electrical Excitation and Contraction in the Heart

A. Coordinated contraction of the whole heart.

Coordinated contraction of the heart is intrinsic to its function as an efficient
pump. Cardiac contraction is initiated by a wave of electrical depolarization that travels
rapidly to every myocyte throughout the heart. This wave of depolarization arises from
the internal cardiac pacemaker, the sinoatrial node (Berne & Levy, 2001). The sinoatrial
node is located on the right atrium, near the superior vena cava. Sinoatrial nodal cells are
functionally distinct cardiac myocytes, which can spontaneously generate action
potentials (Berne & Levy, 2001). The action potentials generated at the sinoatrial node
are propagated throughout the heart, and the associated depolarization of cardiac
myocytes initiates coordinated contraction of the heart (Mohrman & Heller, 1997).

Depolarization is conducted rapidly throughout the heart by a specialized
electrical conducting system (Mohrman & Heller, 1997). First the wave of depolarization
spreads across the atria. It then reaches the atrioventricular node located at the junction
of the atria and the ventricles. The atria and ventricles are electrically separated by a
cartilaginous structure, which supports the tricuspid and mitral valves of the heart (Berne
& Levy, 2001). The lower portion of the atrioventricular node, the bundle of His,
consists of parallel fibers that bridge the cartilage and are the primary conductor of the
action potential from the atria to the ventricles. Once excitation has reached the
ventricles, the action potential is conducted by the large diameter Purkinje fibers, which are found in the interventricular septum (Berne & Levy, 2001). The Purkinje fibers are specialized muscle fibers that rapidly conduct the cardiac action potential down the ventricular septum and laterally into the ventricular myocytes (Berne & Levy, 2001). Finally, the cardiac action potential is propagated from myocyte to myocyte by intercellular communications known as gap junctions (Berne & Levy, 2001).

The conduction of the action potential through the ventricles by the Purkinje system, and then from cell to cell, leads to nearly simultaneous excitation of all ventricular cells. Thus, in response to electrical excitation at the level of the sinoatrial node, the millions of individual cardiac myocytes in the heart function as a collective and contract in a coordinated manner (Coraboeuf & Escande, 1990). The initiation of a contractile response by electrical excitation of the membrane of a cardiac myocyte is referred to as excitation-contraction coupling. A discussion of the cardiac myocyte cell structures important to excitation-contraction coupling, the organization of those structures, and the mechanism of excitation-contraction coupling at the level of the cardiac myocyte follows.

B. Cardiac contraction at the cellular level.

i. Isolated cardiac myocyte structure

The cardiac action potential is propagated throughout the heart, and excitation of cardiac myocytes leads to contraction of those myocytes in concert. Individual ventricular cardiac myocytes can be isolated through enzymatic digestion of ventricular
tissue (Mitra & Morad, 1985; Yazawa et al., 1990). The electrical and contractile properties of ventricular myocytes can be studied following enzymatic dissociation (Mitra & Morad, 1985; Yazawa et al., 1990; Capogrossi et al., 1986). Isolated myocytes make it possible to study the mechanisms that produce contractile responses in cardiac muscle under close control (Lew et al., 1994), while recording ionic currents (Yazawa et al., 1990). The contractile properties and responses of isolated myocytes remain similar to responses recorded in intact muscle preparations from the same species (Capogrossi et al., 1986). As such, the isolated cardiac myocyte has become a powerful tool with which to examine the factors that influence positive inotropy in cardiac muscle.

Cardiac myocyte structure is highly organized, and much of this organization remains intact in isolated myocytes (Langer, 1997). The myofilaments are the contractile proteins of the cardiac myocyte, and can be visualized under light microscopy as striations in isolated cardiac myocytes (Langer, 1997), as shown in figure 1. Myosin (Cantino & Squire, 1986) and actin (Goldstein et al. 1989; Isobe et al., 1988) make up the interdigitating thick and thin filaments respectively. Other important contractile proteins include the troponin complex (troponin C, troponin I and troponin T) and tropomyosin (Zot & Potter, 1987; Solaro & Rarick, 1998). The actin-myosin interaction is the primary mediator of cardiac force generation (Zot & Potter, 1987; Solaro & Rarick, 1998). Tropomyosin is an alpha-helical protein which is closely associated with the actin thin filament. In the resting state, the troponin-tropomyosin complex is bound to actin. Troponin and tropomyosin, when bound to actin, sterically hinder the binding of myosin to actin (Salaro & Rarick, 1998). The interaction between actin and the troponin complex is disrupted by Ca\(^{2+}\) binding to troponin C (Zot & Potter, 1987; Solaro &
Figure 1: Digital photomicrograph of an isolated guinea pig ventricular myocyte.

Differential interference contrast microscopy was used to visualize the myocyte. The isolated myocyte is roughly rectangular and the edges are clearly defined. The grid-like delineations within the myocyte are the striations, a visible indication of the highly organized intracellular structure of the cardiac myocyte.
Rarick, 1998). $\text{Ca}^{2+}$ ions binding to troponin C produce a conformational change in the troponin-tropomyosin complex, and the myosin-binding site on actin becomes available. Thus, in the presence of $\text{Ca}^{2+}$, the inhibition of the actin-myosin interaction is removed and force production, or cell shortening, can occur (Zot & Potter, 1987; Solaro & Rarick, 1998). The force required for contraction or cell shortening is generated through hydrolysis of adenosine triphosphate (ATP) by the myosin ATPase. The myofilaments give direct visual evidence of the highly organized nature of the cardiac myocytes, and myofilament organization is central to force production.

The arrangement of the myofilaments is not the only highly organized component of the cardiac myocyte. The cardiac myocyte sarcolemma (SL) forms deep invaginations that are oriented in alignment with the contractile proteins of the cell (Brette & Orchard, 2003). These deep invaginations are known as the transverse or t-tubules, although they are a complex system of tubules which branch in both transverse and longitudinal directions (Brette & Orchard, 2003). The t-tubules are both structurally and functionally distinct components of the SL. Specific membrane proteins are localized in, or show higher densities in the t-tubular membrane (Scriven et al., 2000; McDonough et al., 1996). The t-tubules have a high density of L-type $\text{Ca}^{2+}$ channels (Scriven et al., 2000), which are SL voltage-gated $\text{Ca}^{2+}$ channels (McDonald et al., 1994). The density of the $\text{Na}^+$/Ca$^{2+}$ exchanger (NCX) also is increased in the t-tubular part of the SL (Scriven et al., 2000). NCX is an electrogenic transporter. NCX can exchange several $\text{Na}^+$ ions for one Ca$^{2+}$ ion, and transports Ca$^{2+}$ using the Na$^+$ gradient produced by the Na$^+$/K$^+$ ATPase pump (Na$^+$ pump; Bers & Weber, 2002). Interestingly, the Na$^+$ pump is distributed uniformly throughout guinea pig myocyte SL (McDonough et al., 1996). The t-tubules
are organized in concert with the myofilaments. Also arranged in close proximity to the
t-tubules of the SL is the endoplasmic reticulum (Franzini-Armstrong et al., 1998). In
muscle, the endoplasmic reticulum is referred to as the sarcoplasmic reticulum (SR).

The SR is the primary $\text{Ca}^{2+}$ store in cardiac myocytes (Bers, 2001). The SR $\text{Ca}^{2+}$
ATPase pump (SERCA) pumps $\text{Ca}^{2+}$ from the cytosol into the SR (Inui et al., 1986).
SERCA actively pumps $\text{Ca}^{2+}$ into the SR against a high concentration gradient (Beard et
al., 2004). The ability of SERCA to pump $\text{Ca}^{2+}$ into the SR efficiently is due in part to
the removal of $\text{Ca}^{2+}$ from the free $\text{Ca}^{2+}$ pool within the SR (Beard et al., 2004). $\text{Ca}^{2+}$ in
the SR binds to a SR $\text{Ca}^{2+}$ binding protein, calsequestrin, decreasing the free $\text{Ca}^{2+}$
concentration within the SR (Beard et al., 2004). Phospholamban is a regulatory protein
found closely associated with SERCA in the SR membrane (Scriven et al., 2000).
Phospholamban can act to alter SERCA function, and plays a role in modulating SR $\text{Ca}^{2+}$
load (Inui et al., 1986).

Storage of $\text{Ca}^{2+}$, however, is not the sole function of the SR. The SR also has the
ability to release $\text{Ca}^{2+}$ through $\text{Ca}^{2+}$ release channels. These $\text{Ca}^{2+}$ release channels
include the ryanodine receptors (RyRs) and the inositol trisphosphate (IP$_3$) receptors
(Marks, 1997), so named because of the ability of the ligands ryanodine, and IP$_3$,
respectively to modulate their function (Marks, 1997). The RyRs are the primary $\text{Ca}^{2+}$
release channels of the cardiac SR. In electron micrographs, the RyRs were initially
identified as "feet" projecting between the SR and the t-tubular SL membranes (Franzini-
Armstrong et al, 1998). The tiny space between the SL and the SR membrane at level of
the t-tubules is referred to as the diadic cleft (Langer & Peskoff, 1997). The L-type $\text{Ca}^{2+}$
channel in the SL is closely associated with the RyR in the SR membrane, as can be
shown by co-localization studies (Scriven et al., 2000). SERCA and phospholamban also are located in the region of the SR that lies in close proximity to the t-tubular SL (Scriven et al., 2000). Thus, the myofilaments, SL and SR show a high degree of organization, and components of these structures are closely associated. As will be seen in the next section, much of this organization is important to the function of cardiac myocytes as contracting muscle cells.

**ii. Excitation-contraction coupling**

SL depolarization, or excitation, of a cardiac myocyte initiates contraction. The coupling of excitation to contraction, or excitation-contraction (EC) coupling, is Ca^{2+} dependent (Ringer, 1883a). The depolarization of the SL is carried deep into the cardiac myocyte by the t-tubules, which are deep invaginations in the SL (Brette & Orchard, 2003). As described previously, the t-tubular SL contains a large number of L-type Ca^{2+} channels (Scriven et al., 2000). Excitation of the cardiac cell membrane causes voltage-gated L-type Ca^{2+} channels to open, and Ca^{2+} to enter the cell as L-type Ca^{2+} current ($I_{Ca-L}$). This Ca^{2+} entry leads to a local increase in the intracellular Ca^{2+} concentration in the diadic cleft (Langer & Peskoff, 1997). L-type Ca^{2+} channels in the t-tubular membrane are functionally coupled to the RyRs in the SR membrane, and Ca^{2+} entering the cell via the L-type Ca^{2+} channel acts at the RyR (Langer & Peskoff, 1997). The RyR open probability is increased markedly in the presence of elevated Ca^{2+} concentrations on the cytosolic side of the SR membrane (Marks, 1997). Thus, extracellular Ca^{2+} ions entering the cell act at the RyRs to induce Ca^{2+} release from the SR.
The process by which extracellular Ca\(^{2+}\) entering the cell after membrane depolarization triggers a larger SR Ca\(^{2+}\) release was first described by Fabiato (Fabiato, 1985a; 1985b; 1985c; Fabiato & Fabiato, 1978), and is termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). The triggering of CICR by I\(_{\text{Ca-L}}\) initiates a large transient increase in the intracellular Ca\(^{2+}\) concentration, or Ca\(^{2+}\) transient (Bers, 2001). The elevation of the intracellular Ca\(^{2+}\) concentration permits Ca\(^{2+}\) to act at the myofilaments to induce contraction (Fabiato, 1985a; 1985b; 1985c). The increase in intracellular Ca\(^{2+}\) concentration, which occurs in response to initiation of CICR, however, is transient and the released Ca\(^{2+}\) is quickly removed from the cytosol. The transient nature of the increase in intracellular Ca\(^{2+}\) is necessary for relaxation of the cardiac myocyte contraction to occur (Bers, 2001; Bassani et al., 1995a). Ca\(^{2+}\) is pumped back into the SR by SERCA, pumped out of the cell by the SL Ca\(^{2+}\) ATPase pump, and extruded from the cell by NCX (Bers, 2001; Bassani et al., 1995a). Co-localization studies have shown that the proteins responsible for the removal of Ca\(^{2+}\) from the cytosol may be closely associated with the t-tubules and the diadic cleft (Scriven et al., 2000). This, and the close association of the RyR and the L-type Ca\(^{2+}\) channels (Scriven et al., 2000) are examples of how the highly organized structure of the cardiac myocyte at the levels of the contractile proteins, SL and SR can facilitate the coupling of electrical excitation to mechanical shortening, and also to relaxation.

A schematic representation of the EC coupling cascade in a cardiac myocyte is shown in figure 2. Figure 2 illustrates the response of the cell following SL depolarization. The voltage gated L-type Ca\(^{2+}\) channels open, and Ca\(^{2+}\) enters the diadic cleft. Ca\(^{2+}\) entering the cell can then act at the RyR to induce SR Ca\(^{2+}\) release, and
Figure 2: Excitation-contraction coupling in a ventricular myocyte. This is a schematic illustration of EC coupling in an isolated cardiac myocyte. Immediately following depolarization of the sarcolemma (SL) Ca\(^{2+}\) enters the cell via the L-type Ca\(^{2+}\) channels. The Ca\(^{2+}\) which enters via the L-type Ca\(^{2+}\) channels acts at the ryanodine receptors to induce further Ca\(^{2+}\) release from the SR. This Ca\(^{2+}\)-induced Ca\(^{2+}\) release leads to a large transient increase in cytosolic Ca\(^{2+}\). This cytosolic Ca\(^{2+}\) can then act at the myofilaments to induce contraction. Contraction is terminated and relaxation occurs when Ca\(^{2+}\) is removed from the cytosol by the SR Ca\(^{2+}\) ATPase pump (SERCA) by the SL Ca\(^{2+}\) ATPase pump, and by the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX).
produce a transient increase in intracellular $\text{Ca}^{2+}$. The $\text{Ca}^{2+}$ released from the SR then acts at the myofilaments to induce contraction (Fabiato, 1985a, 1985b & 1985c). The intracellular $\text{Ca}^{2+}$ is then returned to diastolic levels, as the $\text{Ca}^{2+}$ released from the SR is removed from the cytosol by the SR and SL $\text{Ca}^{2+}$ ATPase pumps and NCX (Bers, 2001; Bassani et al., 1995a).

As its name implies, CICR would appear to be a self-propagating mechanism, which would always produce the same response to any stimulus. The magnitude of the CICR transient, however, is graded by the amplitude of $I_{\text{Ca-L}}$, which is the trigger for SR $\text{Ca}^{2+}$ release. Thus, CICR is not an all-or-none response; rather the amplitude of $I_{\text{Ca-L}}$ can grade the amplitude of the $\text{Ca}^{2+}$ transient. The grading of CICR by $I_{\text{Ca-L}}$ amplitude has been explained by the theory of local control of $\text{Ca}^{2+}$ release (Stern, 1992; Cheng et al., 1993; Cannell et al., 1995). SR $\text{Ca}^{2+}$ is released in units called $\text{Ca}^{2+}$ sparks (Cheng et al., 1993; Cheng et al., 1996). $\text{Ca}^{2+}$ sparks represent the quantal release of $\text{Ca}^{2+}$ from the SR (Cannell et al., 1995). A number of RyRs opening in a coordinated manner function as a

$\text{Ca}^{2+}$ release unit (Cheng et al., 1993; Cheng et al., 1996). A single $\text{Ca}^{2+}$ release unit is activated by $\text{Ca}^{2+}$ entry through a single L-type $\text{Ca}^{2+}$ channel (Cheng et al., 1993; Cannell et al., 1995; Bers, 2001). However, a $\text{Ca}^{2+}$ release unit is not normally activated by $\text{Ca}^{2+}$ released by neighboring groups of RyRs (Cheng et al., 1996; Bers, 2001; Stern, 1992). Therefore, the number of L-type $\text{Ca}^{2+}$ channels that allow $\text{Ca}^{2+}$ entry determines the number of local $\text{Ca}^{2+}$ release units activated by SL depolarization (Cannell et al., 1995; Bers, 2001; Stern, 1992). It is the $\text{Ca}^{2+}$ released by CICR that acts as the coupler between electrical excitation of the cardiac SL and cardiac contraction. Thus, the amplitude of
contraction in an isolated myocyte is graded by the magnitude of CICR, as described by the amplitude of the \( \text{Ca}^{2+} \) transient. Without local control of \( \text{Ca}^{2+} \) release, CICR would be an all-or-none response and modulation of the strength of contraction would not be possible.

3. Cardiac Contractility in the Intact Heart

In order to respond to internal and external stimuli such as postural changes, exercise and altitude, the heart must be able to alter the rate at which blood is pumped. Stimuli such as exercise, which make large energetic demands on the body, require the heart to respond by moving more blood to the lungs and extremities, and therefore to increase cardiac output (Mohrman & Heller, 1997). As discussed previously, increasing either heart rate or SV can increase cardiac output (Mohrman & Heller, 1997). Physiological factors or pharmacological interventions that influence heart rate produce a positive chronotropic response, while interventions that alter SV by influencing cardiac contractility are said to produce positive inotropy (Bers, 2001).

Positive inotropy occurs in response to several types of stimuli. First, by increasing stretch on the ventricle, elevated end diastolic volume can increase contractility. E.H. Starling demonstrated that contractility increases in response to increased muscle length (Starling, 1918). This mechanism, known as the Frank-Starling mechanism (Bers, 2001), is beneficial to the body as it guarantees the maintenance of SV in the face of elevated arterial pressure (Fleg et al., 1995; Rodeheffer et al., 1984). Studies of the cellular mechanisms of force generation have demonstrated that the Frank-
Starling mechanism functions in isolated cardiac muscle, such as rat trabeculae (Kentish & Wrzosek, 1998) and even in isolated myocytes (Roos, 1997).

The Frank-Starling effect of muscle length arises through the contributions of two different mechanisms which can increase force production in cardiac muscle. First, as muscle is lengthened, the number of actin-myosin crossbridges that can cycle increases, increasing the amount of force that can be generated (length-tension relationship; Bers, 2001). Thus an increase in end diastolic volume could increase the amplitude of contraction by increasing muscle length. Second, as muscle length is increased, the Ca\(^{2+}\) sensitivity of troponin C also increases (Fuchs & Wang, 1991; Hoffman & Fuchs, 1988). Elevated Ca\(^{2+}\) sensitivity increases the probability of Ca\(^{2+}\) binding to troponin C and removes the inhibition of myosin binding (Fuchs & Wang, 1991; Hoffman & Fuchs, 1988). This elevation in Ca\(^{2+}\) sensitivity appears to be the primary component of the Frank-Starling relationship, as the length tension curve in cardiac myocytes is very steep and represents a small fraction of Frank-Starling related positive inotropy (Bers, 2001). Thus, increased muscle length promotes the actin-myosin interaction responsible for contraction. There also is some evidence that, while the Frank-Starling relationship explains rapid adaptation to changes in muscle length, contractile force also can be increased over a longer time course by stretch-related changes in the configuration of the action potential (Kentish & Wrzosek, 1998).

Increases in cardiac contractility also can arise in response to positive chronotropy. Early experiments showed that progressive increases in the force of contraction occur in response to progressive increases in the rate of stimulation in the hearts of many species (Woodworth, 1902; Singal, 1985). Sympathetic nerve stimulation
and circulating catecholamines are key modulators of chronotropy, and thus inotropy (Mohrman & Heller, 1997). Sympathomimetic signals also can increase cardiac contractility in the absence of changes in rate. The effects of catecholamines on the strength of contraction are due to the influence of cyclic adenosine monophosphate (cAMP) second messenger phosphorylation pathways on components of EC coupling including $I_{Ca-L}$ (Viatchenko-Karpinski & Gyorke, 2001; reviewed by MacDonald et al., 1994), $Ca^{2+}$ transient amplitude (Spurgeon et al., 1990), the RyRs (Valdivia et al., 1995; Marx et al., 2000), SR $Ca^{2+}$ content (Kirchberger et al., 1974) and the myofilaments themselves (Ray & England, 1976).

Cardiac contractility also is influenced by other hormones and circulating factors. For example, thyroid hormone can enhance contractility by increasing the rate of hydrolysis of ATP by SERCA (Chang et al., 1997). It also can cause shifts in contractile protein expression (Chang et al., 1997), can up-regulate $Na^{+}$ pump expression (Awais et al., 2000), and may also influence beta adrenergic receptor expression in the heart (Bahouth, 1991). Insulin and glucagon also have positive inotropic effects on the heart (Berne & Levy, 2001). Thus, sympathomimetic hormones, nuclear hormones such as thyroid hormone, and metabolic hormones such as insulin all can influence cardiac contractility.

Finally, hypothermia has been shown to produce positive inotropy (Suga, 1988; Langer & Brady, 1968). Studies employing computer derived models of myocyte function suggest that cooling increases the $Ca^{2+}$ sensitivity of the myofilaments, increasing the magnitude of the contractile response in the absence of changes in $Ca^{2+}$ transient amplitude (Mikane et al., 1997). Bers (2001), however, suggests that there may
be little if any change in myofilament Ca\textsuperscript{2+} sensitivity in response to moderate hypothermia, and that hypothermia reduces myofilament Ca\textsuperscript{2+} sensitivity. Hypothermia increases the probability and duration of RyR openings (Sitsapesan et al., 1991), which could contribute to greater SR Ca\textsuperscript{2+} release in response to extracellular Ca\textsuperscript{2+} entry. In addition, the Na\textsuperscript{+} pump is inhibited by at lower temperatures (Eisner & Lederer, 1980), which could shift NCX activity towards Na\textsuperscript{+} extrusion, and consequently the accumulation of Ca\textsuperscript{2+} in the cytosol (Bers, 1987; Eisner & Lederer, 1980). Thus, temperature is a potent modulator of cardiac contractility.

As discussed earlier, many factors, both internal and external, can lead to increases in contractility. The ability of the heart to increase the strength of contraction is important to the survival of the organism in situations of increased metabolic demand (Berne & Levy, 2001). Therefore, there are many factors that influence contractility, and many of these factors show significant interplay. One of the difficulties associated with measuring cardiac contractility in an intact organism or in the whole heart is the redundancy within the system. Many stimuli can induce the same response, in this case positive inotropy, through several different convergent pathways (Berne & Levy, 2001; Mohrman & Heller, 1997). This redundancy does not allow the individual mechanisms by which positive inotropy in any one pathway arises to be easily separated from the influences of the other convergent stimuli. For example, hormones that increase contractility also can increase venous tone and venous return (Berne & Levy, 2001; Mohrman & Heller, 1997). Increased venous return also increases cardiac contractility through the Frank-Starling mechanism (Berne & Levy, 2001; Mohrman & Heller, 1997). Therefore, it becomes important to look at positive inotropy in isolated tissue, and single
cardiac myocytes, where factors such as circulating hormones and temperature can be tightly controlled.

4. Inotropy in the Isolated Myocyte

A. The isolated myocyte.

In the isolated myocyte the strength of contraction is usually equated to the amplitude of contraction, and positive inotropy is said to occur when the amplitude of contraction increases (Brady, 1991). Single isolated cardiac myocytes decrease in length, or shorten, in response to depolarization. However, the cells are no longer connected to other myocytes, and therefore no longer experience resistance to contraction, or a load (Brady, 1991). Conventionally, only unloaded cell shortening and the velocity of unloaded cell-shortening are measured in most isolated myocyte preparations (Brady, 1991). Although cell-shortening and velocity of shortening do not definitively describe contractility, they are good estimates of contractility, and responses recorded from unloaded cells parallel responses measured from loaded cells and tissues (Brady, 1991). Therefore measurement of unloaded cell-shortening provides a reasonable estimate of cardiac contractile function in isolated myocyte models.

The model of EC coupling in figure 2 shows that there are several points at which a positive inotropic effect could arise at a cellular level. First, changes in the Ca\textsuperscript{2+} sensitivity and energy dependence of the myofilaments can influence contraction amplitude in the absence of changes in other components of the EC coupling pathway (Bers, 2001). Second, the amplitude of the Ca\textsuperscript{2+} transient also could influence
contraction amplitude by making more or less Ca\textsuperscript{2+} available to the myofilaments (Bers, 2001). Several different factors can influence the amplitude of the Ca\textsuperscript{2+} transient, including the amplitude of I_{Ca-L}, the magnitude of SR Ca\textsuperscript{2+} release, and the SR Ca\textsuperscript{2+} content (Eisner et al., 2000; Bers, 2001). The following discussion will describe the factors that can influence myofilament Ca\textsuperscript{2+} sensitivity, Ca\textsuperscript{2+} transient amplitude, the amplitude of I_{Ca-L}, SR Ca\textsuperscript{2+} content and SR Ca\textsuperscript{2+} release.

B. Factors influencing myofilament Ca\textsuperscript{2+} sensitivity.

The velocity and amplitude of cardiac contraction can be directly altered by changes in the sensitivity of the myofilaments to Ca\textsuperscript{2+} (Fuchs & Wang, 1991; Hoffman & Fuchs, 1988; Roos, 1997). As discussed in the section on contractility in the whole heart, myofilament Ca\textsuperscript{2+} sensitivity can be altered by phosphorylation events (Ray & England, 1976), stretch (Kentish & Wrzosek, 1998; Fuchs & Wang, 1991; Hoffman & Fuchs, 1988) and temperature (Bers, 2001; Mikane et al., 1997). The isoforms of myofilament proteins expressed in the heart can be altered by factors that influence transcription (Chin et al., 1998), including circulating hormones such as the thyroid hormones (Chang et al., 1997). Other factors that influence myofilament Ca\textsuperscript{2+} sensitivity include: pH (Fabiato & Fabiato, 1978; Blanchard & Solaro, 1984), ionic strength of buffers (Kentish, 1984), the magnesium ion concentration (Fabiato & Fabiato, 1975) and drugs, such as caffeine (Wendt & Stephenson, 1983). Therefore, many factors can influence myofilament Ca\textsuperscript{2+} sensitivity in the isolated cardiac myocyte. It is important to be conscious of factors, such as temperature, ionic composition of buffers and pH, which could influence the Ca\textsuperscript{2+} sensitivity of the cardiac myocyte myofilaments, when designing experiments to
investigate mechanisms of positive inotropy.

C. Factors influencing the amplitude of the Ca\textsuperscript{2+} transient.

Positive inotropy also can arise from increases in the amplitude of the intracellular Ca\textsuperscript{2+} transient (Bers, 2001). Stimuli which increase the amplitude of the trigger for CICR, I\textsubscript{Ca-L}, can increase Ca\textsuperscript{2+} transient amplitude, and thereby increase contraction amplitude. Factors that influence SR Ca\textsuperscript{2+} content and SR Ca\textsuperscript{2+} release also can have inotropic effects by increasing the amplitude of the Ca\textsuperscript{2+} transient. Each of these situations will be discussed individually below.

i. Factors influencing the amplitude of I\textsubscript{Ca-L}

Depolarization of the cardiac myocyte SL triggers the opening of L-type Ca\textsuperscript{2+} channels. L-type Ca\textsuperscript{2+} channels are voltage gated channels that inactivate with either Ca\textsuperscript{2+} or voltage (McDonald et al., 1994). Ca\textsuperscript{2+} dependent inactivation is rapid and is the primary form of inactivation when Ca\textsuperscript{2+} is present (McDonald et al., 1994). An increase in the amplitude of the whole cell Ca\textsuperscript{2+} current means that, at the single channel level, either more Ca\textsuperscript{2+} channels are open, Ca\textsuperscript{2+} channels are open longer, or more Ca\textsuperscript{2+} is moving through the channels per opening (McDonald et al., 1994). The number of Ca\textsuperscript{2+} ions entering a cell as I\textsubscript{Ca-L} also is dependent on the rate of inactivation of the L-type Ca\textsuperscript{2+} channels (McDonald et al., 1994). If rates of inactivation are slowed, more Ca\textsuperscript{2+} can enter the cell at later time points during the depolarization. Thus, Ca\textsuperscript{2+} current amplitude can be influenced by the characteristics of the L-type Ca\textsuperscript{2+} channel.
The number of open channels, and the amount of Ca\textsuperscript{2+} moving through the
channels is determined by the voltage at which the current is elicited. Voltage influences
channel gating and ionic movement. Approximately 50% of available L-type Ca\textsuperscript{2+}
channels are activated at membrane potentials between -20 and 20 mV, depending on the
tissue type (Hille, 2001). Although the majority of available channels are activated at
positive voltages, there is little driving force for Ca\textsuperscript{2+} through the channels (McDonald et
al., 1994). At negative voltages, where driving force is higher, few channels are activated
(McDonald et al., 1994). An increase in the extracellular Ca\textsuperscript{2+} concentration also can
influence the amplitude of I\textsubscript{Ca-L}. Elevation of extracellular Ca\textsuperscript{2+} increases the driving
force for Ca\textsuperscript{2+} through L-type Ca\textsuperscript{2+} channels. Thus, elevated extracellular Ca\textsuperscript{2+} causes an
increase in the peak amplitude of I\textsubscript{Ca-L} (Janczewski et al., 2000). Interestingly, although
elevation of extracellular Ca\textsuperscript{2+} increases the amplitude of I\textsubscript{Ca-L}, elevated intracellular Ca\textsuperscript{2+}
concentrations depress I\textsubscript{Ca-L} (Tseng & Boyden, 1991). Tseng and Boyden (1991)
reported that depression of I\textsubscript{Ca-L} by elevated intracellular Ca\textsuperscript{2+} likely was due to Ca\textsuperscript{2+}
dependent inactivation of L-type Ca\textsuperscript{2+} channels. Therefore factors that alter the electrical
and chemical gradients for Ca\textsuperscript{2+} movement through the L-type Ca\textsuperscript{2+} channel, such as
voltage and extracellular Ca\textsuperscript{2+} concentration, and factors that influence the activation and
inactivation of the L-type Ca\textsuperscript{2+} channels, such as intracellular Ca\textsuperscript{2+} concentration and
voltage, are important modulators of the amplitude of I\textsubscript{Ca-L}.

Phosphorylation of the L-type Ca\textsuperscript{2+} channel by cAMP dependent second
messenger pathways also leads to significant elevation of the amplitude of I\textsubscript{Ca-L} (Hartzell
et al., 1991; Kameyama et al., 1985). cAMP - PKA pathway mediated phosphorylation
of the L-type Ca\textsuperscript{2+} channel increases current amplitude by increasing the open probability
of the L-type Ca\textsuperscript{2+} channels (Kameyama et al., 1986; Yue et al., 1990). Agents that lead to increases in PKA mediated phosphorylation include catecholamines, glucagon, serotonin and isoproterenol (Bers, 2001). Phosphorylation events also play an important role in modulating Ca\textsuperscript{2+} transient amplitude through effects on SR Ca\textsuperscript{2+} load and SR Ca\textsuperscript{2+} release as will be discussed further in later sections.

Many processes within cardiac myocytes, including PKA-dependent phosphorylation of intracellular proteins, are energy dependent. The primary source of energy for energy dependent processes in cardiac tissue is hydrolysis of ATP (Bers, 2001). ATP is important for pumping Na\textsuperscript{+} and Ca\textsuperscript{2+} to maintain concentration gradients required for excitability and relaxation (Bers, 2001). ATP and its metabolite/precursor, adenosine, also have been shown to have direct effects on Ca\textsuperscript{2+} channels. Extracellular adenosine has been shown to decrease I\textsubscript{Ca-L} amplitude (Qu et al., 1993a), as has extracellular ATP (Qu et al., 1993b). However, others report increased amplitude of I\textsubscript{Ca-L} in ventricular myocytes (Scamps et al., 1993) in the presence of extracellular ATP. Thus, ATP and adenosine, which are important to the energy dependent components of cardiac contraction, also can influence the amplitude of I\textsubscript{Ca-L}.

Some studies point to an influence of stimulation rate on I\textsubscript{Ca-L} amplitude (Lee, 1987; Kaspar & Pelzer, 1995), although it is not clear whether increasing stimulation rate increases amplitude of I\textsubscript{Ca-L}, or decreases it. Early researchers (Woodworth, 1902; Niedergerke, 1956) showed that increases in stimulation rate increased contractility in intact cardiac muscle. Niedergerke (1956) reported that the action potential duration does not change during these increases in contraction amplitude. This may indicate that changes in ionic currents across the membrane do not influence the positive inotropy
reported by Niedergerke (1956). However, others have shown that increasing stimulation rate changes ionic flux across the membrane. Lee (1987) determined that the amplitude of $I_{\text{Ca-L}}$ increased with increasing rate of stimulation, which would be expected to produce positive inotropy. Conversely, Kaspar & Pelzer (1995) report that increasing stimulation rate decreases $I_{\text{Ca-L}}$ amplitude. Therefore, an interesting controversy exists surrounding the role of $I_{\text{Ca-L}}$ amplitude, and by extension Ca$^{2+}$ transient amplitude, in the positive inotropy which arises when stimulation rate is increased.

Hypothermia is a positively inotropic stimulus. Curiously, hypothermia also has been shown to decrease peak amplitude of $I_{\text{Ca-L}}$ (Cavalie et al., 1985). A decrease in the amplitude of $I_{\text{Ca-L}}$ would be expected to produce negative inotropic effects. However, as discussed earlier, myofilament Ca$^{2+}$ sensitivity may be altered in response to hypothermia (Mikane et al., 1997) and, as will be discussed further, hypothermia also influences SR Ca$^{2+}$ content and release (Sitsapesan et al., 1991). Interestingly, although peak current amplitude is reduced, inactivation of the L-type Ca$^{2+}$ channel is slowed by hypothermia (Puglisi et al., 1999). When inactivation is slowed, more Ca$^{2+}$ can enter for a given period of depolarization as the channels remain open longer. Thus, slowed inactivation allows further Ca$^{2+}$ entry later in the depolarization, and so the number of Ca$^{2+}$ ions entering the cell for a given period of depolarization may remain constant, despite a decrease in the peak current amplitude (Puglisi et al., 1999). The reduction in peak current amplitude, therefore, does not necessarily reflect a reduction in Ca$^{2+}$ influx, nor does it necessarily relate to a reduction in the number of Ca$^{2+}$ release units activated by Ca$^{2+}$ entry (Puglisi et al., 1999). The reduction in peak $I_{\text{Ca-L}}$ amplitude associated with hypothermia, therefore, may not influence SR Ca$^{2+}$ release (Puglisi et al., 1999).
Although it is likely that other contributors to positive inotropy, such as SR Ca\(^{2+}\) content (Puglisi et al., 1996), SR Ca\(^{2+}\) release (Sitsapesan et al., 1991) and myofilament Ca\(^{2+}\) sensitivity (Mikane et al., 1997) are responsible for much of the positive inotropy associated with hypothermia, the amount of Ca\(^{2+}\) entering the cell through the L-type Ca\(^{2+}\) channels also may contribute.

Finally, the amplitude of I\(_{Ca-L}\) can be manipulated by pharmacological interventions. Drugs which influence I\(_{Ca-L}\) are used in the treatment of cardiovascular disorders such as cardiomyopathy, angina and congestive heart failure (Katzung & Chatterjee, 2004). One class of drugs which influence Ca\(^{2+}\) channel function is the dihydropyridine family of Ca\(^{2+}\) channel antagonists and agonists (Hess et al., 1985). The dihydropyridine receptor antagonists stabilize the L-type Ca\(^{2+}\) channel in mode 0, the state in which the channel is not available to be opened (Hess et al., 1985). Dihydropyridine family Ca\(^{2+}\) channel agonists promote longer single channel openings and larger peak current amplitudes (Hess et al., 1985). These pharmacological agents have been used both as tools to investigate L-type Ca\(^{2+}\) channel function, and as interventions in disease (Hess et al., 1985; Katzung & Chatterjee, 2004). Their use underscores the importance of I\(_{Ca-L}\) as the trigger for CICR, and as a modulator of Ca\(^{2+}\) transient amplitude.

Increases in the amplitude of I\(_{Ca-L}\) produce an inherent positively inotropic effect. First, an increase in the amplitude of I\(_{Ca-L}\) should lead to an increase in the intracellular Ca\(^{2+}\) concentration because more Ca\(^{2+}\) has entered the cell from the extracellular space (Bers, 2001). Second, an increase in the number of Ca\(^{2+}\) ions entering the cell, or an increase in the number of Ca\(^{2+}\) channels that are open should increase the likelihood of
Ca$^{2+}$ entering the cell to activate a Ca$^{2+}$ release unit (Cannell et al., 1995). The more Ca$^{2+}$ release units activated in a coordinated manner, the larger the Ca$^{2+}$ transient should be (Cannell et al., 1995). Finally, a larger Ca$^{2+}$ transient should induce a larger contraction, and thus will produce positive inotropy.

ii. Changes in the gain of SR Ca$^{2+}$ release

In the previous section, the modulation of Ca$^{2+}$ release by the amplitude of I$_{Ca-L}$ was discussed. The amplitude of the Ca$^{2+}$ transient also can change in the absence of a change in I$_{Ca-L}$ amplitude. Such a change is referred to as a change in the "gain" of SR Ca$^{2+}$ release. Thus, the amplitude of contraction also can be modulated by changes in the gain of SR Ca$^{2+}$ release.

The gain of SR Ca$^{2+}$ release can be increased by many factors that influence the RyRs, which are the Ca$^{2+}$ release channels of the SR (Marks, 1997). These factors can act to increase the open probability of the RyRs (Valdivia et al., 1995; Patel et al., 1995; Sitsapesan et al., 1995; Sitsapesan & Williams, 1994; Gyorke & Gyorke, 1998; Xu & Meissner, 1998), increase the duration of RyR openings (Sitsapesan et al., 1991) or increase the driving force for Ca$^{2+}$ through RyRs (ZhuGe et al., 1999; Bers, 2001). The gain of SR Ca$^{2+}$ release can be elevated by an increase in the open probability of the RyR following phosphorylation of the RyR (Valdivia et al., 1995). Thus, Ca$^{2+}$ transient amplitude can be increased by phosphorylation of the RyR in the absence of changes in the amplitude of the trigger (Patel et al., 1995). Increases in SR Ca$^{2+}$ content can increase the driving force for Ca$^{2+}$ through the RyR (ZhuGe et al., 1999; Bers, 2001), as well as RyR open probability (Sitsapesan & Williams, 1994; Gyorke & Gyorke, 1998; Xu &
Meissner, 1998). Increases in cytosolic Ca\textsuperscript{2+} increase the open probability of the cardiac RyR (Sitsapesan et al., 1995). Cytosolic ATP also increases open probability, although apparently though an interaction with a binding site separate from the Ca\textsuperscript{2+} binding site (Sitsapesan et al., 1995). Thus, many factors inside the cell can influence the open probability of the RyR or the duration of RyR openings, and many factors, therefore, can modulate the gain of Ca\textsuperscript{2+} release in the intact myocyte.

External stimuli, such as ryanodine, caffeine and hypothermia, however, also can influence RyR open probability and the durations of those openings. These increases in RyR open probability and RyR open times increase the gain of SR Ca\textsuperscript{2+} release (Bers, 2001). The pesticide ryanodine has long been used as a research tool. Ryanodine has been reported to have opposing effects on RyRs at different concentrations. At low concentrations ryanodine enhances Ca\textsuperscript{2+} release from the SR, while at high concentrations (> 100 μM) ryanodine blocks SR Ca\textsuperscript{2+} release (Rousseau et al., 1987). Ryanodine can be used to remove the SR component of EC coupling to more easily study the contributions of other components such as I\textsubscript{Ca-L}, NCX and diastolic Ca\textsuperscript{2+} (Hattori et al., 1991; Stemmer & Akera, 1986). Another agent which also is used as a research tool is caffeine, which dramatically increases the open probability of the RyR (O’Neill et al., 1990; Bers, 2001). Rapid application of 10 mM caffeine solution to isolated myocytes can be used to assess total SR Ca\textsuperscript{2+} content (O’Neill et al., 1990; Bers, 2001). Finally, rapid cooling of isolated myocytes also is a method with which SR Ca\textsuperscript{2+} load can be assessed in isolated myocytes (Hryshko et al., 1989; Bers, 2001). This is possible because hypothermia increases the duration of RyR openings (Sitsapesan et al., 1991). Thus, temperature also is a potent modulator of RyR function. Therefore, ryanodine,
caffeine, and hypothermia all can increase the gain of Ca\(^{2+}\) release from the SR.

Increasing the gain of SR Ca\(^{2+}\) release with drugs or temperature, or blocking SR release, therefore, can be used as important tools to determine SR Ca\(^{2+}\) content, and the contribution of the SR to changes in intracellular [Ca\(^{2+}\)]. Increasing the gain of SR release by altering SR Ca\(^{2+}\) load and RyR Ca\(^{2+}\) sensitivity also is important to increasing the strength of contraction during physiological challenges, such as increased metabolic demand, or beta adrenergic stimulation (Bers, 2001). The gain of SR Ca\(^{2+}\) release is at the center of a complex web of factors which can act in concert to increase Ca\(^{2+}\) transient amplitude, and thus the amplitude of contraction.

As described above, the amplitude of the Ca\(^{2+}\) transient can be altered by changes in RyR function. Increases in the gain of SR Ca\(^{2+}\) release can increase Ca\(^{2+}\) transient amplitude and thereby lead to positive inotropy in the absence of changes in I\(_{\text{Ca,L}}\) amplitude. However, many of the factors that appear to influence the gain of Ca\(^{2+}\) release, such as temperature, phosphorylation, and purines, also may alter the amplitude of I\(_{\text{Ca,L}}\). Therefore, complex interactions exist between I\(_{\text{Ca,L}}\) and the gain of SR Ca\(^{2+}\) release. The factors which influence the amplitude of the Ca\(^{2+}\) transient by increasing I\(_{\text{Ca,L}}\) amplitude, such as phosphorylation (Kameyama et al., 1986; Yue et al., 1990), also can influence the gain of SR Ca\(^{2+}\) release (Patel et al., 1995; Valdivia et al., 1995) making separation of the different components of positive inotropy difficult. SR Ca\(^{2+}\) content also interacts with I\(_{\text{Ca,L}}\) and the gain of SR Ca\(^{2+}\) release to determine the amplitude of the Ca\(^{2+}\) transient. As indicated above, increased SR Ca\(^{2+}\) content can increase the gain of SR Ca\(^{2+}\) release. However, an increase in the gain of Ca\(^{2+}\) release can lead to depletion of the SR and an accompanying decrease in the total amount of Ca\(^{2+}\) available for release.
from the SR with each beat (Trafford et al., 2001; Eisner et al., 2000). The modulation of SR Ca\(^{2+}\) content, which can contribute to the gain of Ca\(^{2+}\) release and Ca\(^{2+}\) transient amplitude, will be discussed in the next section.

### iii. Changes in SR Ca\(^{2+}\) load

Increases in SR Ca\(^{2+}\) load have been shown experimentally to increase the amplitude of Ca\(^{2+}\) transients (Janczewski et al., 2000; ZhuGe et al., 1999). Increases in SR Ca\(^{2+}\) content increase Ca\(^{2+}\) conductance through the RyR (ZhuGe et al., 1999; Bers, 2001). Elevated SR Ca\(^{2+}\) load also increases the open probability of the RyR (Sitsapesan & Williams, 1994, 1997; Gyorke & Gyorke, 1998; Xu & Meissner, 1998). Therefore, increased SR Ca\(^{2+}\) load can increase the gain of SR Ca\(^{2+}\) release, and SR Ca\(^{2+}\) load is an important modulator of Ca\(^{2+}\) transient amplitude. SR load can be modulated by factors that influence the rate of Ca\(^{2+}\) pumping into the SR, the rate of Ca\(^{2+}\) leak from the SR, the amount of Ca\(^{2+}\) release being stimulated by I\(_{\text{Ca-L}}\), the amount of Ca\(^{2+}\) entering the cell as I\(_{\text{Ca-L}}\), and the activity of NCX (Bers, 2001). Thus, changes in the rates of pumping and leak, changes in Ca\(^{2+}\) release, and changes in Ca\(^{2+}\) flux across the SL all can influence SR Ca\(^{2+}\) content, and therefore Ca\(^{2+}\) transient amplitude.

cAMP dependent phosphorylation has been shown to increase SR Ca\(^{2+}\) ATPase pumping rates (Kirchberger et al., 1974). More recently, this has been shown to be due to phosphorylation of phospholamban, an SR membrane protein closely associated with SERCA (Inui et al., 1986). When not phosphorylated, phospholamban associates with SERCA, and can slow the rate of active transport of Ca\(^{2+}\) into the SR (Inui et al., 1986). PKA dependent phosphorylation of phospholamban, or phosphorylation by another
kinase, calmodulin kinase, leads to increased SERCA efficiency (Inui et al., 1986). However, not all stimuli which promote PKA mediated phosphorylation will increase SR Ca\(^{2+}\) stores. For example, isoproterenol is known to induce phosphorylation through PKA dependent pathways, yet Viatchenko-Karpinski and Gyorke (2001) failed to see an elevation in SR Ca\(^{2+}\) stores in the presence of isoproterenol. The lack of a significant effect on SR Ca\(^{2+}\) load could be due to an isoproterenol-induced increase in Ca\(^{2+}\) transient amplitude. Once Ca\(^{2+}\) transient amplitude is elevated, Ca\(^{2+}\) loss from the SR Ca\(^{2+}\) pool can occur as a result of Ca\(^{2+}\) extrusion or exchange out of the cytosol, rather than Ca\(^{2+}\) reuptake into the SR. Although isoproterenol-mediated phosphorylation increases SERCA pump efficiency (Inui et al., 1986), phosphorylation also increases I\(_{\text{Ca-L}}\) amplitude (Yue et al., 1990) and the gain of SR Ca\(^{2+}\) release (Patel et al., 1995; Sitsapesan et al., 1995). If the increase in Ca\(^{2+}\) transient amplitude mediated by isoproterenol-induced phosphorylation exceeds the increase in SERCA efficiency, then loss of Ca\(^{2+}\) from the SR Ca\(^{2+}\) pool, through extrusion and exchange, could exceed Ca\(^{2+}\) reuptake into the SR. Therefore, although factors such as isoproterenol may modulate SERCA function, their influence on SR Ca\(^{2+}\) load may be blunted by their effects on other components of EC coupling.

SR loading can be altered by changes in the rate of stimulation of the cardiac myocyte. Increases in stimulation rate lead to increases in SR Ca\(^{2+}\) content due to repeated activation of I\(_{\text{Ca-L}}\), which can promote loading of the SR (Fabiato, 1985c). Increases in stimulation rate also increase the amount of Na\(^{+}\) entering the cell, and leave less time in diastole for Na\(^{+}\) to be pumped out of the cell (Frampton et al., 1991a). Thus, increased stimulation rate may elevate diastolic Na\(^{+}\) and Ca\(^{2+}\) concentrations (Frampton
et al., 1991a). Elevation of diastolic Na\(^+\) or Ca\(^{2+}\) can increase SR Ca\(^{2+}\) load. First, elevated intracellular Na\(^+\) can increase intracellular Ca\(^{2+}\) by promoting Ca\(^{2+}\) entry into the cell via NCX (Frampton et al., 1991a). Second, elevated diastolic Ca\(^{2+}\) can decrease the gradient against which Ca\(^{2+}\) must be pumped into the SR (Frampton et al., 1991a). Hypothermia has been shown to increase SR Ca\(^{2+}\) load (Puglisi et al., 1996), and is believed to do so through elevated intracellular [Na\(^+\)] which arises due to hypothermia induced inhibition of Na\(^+\) pump function (Bers, 2001; Eisner & Lederer, 1980).

Although NCX can contribute to increases in SR Ca\(^{2+}\) load, extrusion of Ca\(^{2+}\) from the cytosol by NCX also can deplete SR Ca\(^{2+}\) load, which can limit positive inotropy, as discussed in the next section.

Ca\(^{2+}\) leak from the SR is another determinant of SR Ca\(^{2+}\) content. In guinea pig ventricular myocytes SR Ca\(^{2+}\) leak is prominent at rest, especially when compared to rat myocytes where SR Ca\(^{2+}\) load increases at rest (Bers, 2001). SR Ca\(^{2+}\) content shows a 50% decline following a 25 second rest period in guinea pig myocytes (Terracciano et al., 1995). SR Ca\(^{2+}\) leak occurs through several different processes. First, SR Ca\(^{2+}\) is released in resting myocytes in the form of spontaneous Ca\(^{2+}\) sparks (Cheng et al., 1996). Sparks may be a component of a self-regulating SR mechanism which reduces Ca\(^{2+}\) overload, as the incidence of spontaneous sparks increases when SR load is elevated (Ferrier et al., 2003; Satoh et al., 1997; Cheng et al., 1993). Second, SR Ca\(^{2+}\) leak can occur through back-flux of Ca\(^{2+}\) through the SERCA pump (Shannon et al., 2000). Third, other Ca\(^{2+}\) release channels such as the inositol trisphosphate receptor also can release SR Ca\(^{2+}\) (Woodcock et al., 1998; Perez et al., 1997), although their function is believed to be primarily atrial rather than ventricular (Lipp et al., 2000). The loss of Ca\(^{2+}\) from the SR
over time will lead to a decrease in the driving force for Ca\(^{2+}\) release from the SR, and may influence the gain of SR Ca\(^{2+}\) release as well.

Thus, increases in SR Ca\(^{2+}\) content contribute to increased gain of SR Ca\(^{2+}\) release. Increases in the gain of SR Ca\(^{2+}\) release and the amplitude of I\(_{\text{Ca-L}}\) both can contribute to positive inotropy. As was shown earlier, there are many mechanisms by which positive inotropy can occur. However, given that many factors can initiate positive inotropy, there must also be tight control of positive inotropy, to maintain a balance between the need to increase contraction amplitude and the necessity of cardiac relaxation and filling of the ventricle for the whole heart to function as a pump.

D. Limits to positive inotropy.

As reviewed in the previous section, the amplitude of I\(_{\text{Ca-L}}\), the gain of SR release, and SR Ca\(^{2+}\) content, all can contribute to positive inotropy in the heart. The three contribute to positive inotropy by increasing the amplitude of the Ca\(^{2+}\) transient. However, mechanisms do exist to limit positive inotropy and control the maximum amplitude of Ca\(^{2+}\) transients.

Some negative feedback regulation of SR Ca\(^{2+}\) content appears to occur in situations where Ca\(^{2+}\) transient amplitude is altered by changes in I\(_{\text{Ca-L}}\) amplitude or gain of SR Ca\(^{2+}\) release. Increases in the amplitude of Ca\(^{2+}\) transients can decrease SR Ca\(^{2+}\) load (Bers, 2001; Trafford et al., 2001). It has been hypothesized that SR depletion occurs because Ca\(^{2+}\) is extruded from the cell via the SL Ca\(^{2+}\) ATPase and NCX rather than being returned to the SR (Bers, 2001; Trafford et al., 2001). Because not all of the Ca\(^{2+}\) released from the SR during a large Ca\(^{2+}\) transient is returned to the SR, when large
Ca$^{2+}$ transients are initiated SR load will decrease. As SR load decreases, a smaller, steady state Ca$^{2+}$ transient amplitude, at which Ca$^{2+}$ release can be balanced by Ca$^{2+}$ reuptake, is reached. This decrease in the amplitude of the Ca$^{2+}$ transient occurs even if the stimulus that led to the larger Ca$^{2+}$ transient is still present (Bers, 2001; Trafford et al., 2001). Thus, in response to a reduction in SR Ca$^{2+}$ content, the gain of Ca$^{2+}$ release decreases. Equally, interventions that decrease the amplitude of Ca$^{2+}$ transients, such as reduced amplitude of I_{Ca-L}, have been shown to increase SR Ca$^{2+}$ content in some studies (Trafford et al., 2001). SERCA efficiency remains constant, although SR release is very small, and therefore Ca$^{2+}$ uptake exceeds Ca$^{2+}$ release on a beat-to-beat basis (Trafford et al., 2001). The increased SR Ca$^{2+}$ uptake to release ratio leads to an accumulation of Ca$^{2+}$ in the SR. If SR Ca$^{2+}$ is sufficiently elevated, the gain of SR Ca$^{2+}$ release may eventually increase. Thus, SR Ca$^{2+}$ load is not only a determinant of the gain of SR Ca$^{2+}$ release, and therefore of Ca$^{2+}$ transient amplitude, but also can be influenced by Ca$^{2+}$ transient amplitude.

Local control of Ca$^{2+}$ release also can act to limit Ca$^{2+}$ transient amplitude in the face of increasing I_{Ca-L} amplitude. Ca$^{2+}$ transient amplitude is graded by the amplitude of I_{Ca-L}, through local control of Ca$^{2+}$ release at the level of the RyRs (Bers, 2001; Cannell et al., 1995; Lopez-Lopez et al., 1995). The numbers of L-type Ca$^{2+}$ channels that open in response to depolarization determine the amplitude of the whole cell current and the magnitude of SR Ca$^{2+}$ release (Bers, 2001; Cannell et al., 1995; Lopez-Lopez et al., 1995). However, it is hypothesized that there are a finite number of Ca$^{2+}$ release units within the SR, and that they are coupled to a finite number of L-type Ca$^{2+}$ channels. If the amplitude of I_{Ca-L} is increased by increasing the driving force for I_{Ca-L}, as would occur
with elevated extracellular Ca\(^{2+}\) concentration, eventually all of the Ca\(^{2+}\) channels coupled to Ca\(^{2+}\) release units should open. The number of Ca\(^{2+}\) release units available for activation also should reach a maximum. Further activation of Ca\(^{2+}\) release units by continuing elevation of I\(_{\text{Ca-L}}\) amplitudes, therefore, should no longer be possible, and Ca\(^{2+}\) transient amplitude should saturate despite increasing trigger amplitude. Thus, local control theory dictates that CICR from the SR should saturate, as the number of release units available for recruitment reaches a maximum despite increasing amplitude of I\(_{\text{Ca-L}}\) (Janczewski et al., 2000; Stern, 1992).

Increasing the amplitude of the Ca\(^{2+}\) transient is an important mechanism by which positive inotropy can be produced in isolated myocytes. However, the ability of Ca\(^{2+}\) transient amplitude to increase without limit would be detrimental to cardiac function. Therefore, these limits to positive inotropy exist.

E. Positive staircases of contraction.

In many species, following a rest period, cardiac contraction amplitude increases progressively with repeated stimulation (apex of dog heart, Woodworth, 1902; guinea pig ventricular myocytes, Hattori et al., 1991; frog ventricle, Niedergerke, 1956; feline ventricular myocytes, duBell & Houser, 1989; but not in rat, Suda & Kokubun, 1994). In his 1902 discussion of contraction in the heart, Woodworth indicated that a staircase in contraction amplitude arose when canine myocardium was stimulated following a quiescent period. This phenomenon was termed a staircase of contraction. In his early investigations of the staircase phenomenon in excised frog ventricle, Niedergerke (1956) showed that progressive increases in the amplitude of contraction following a rest period
occurred without changes in the size or duration of the action potential. Niedergerke (1956) also determined that similar increases in the amplitude of contraction could be elicited, not by repetitive stimulation, but rather by changes in extracellular Ca\(^{2+}\) concentration. From this finding, it was inferred that the strength of contraction is controlled by Ca\(^{2+}\) concentration in a discrete, superficial region of the heart cell (Niedergerke, 1956). Although our understanding of the mechanism by which Ca\(^{2+}\) influences cardiac contraction is now more complete, controversy remains as to the components of EC coupling that are responsible for the positive inotropic staircase following rest.

In guinea pig ventricular myocytes, SR Ca\(^{2+}\) content declines with increasing rest interval (Terracciano et al., 1995). SR Ca\(^{2+}\) content is decreased by 50% following a 25 second rest period when compared to a 2 sec rest period (Terracciano et al., 1995). The decline in SR Ca\(^{2+}\) content following a rest period is believed to contribute to positive inotropic staircases (Bers, 2001), at least in guinea pig ventricular myocytes. Lee (1987) has reported progressive increases in the amplitude of I\(_{\text{Ca-L}}\) with increasing beat number when a 10 second rest was allowed prior to the stimulation train. The progressive increase in the amplitude of I\(_{\text{Ca-L}}\) with increasing beat number also could contribute to positive staircases. duBell and Houser (1989) reported that Ca\(^{2+}\) transient amplitude increased with increasing beat number following a rest period in feline ventricular myocytes at room temperature. They attributed this increase in Ca\(^{2+}\) transient amplitude to increases in SR Ca\(^{2+}\) load, due to repetitive stimulation and frequent repetitive I\(_{\text{Ca-L}}\) influx (duBell & Houser, 1989). Thus, positive inotropic staircases appear to arise as a result of progressive increases in Ca\(^{2+}\) transient amplitude, as SR Ca\(^{2+}\) content increases
with increasing beat number.

Interestingly, Hattori et al. (1991) showed that increases in diastolic $\text{Ca}^{2+}$ concentration may play a direct role in positive staircases in guinea pig ventricular myocytes studied at room temperature. While $\text{Ca}^{2+}$ transient amplitude increases in positive staircases, the increase does not correspond closely to the increase in contraction amplitude, but rather, contraction amplitude is directly related to progressive increases in diastolic $\text{Ca}^{2+}$ concentration (Hattori et al., 1991). Positive inotropic staircases were seen in both rat and guinea pig ventricular myocytes in the presence of ryanodine (Hattori et al., 1991). Ryanodine should remove the contribution of SR $\text{Ca}^{2+}$ release to staircases (Hattori et al., 1991), and therefore this observation suggests a role for changes in diastolic $\text{Ca}^{2+}$ in generation of staircases. However, most studies which have examined staircases of contraction have not examined the role of diastolic $\text{Ca}^{2+}$ in contributing to the staircase (i.e. duBell & Houser, 1989; Suda & Kokubun, 1994). Also, many investigations of staircases in isolated myocytes have examined the staircases at room temperature (duBell & Houser, 1989; Hattori et al., 1991; Suda & Kokubun, 1994). As indicated throughout this introduction, hypothermia can have potent effects on SR $\text{Ca}^{2+}$ load, the gain of SR $\text{Ca}^{2+}$ release, myofilament $\text{Ca}^{2+}$ sensitivity and $I_{\text{Ca-L}}$. Further investigations of the role of diastolic $\text{Ca}^{2+}$ in positive staircases, and in other interventions which produce positive inotropy, therefore are warranted.

F. Stimulation rate.

As discussed previously, positive chronotropy can lead to positive inotropy in the heart. The generation of positive inotropy in response to increasing heart rate, or a
positive force-frequency relationship, is believed to be an important survival mechanism (Berne & Levy, 1997). Increasing the strength of contraction in concert with increases in heart rate, increases the cardiac output significantly, and enables rapid delivery of nutrients to, and removal of metabolites from, working skeletal muscle and other tissues during periods of elevated metabolic demand (Mohrman & Heller, 1997). As with whole heart, and multi-cellular muscle preparations, evidence shows that increases in stimulation rate increase contraction amplitude in isolated cardiac myocytes (Frampton et al., 1991a). Although force is not normally recorded in isolated myocytes (Brady, 1991), this relationship is often referred to as a positive force-frequency relationship, as it appears to mirror the situation in intact muscle. Increasing the rate of stimulation is a classical example of a positively inotropic stimulus, and has been studied extensively. Although some conflict remains, the mechanism by which positive inotropy occurs has been investigated extensively.

Increasing stimulation rate in cardiac muscle preparations produces positive force-frequency relationships in many species (Kennedy et al., 1986; Woodworth, 1902; Bers 2001). With each depolarization, Ca^{2+} enters the cell in the form of I_{Ca-L} (McDonald, 1994). An increase in stimulation rate leads to an increase in Ca^{2+} influx into the cell per unit time, which elevates diastolic Ca^{2+} and promotes SR Ca^{2+} loading (Fabriato, 1985c; Bers, 2001; Bers et al., 2000). Repeated stimulation also elevates intracellular Na^{+} concentrations, and elevated intracellular Na^{+} may promote elevation of diastolic Ca^{2+} through NCX (Frampton et al., 1991a). Elevations in SR Ca^{2+} load, diastolic Ca^{2+} and systolic Ca^{2+} concentrations have been recorded experimentally in response to elevated stimulation rates in isolated rat cardiac myocytes (Frampton et al.,
1991a). Studies also have shown that phospholamban plays a role in mediating positive force-frequency relationships (Bluhm et al., 2000; Meyer et al., 1999). Enhanced expression of phospholamban appears to promote positive force-frequency (Meyer et al., 1999). Thus, increased Ca\(^{2+}\) flux, elevated diastolic Ca\(^{2+}\), and up-regulation of SERCA function by phospholamban all act in concert to increase SR Ca\(^{2+}\) load. The increase in SR Ca\(^{2+}\) load, therefore, is believed to be responsible for the increase in contraction amplitude associated with elevated stimulation rate that occurs in isolated cardiac myocytes.

Although the positive-force frequency relationship has been studied extensively, the factor or factors that contribute to positive inotropy during positive force frequency are not clear. Combining the results of various studies does not necessarily draw a definitive picture, likely due to differences in species, muscle preparations and other experimental conditions. Some investigators have indicated that the amplitude of I\(_{Ca-L}\) increases progressively in response to increasing stimulation rate (Lee, 1987), and therefore can contribute to increasing contraction amplitude. Others, however, have shown decreases in I\(_{Ca-L}\) in response to increasing stimulation rate (Kaspar & Pelzer, 1995). Cardiac muscle preparations from guinea pig (Kurihara & Sakai, 1985) and rabbit show positive force frequency relationships (Bers, 2001), however, force-frequency relationships are negative in rat and mouse heart, as are those of failing human myocardium (Bers, 2001). Interestingly, Frampton et al. (1991a) noted that 40% of rat ventricular myocytes exhibit positive inotropy in response to increased stimulation rate, while approximately 60% exhibit negative inotropy. Further, Borzak et al. (1991) have reported that the rat force-frequency relationship is biphasic, with a very negative
relationship at low stimulation frequencies, and a slight positive relationship at more physiological frequencies. Thus, even within a single species, controversy exists as to the type of force-frequency relationship which can be elicited, especially when different tissues or single cell preparations are examined. These differences may account, in part, for the controversy surrounding potential contributors to positive force-frequency relationships such as $I_{\text{Ca-L}}$ amplitude, intracellular Na$^+$ concentrations, SERCA pump function and SR Ca$^{2+}$ load.

Interestingly, in isolated rat cardiac myocytes that exhibit positive force-frequency relationships, Frampton et al. (1991a) also showed that increasing stimulation rate elevated the diastolic Ca$^{2+}$ concentration, and decreased the diastolic cell length. Frampton et al. (1991a) reported that increasing stimulation rate increased SR Ca$^{2+}$ content, and that that increase was likely mediated by the elevated diastolic Ca$^{2+}$ concentration. Elevated diastolic Ca$^{2+}$ however, also could have positive inotropic effects in the absence of changes in SR Ca$^{2+}$ load. Elevated diastolic Ca$^{2+}$ could contribute to positive force-frequency relationships by elevating peak systolic Ca$^{2+}$. Such a mechanism would be similar to the positive diastolic Ca$^{2+}$ staircase noted by Hattori et al. (1991) during positive staircases of contraction. Although rat and mouse myocytes traditionally exhibit negative force frequency relationships (Stemmer & Akera, 1986; Frampton et al., 1991a), Stemmer and Akera (1986) reported that positive force-frequency relationships could be elicited from rat and mouse cardiac myocytes when SR function was depressed by ryanodine. These results were similar to the findings of Hattori et al. (1991) who showed that ryanodine treatment could produce positive staircases in rat myocytes, which normally exhibit negative inotropic staircases from rest.
Stemmer and Akera (1986) concluded that the positive force-frequency relationship exists in all species. These positive force-frequency relationships can be masked by contributions of SR Ca\(^{2+}\) load, which can produce negative force-frequency relationships. Negative force frequency relationships can arise in species such as rat and mouse because the Ca\(^{2+}\) load in the SR of these species increases at rest (Bers, 2001). Therefore, the longer inter-stimulus interval at slower rate of stimulation leads to elevation of SR Ca\(^{2+}\) load. Elevated SR Ca\(^{2+}\) load increases the gain of SR Ca\(^{2+}\) release. Therefore, in rat and mouse myocytes, contraction and Ca\(^{2+}\) transient amplitudes increase at lower stimulation rates, producing negative force-frequency relationships. If, however, more physiological rates of stimulation are employed (Borzak et al., 1991), or if the SR component of the Ca\(^{2+}\) transient is inhibited (Stemmer & Akera, 1986), then positive force-frequency relationships are revealed. Thus, although SR Ca\(^{2+}\) load is an important determinant of the type of force-frequency relationship which arises, it may not be an important component of the positive force-frequency relationship.

As with positive staircases (Hattori et al., 1991), it is possible that the elevation of diastolic Ca\(^{2+}\) in positive force-frequency relationships is responsible, in part, for the positive inotropy associated with increasing stimulation rate. Thus, it is possible that diastolic Ca\(^{2+}\) also plays an important role in positive force-frequency relationships. However, the different components of EC coupling are interconnected in a complex manner, and differentiating the contributions of different components to positive force-frequency relationships remains difficult.
G. Elevation of extracellular Ca\(^{2+}\) concentration.

Extracellular Ca\(^{2+}\) also can modulate contraction amplitude through several different components of the EC coupling pathway. Changes in extracellular Ca\(^{2+}\) change the driving force for \(I_{\text{Ca-L}}\) (Hille, 2001), and thereby grade the amplitude of the Ca\(^{2+}\) transient. Further, elevated extracellular Ca\(^{2+}\) concentration can increase SR Ca\(^{2+}\) content (Frampton et al., 1991b; Schror et al, 1979). Elevated extracellular Ca\(^{2+}\) can increase the amount of Ca\(^{2+}\) entering the cell in the form of \(I_{\text{Ca-L}}\) (Janczewski et al., 2000), and reduce Ca\(^{2+}\) extrusion by NCX (Frampton et al., 1991a). Both greater Ca\(^{2+}\) entry into, and reduced Ca\(^{2+}\) loss from, the cytosol can promote Ca\(^{2+}\) uptake into the SR, and produce elevated SR Ca\(^{2+}\) load (Frampton et al., 1991a). Therefore, elevation of extracellular Ca\(^{2+}\) should be positively inotropic, and could increase SR Ca\(^{2+}\) load, due to the increase in the amplitude of \(I_{\text{Ca-L}}\).

Interestingly, as noted in the section discussing limits to positive inotropy, changes in \(I_{\text{Ca-L}}\) and Ca\(^{2+}\) transient amplitudes also can influence SR Ca\(^{2+}\) load through negative feedback regulation. Trafford et al. (2001) determined that, by decreasing Ca\(^{2+}\) transient amplitude, decreased extracellular Ca\(^{2+}\) concentration can increase SR Ca\(^{2+}\) load. This can occur because decreasing the quantity of SR Ca\(^{2+}\) released on a beat-to-beat basis will decrease the loss of Ca\(^{2+}\) through extrusion and exchange. However, the efficiency of Ca\(^{2+}\) reuptake into the SR will remain constant, producing an elevation in the fraction of released Ca\(^{2+}\) taken back up into the SR and therefore increasing SR Ca\(^{2+}\) load (Trafford et al., 2001). Also, increasing transient amplitude may deplete SR Ca\(^{2+}\) stores, and eventually decrease the gain of SR Ca\(^{2+}\) release (Diaz et al., 2005). When extracellular Ca\(^{2+}\) is elevated, \(I_{\text{Ca-L}}\) amplitude is increased. Ca\(^{2+}\) transient
amplitude initially increases in response to the increase in $I_{\text{Ca-L}}$ trigger amplitude.

However, the fraction of the released SR Ca\(^{2+}\) that will be extruded from the cytosol will be greater than when the Ca\(^{2+}\) transient is small (Diaz et al., 2005). Also, the efficiency of SERCA will not be greatly enhanced. Thus, Ca\(^{2+}\) loss from the SR Ca\(^{2+}\) pool may exceed Ca\(^{2+}\) reuptake into the SR, leading to depletion of SR Ca\(^{2+}\) (Diaz et al., 2005). Decreases SR Ca\(^{2+}\) load will decrease the gain of SR Ca\(^{2+}\) release, therefore decreasing the Ca\(^{2+}\) transient amplitude (Bers, 2001). Thus, although elevation of extracellular Ca\(^{2+}\) may be positively inotropic due to effects on $I_{\text{Ca-L}}$ amplitude, the positively inotropic influence may be attenuated by decreased SR Ca\(^{2+}\) load and gain of SR Ca\(^{2+}\) release.

Some studies also point to a potential contribution of diastolic Ca\(^{2+}\) to the positive inotropy associated with elevated extracellular Ca\(^{2+}\). Frampton et al. (1991b) reported that when extracellular Ca\(^{2+}\) is increased, diastolic Ca\(^{2+}\) concentration increases. However, Frampton et al. (1991b) reported that the positive inotropic influence of elevated diastolic Ca\(^{2+}\) was due to increased SR Ca\(^{2+}\) load. In contrast, although Suda and Kokubun (1994) detected increases in diastolic Ca\(^{2+}\) when they elevated the extracellular Ca\(^{2+}\) concentration, they also reported that diastolic Ca\(^{2+}\) did not play a role in determining contraction amplitude during positive and negative staircases. Thus, although some studies show that positive inotropy occurs when extracellular Ca\(^{2+}\) is elevated (Frampton, 1991b; Janczewski et al., 2000), little is known about how diastolic Ca\(^{2+}\) changes during exposure to different extracellular Ca\(^{2+}\) concentrations.

Elevation of extracellular [Ca\(^{2+}\)] may be positively inotropic. However, as noted in the preceding discussion of changes in extracellular [Ca\(^{2+}\)], the mechanism for positive inotropy arising from elevated extracellular [Ca\(^{2+}\)] remains unclear. While Frampton et
al. (1991a) report a role for elevated intracellular [Ca$^{2+}$] in elevating SR Ca$^{2+}$ load, Trafford et al. (2001) and Diaz et al. (2005) appear to hypothesize that SR Ca$^{2+}$ load and the gain of SR Ca$^{2+}$ release both will be decreased by elevation of extracellular [Ca$^{2+}$]. The contribution of diastolic Ca$^{2+}$ to positive inotropy, when extracellular Ca$^{2+}$ is elevated, or following other positively inotropic stimuli such as stimulation from rest or increased stimulation rate, is poorly understood, and the influence of any of these interventions on diastolic Ca$^{2+}$ concentrations is rarely, if ever, reported. Therefore, the objective of this thesis was to examine factors that influence inotropy in isolated cardiac ventricular myocytes, with a specific focus on the role of diastolic Ca$^{2+}$ in positive inotropy.

5. Hypothesis & Objectives

A. Hypothesis.

There is strong evidence that changes in the amplitude of I$_{Ca-L}$ and SR Ca$^{2+}$ load are important mediators of inotropic effects in the heart. However, several lines of evidence suggest that diastolic Ca$^{2+}$ levels also may play a role in regulation of the amplitude of cardiac contraction. Hattori et al. (1991) showed that increases in diastolic Ca$^{2+}$ might play a direct role in mediating positive staircases, which were generated by stimulating cardiac myocytes following a period of rest. Frampton et al. (1991a & 1991b) reported that, when either stimulation rate or extracellular Ca$^{2+}$ is increased, diastolic Ca$^{2+}$ concentrations increase. In contrast, other studies show that positive staircases (duBell & Houser, 1989), positive force frequency, and inotropic responses to
elevation of extracellular [Ca$^{2+}$] (Janczewski et al., 2000) occur in the absence of changes in diastolic Ca$^{2+}$. However, those studies do not eliminate the possibility that diastolic Ca$^{2+}$ could contribute to positive inotropy in some situations. Furthermore, many of these studies have been performed under hypothermic conditions, at room temperature, rather than at physiological temperatures (duBell & Houser, 1989; Janczewski et al., 2000; Frampton et al., 1991a & 1991b; Hattori et al., 1991). However, hypothermia is a potent positively inotropic stimulus which can influence SR Ca$^{2+}$ load (Puglisi et al., 1996), RyR open probability (Sitsapesan et al., 1991), myofilament Ca$^{2+}$ sensitivity (Mikane et al., 1997), amplitude of I$_{Ca-L}$ (Puglisi et al., 1999; Cavallie et al., 1985) and Na$^+$ pump function (Eisner & Lederer, 1980). Therefore the role of diastolic Ca$^{2+}$ in positive inotropy has not been thoroughly investigated, at physiological temperature, in the absence of hypothermia-induced positive inotropy. The specific hypothesis to be tested in this thesis, therefore, is that diastolic Ca$^{2+}$ makes an important contribution to inotropic effects in guinea pig ventricular myocytes at physiological temperature.

**B. Objectives.**

As stated previously, the focus of the thesis is the role of diastolic Ca$^{2+}$ in positive inotropy. The overall objective of this thesis is to examine factors that influence inotropy in isolated cardiac ventricular myocytes. The specific objectives of the thesis are:

1. To determine if diastolic Ca$^{2+}$ concentration plays a role in producing positive inotropy in response to two classical positively inotropic stimuli, stimulation from rest, and changes in stimulation rate.

2. To determine if diastolic Ca$^{2+}$ concentration can contribute to changes in
contraction amplitude arising from changes in extracellular Ca²⁺ concentration, and to separate the individual contributions of I_{Ca-L} and SR Ca²⁺ load to Ca²⁺ transient amplitude under these conditions.

3. To determine whether diastolic Ca²⁺ and Ca²⁺ transient amplitude can contribute to positive inotropy both separately and in concert.
CHAPTER 2: METHODS

1. Animals

All studies presented within this thesis used guinea pig ventricular myocytes. Albino guinea pigs (85-95% male) were purchased from Charles River Canada (Saint-Constant, QC). Guinea pigs were housed on a 12 hour sleep/wake cycle in the Carleton Campus Animal Care facility. Guinea pigs were co-housed (1-5 animals/cage) in large solid bottom plastic tubs with food and water freely available. At all times a sentinel animal was present in the guinea pig housing area to ensure the health of the environment in the animal care facility. All guinea pigs were allowed a minimum of 24 hours to acclimate in the animal care facility prior to the acute isolation of cardiac myocytes. All experimental protocols involving animals were approved by the Dalhousie University Committee on Laboratory Animal Care, in accordance with Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 1993).

2. Myocyte Isolation

Guinea pigs were selected at random, removed from the animal care facility in a covered cage and transported to the laboratory for weighing and acclimation. After a 30 to 120 minute period of acclimation, sodium pentobarbital was co-administered with heparin by intraperitoneal injection. Sodium pentobarbital dosage was 120 mg/kg. Heparin dosage was 3300 IU/kg. The animal was then returned to the covered cage to
await induction of anesthesia (5-10 minutes). Induction of anesthesia was determined by pinching of the forefoot and chest and also confirmed by a lack of blinking reflex.

Following induction of anesthesia, the animal was laid in a supine position and fixed in that position with clamps. The thorax was bathed in alcohol to minimize contamination of the open chest cavity. The ventral thorax was then opened at the level of the attachment of the diaphragm to the ribs. Next the left and right lateral ribs were cut rostrally. Then the ventral rib cage was folded back to expose the heart. A silk suture was passed around the ascending aorta, and moved to the aortic trunk. This suture was used as both a landmark and, later, to attach the aorta to the cannula. The aorta was then cut, and the cannula was inserted into the aorta. The silk suture was tied, affixing the heart to the cannula and the cannulated heart was lifted from the chest cavity to the perfusion apparatus (perfusion apparatus shown in Figure 3).

The isolated heart was perfused with a nominally Ca$^{2+}$ free oxygenated (100% O$_2$, Praxair, Halifax NS) isolation buffer (in mM: 120.5 NaCl, 4 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 10 HEPES, 12 glucose, pH to 7.4 with NaOH). Solutions were warmed by a water bath (Isotemp 3016H, Fisher Scientific, Ottawa ON), and passed through a water-jacketed heating coil (Radnoti Glass Technology Inc., Monrovia CA, USA), so that all solutions had a temperature of 37°C when measured at the cannula. Perfusion solution was pumped into the coronary circulation via perfusion of the aorta at a rate of 12-20 mL/min with a Piper model P peristaltic pump (Fred A. Dungey Inc., Agincourt ON). Bubbles were removed from the perfusion system by use of a bubble trap. The bubble trap was a component of the heating coil, specifically a bifurcation of the perfusate carrying part of the heating coil. Perfusion pressure also was measured at the top of the
bifurcation (illustration of perfusion apparatus shown in figure 3).

When the blood had been washed from the heart, and spontaneous contractions had ceased, the isolation buffer was replaced with a digestion buffer. This buffer had the same composition as the initial perfusion buffer but with the addition of collagenase (Worthington Type II, 16-23 mg/50 mL) and protease (Sigma, 4 mg/50 mL). Perfusion pressure was measured with a low pressure gauge (5 psi, Ashcroft, Stratford CT, USA) and the pressure changes which occurred in response to enzyme digestion were noted. Digestion was considered to be complete when the appearance of the tissue changed from opaque to translucent, and the perfusion pressure dropped from approximately 1 psi to < 0.5 psi. This typically occurred within 5 to 7 minutes of perfusion with the digestion buffer.

Following digestion, the ventricles were excised and placed in a Petri dish containing a high K⁺ buffer (in mM: 30 KCl, 3 MgSO₄, 50 L-glutamic acid, 30 KH₂PO₄, 20 taurine, 0.5 EGTA, 10 HEPES, 10 glucose, pH to 7.4 with KOH), then minced. In some cases, the heart was perfused with the high K⁺ buffer to arrest digestion prior to excision of the ventricles. Single cells were dissociated from the tissue by gentle, circular, manual agitation. The supernatant was then filtered through a 225 μm filter (Spectra/Mesh) to exclude large aggregates of poorly dissociated cells. Approximately 1 mL of dissociated, filtered, isolated myocytes suspended in high K⁺ buffer was decanted into a 1.5 mL tube for incubation with fura-2 AM. Cell suspensions were incubated for 15-20 minutes, at room temperature in the dark, with 5 μM fura-2 AM (fura-2 AM stock solution in anhydrous DMSO, 0.2% DMSO in cell suspension). Myocytes were then transferred to an experimental chamber installed on the stage of an inverted microscope.
Figure 3: Schematic illustration of perfusion apparatus for isolation of ventricular myocytes. From left to right: The heating bath maintains the perfusate at 37°C by circulating heated water through the water-jacket surrounding the heating coil. The cannula and cannulated heart are attached to the bottom of the heating coil, while a pressure gauge is attached at the top of the coil. The perfusate is pumped by a peristaltic pump, at a rate of 12-20 mL/min. The perfusate is pre-warmed in a 37°C water bath to facilitate heating to 37°C in the heating coil and oxygenated with pressurized O₂. Further details of the isolation procedure can be found in the text.
The experimental chamber was a custom designed, flow-through plexi-glass bath chamber (shown in Figure 4). The actual bath consisted of an approximately one by two cm hole, to the bottom of which was affixed a custom fitted optical grade glass coverslip (No. 1, 22 x 40 mm VWR International, Montréal QC). An outflow channel and a small well for ground solution were routed out of the plexi-glass (Figure 4). The solution delivery system included a Gilson Minipuls 3 peristaltic pump (3 ml/min, Mandel Scientific, Guelph ON), a drip chamber to decrease electrical noise conduction and, as shown in figure 4, a custom designed heat exchanger was positioned just before the solution inflow tube to warm the perfusate to 37°C. The heat exchanger temperature was maintained by an immersion circulator (polystat 12112-10, Cole-Parmer, Anjou QC). The temperature gradient across the bath was 2 to 4°C when perfusing at a rate of 3 mL/min. The solution inflow tube was affixed to the plexi-glass chamber opposite the outflow channel (Figure 4, component γ).

Fura-2 loaded myocytes suspended in high potassium buffer were allowed to settle on the glass cover-slip at the bottom of the chamber. Once they had settled (5-15 minutes) at the bottom of the chamber the myocytes were superfused with a physiological buffer warmed to 37°C. A 145 mM Na⁺ buffer (Table 1) was used for field stimulation experiments. In most voltage clamp experiments a reduced (45 mM) Na⁺ physiological buffer (Table 2) was used, however in some experiments a 145 mM Na⁺ physiological buffer (Table 3) was used instead.
Figure 4: Schematic illustration of bath chamber, perfusion and recording apparatus. Labels refer to the following components:

\( \alpha \) - Axon HS-2A Headstage amplifier (Gain x 0.1 LU).

\( \beta \) - Axon instruments HL-U microelectrode holder.

\( \chi \) - 2.7 M KCl filled glass microelectrode with a resistance of 16-26 M\( \Omega \)

\( \delta \) - Silver chloride coated silver ground wire, immersed in a well containing 2.7 M KCl.

\( \varepsilon \) - Agar bridge, filled with 1\% agar in 2.7 M KCl.

\( \phi \) - Heat exchanger.

\( \gamma \) - Solution inlet.

\( \eta \) - Bath chamber.

\( \tau \) - Solution outflow.
Table 1: Physiological (145 mM Na⁺) buffer used for superfusion while recording from myocytes during field stimulation experiments.

<table>
<thead>
<tr>
<th>Constituent</th>
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<td>NaCl,</td>
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<td>HEPES</td>
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<td>Glucose</td>
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<td>KCl,</td>
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<td>MgCl₂</td>
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<tr>
<td>CaCl₂</td>
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Table 2: Reduced [Na⁺] physiological buffer used to superfuse myocytes during most voltage clamp experiments.

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<tr>
<td>Choline Chloride</td>
<td>100</td>
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<td>CaCl₂</td>
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<td>Lidocaine</td>
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Table 3: 145 mM Na\textsuperscript{+} physiological buffer used to superfuse myocytes during some voltage clamp experiments.

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<td>HEPES</td>
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<td>KCl,</td>
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<td>MgCl\textsubscript{2}</td>
<td>1</td>
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<tr>
<td>CaCl\textsubscript{2}</td>
<td>1</td>
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<tr>
<td>Lidocaine</td>
<td>0.3</td>
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3. Field Stimulation

In some experiments, fura-2 loaded cells were field stimulated with silver electrodes. Electrodes were lowered into place, with a micromanipulator (Fine Science Tools Inc., North Vancouver BC), so that they were flat on the glass cover-slip at the bottom of the experimental chamber, and so that they lay on either side of, but did not obscure, the field of view of the 40x oil immersion lens (Nikon S-Fluor 40x, numerical aperture 1.30). Stimulation electrodes were custom made, and consisted of 200 μm enamel coated silver wire (Narishige Scientific Instrument Lab, Tokyo, Japan), stripped of the enamel for 25 to 50 mm at the tips and soldered to insulated copper wire (Radio Shack, Canada) at the other ends. The copper wire extended for 2-4 ft, ending in standard “banana” plugs (Radio Shack, Canada). Myocytes were stimulated at rates between 0.5 and 2.0 Hz by 3-5 msec pulses generated by a Grass SD9 Stimulator (Grass Medical Instruments, Quincy Massachusetts USA). Threshold for stimulation was determined and myocytes were stimulated at 150% of the threshold for stimulation to a maximum of 100 V. Intracellular [Ca^{2+}] (fura-2 fluorescence) and cell shortening (change in cell length) were measured simultaneously, as discussed later in the methods.

Myocytes which responded to field stimulation were selected for recording only if they had clear striations, well defined borders, were free of blebs and cellular debris, and did not generate spontaneous waves in the absence of stimulation. In order to record intracellular [Ca^{2+}] and cell shortening from a chosen cell, the cell was aligned so that it lay within the field of view of the closed circuit television camera (custom built CCD camera, Crescent Electronics, Sandy UT) required to record cell shortening. It was
possible to rotate the camera to accommodate cells with different orientations, so that all
cells lay on their long axis when pictured on the closed circuit television monitor
(Electrohome Ltd., Kitchener ON). Then, an aperture was used to isolate the fura-2
loaded cell in the fluorescence sampling window. Myocyte contractions and Ca^{2+}
transients were allowed to reach steady state and recorded for a minimum of 10 sec
before the stimulation rate was changed.

4. Voltage Clamp

Electrodes were pulled (resistance 16-24 MΩ) from borosilicate glass capillary
tubing with a filament (outer diameter 1.2 mm, inner diameter 0.69 mm, Sutter
Instruments Co., Novato, California, USA) by a Model P-97 Flaming/Brown
Micropipette Puller (Sutter Instruments Co., Novato, California, USA). The capillary
glass was cleaned with 99.9% ethanol before it was placed in the micropipette puller, and
electrodes were used only on the same experimental day as they were pulled. Electrodes
were filled with 2.7 M filtered KCl. Briefly, the tips were allowed to fill by capillary
action. The remainder of the first one-third to one-half of the shaft behind the tip was
filled using a syringe fitted with a syringe driven filter (0.22 μm Milllex-GV, Millipore,
Cambridge ON) and needle (fine, 30, gauge). The electrode was then affixed to an Axon
Instruments HL-U microelectrode holder (Figure 4, components β and χ). A silver wire
extended from the electrode holder into the KCl with which the electrode was filled. The
tip of the silver wire was coated daily with silver chloride by soaking the tip of the wire
in 5.25% sodium hypochlorite (household bleach). The electrode and holder were then
inserted into the jack at the front of the headstage (Axon HS-2A Headstage, Gain x 0.1 LU) amplifier (component α of Figure 4). The headstage was mounted on a micromanipulator (Leitz Inc., Wetzlar, Germany) to allow for precise movement of the electrode.

In order to complete the circuit that was created when the electrode tip was inserted into the bath solution, a salt bridge (component ε of figure 4) made up of 1% agar in 2.7 M KCl in a “C” shaped glass tube was used to ground the experimental chamber. The agar bridge was placed with one side in the buffer perfusing the experimental chamber, and the other side in a well of 2.7 M KCl, which also contained a chloride coated silver ground wire (component δ of figure 4). The ground wire was made up of silver wire, which was then treated regularly with sodium hypochlorite to produce a silver chloride coating. The opposite end of the silver wire was soldered to insulated copper wire, with a brass pin at its terminal. This brass pin was inserted into the grounding jack at the back of the headstage.

Once the electrode was filled and the holder was inserted into the pin jack at the front of the headstage, a myocyte was selected. As with the field stimulation experiments the selected myocyte had clear striations, and well defined borders. The electrode was then used to impale the myocyte. Briefly, the headstage was lowered into the bath until the tip of the electrode was in the solution. The Bertrand lens of the Nikon TE-200 inverted microscope was used to position the electrode in the bath to a depth just above the focal distance for the 40x oil immersion lens. The Bertrand lens was then switched out of position and the electrode was slowly lowered until it appeared in the field of view for the oil immersion lens. The electrode was then lowered into position using the
micromanipulator so that it was centered over the cell. Once the electrode was in position, the cell was aligned for recordings of cell length and/or contraction amplitudes, and masked for recording of intracellular Ca\(^{2+}\) as described in the field stimulation section.

Voltage clamp was carried out with standard single electrode voltage clamp techniques and an Axoclamp 2B current and voltage clamp amplifier (Axon Instruments Foster City, CA). A schematic of a standard single electrode voltage clamp amplifier circuit is shown in figure 5. Before the electrode could be used to apply current and record voltage, the sum of the junction potentials, and the resistance of the electrode had to be neutralized. This allowed measurement of the voltage drop across the membrane without contamination from the electrode itself (Fein, 1977). The neutralization of the voltage drop across the electrode was part of a process referred to as “zeroing the electrode”. First the sum of the liquid-liquid, liquid-metal and metal-metal junction potentials needed to be accounted for and canceled with the input offset of the amplifier. The KCl-filled agar bridge reduced changes in liquid junction potential arising from changes in the composition of the bath solution (Neher, 1992). Then the resistance of the electrode was determined by passing one nA of current, and neutralized by balancing the bridge control. Finally, a capacitance compensation was made to improve the speed of recording. To optimize the capacitance compensation, the rate of capacitive charging and decay, shown on an oscilloscope (Kikusui Model COR5541U, Kikusui Electronic Corp., Japan), were made as rapid as possible. Together this series of steps allowed for accurate, rapid recording of the current flowing across the membrane, and good control of membrane potential.
Figure 5: Schematic illustration of the voltage clamp circuit. The sample and hold circuit samples the membrane potential ($V_m$). $V_m$ is then compared to the user determined command potential, and current (positive or negative) is injected to bring $V_m$ closer to the command potential. The membrane potential and currents arising in response to changes in the command potential are then recorded, another sample of $V_m$ is taken, and the sample and hold cycle begins again.
An electrode, which was put though the process of "zeroing the electrode", as described above, was then used to impale a cardiac myocyte. Impalement of the cardiac myocyte involved lowering the electrode slowly downward with the micromanipulator until the electrode touched or dimpled the myocyte membrane. A brief, high frequency oscillatory current was then applied through the electrode in order to facilitate penetration of the cell by the electrode. The oscillatory current was produced by pressing the "buzz" button. Once the myocyte was impaled, the electrode measured the membrane potential, in its voltage recording capacity. If the myocyte membrane potential was not near -80 to -90 mV, negative current was injected until the membrane potential stabilized near -90 mV. Normally, the myocytes recovered a normal resting potential and no longer required injected current. Cells that required more that 1.0 nA of applied current for a sustained period of time, and that did not attain resting potentials near -90 mV were excluded.

Following successful impalement, the Axoclamp 2B current and voltage clamp amplifier was switched into discontinuous single electrode voltage clamp mode. Finkel and Redman (1984) provide a thorough description of single electrode voltage clamp techniques. The discontinuous single electrode voltage clamp amplifier circuit is illustrated in figure 5. In this mode, the differential amplifier (Figure 5) controlled the membrane potential while recording voltage and current. This is accomplished by switching between voltage recording and current injecting cycles. Briefly, the membrane potential was sampled by the headstage amplifier (Figure 5), then held by the sample and hold circuit until another sample was taken. Meanwhile, the sampled membrane potential was compared to the user determined command voltage at a second, differential amplifier. If the sampled voltage differed from the command potential, then the output of
the second amplifier induced the controlled current source within the circuit to apply
either positive or negative current to the myocyte, via the electrode in its current passing
capacity (Figure 5). This injected current changed the sampled membrane potential so
that it was closer to the command potential. At the end of a current injecting cycle, the
controlled current source stopped applying current to the electrode, and voltage was
recorded by the electrode. Initially the voltage recorded reflected the decay of the current
that had been injected. The gain and phase lag adjustments were manipulated to increase
the rate of decay of the injected current and to minimize the effects of this decay on
voltage and current recordings. However, care was taken not to excessively increase
gain, as this could induce oscillations in the circuit and lead to loss of voltage control. At
the end of the voltage recording cycle, another sample of the membrane potential was
taken, and compared to the command voltage once again. Each voltage-recording cycle
was twice as long as each current-injecting cycle. The recording and injection cycles
were monitored continuously on an oscilloscope, and the phase lag and gain adjusted
when oscillations occurred. Through a number of current-injecting and voltage-recording
cycles, the actual measured membrane potential approached the command potential set
by the user (Finkel & Redman, 1984). During voltage clamp experiments, the switching
rates for cycles of current-injecting and voltage-recording were between 7 and 10 kHz.

The initial user defined holding potential was -80 mV. Voltage clamp protocols
were used to set the command potentials. The voltage clamp protocols were designed in
and delivered by ClampEx software (version 8.1, Axon Instruments Foster City, CA).
All test steps were preceded by trains of five or ten 200 msec long conditioning pulses
(CP) from -80 to 0 mV, delivered at a rate of 2 Hz. The delivery of CPs is important to
maintain common activation histories and SR Ca\(^{2+}\) content in cells that may have experienced different periods of rest or other treatments. Following CP trains, in most protocols, a holding potential of either -60 or -50 mV preceded the test step to facilitate inhibition of Na\(^+\) current by lidocaine (300 \(\mu\)M). Exact details of specific activation protocols are shown in the appropriate RESULTS section.

In some experiments myocytes were voltage clamped with patch pipettes rather than high resistance microelectrodes. Patch pipettes were pulled with a Model P-87 Flaming/Brown Micropipette Puller (Sutter Instruments Co., Novato, California, USA) from 1.65 mm outer diameter (1.20 mm inner diameter) borosilicate glass capillary tubes (A-M Systems Inc., Carlsborg WA USA). Patch pipettes had a resistance of approximately 1-2 M\(\Omega\). Patch pipettes were filled by capillary action with an intracellular solution of the following composition (in mM): 70 KCl, 70 potassium aspartate, 1 MgCl\(_2\), 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 2.5 KH\(_2\)PO\(_4\), 0.12 CaCl\(_2\) and 0.05 8-bromo cAMP (Ferrier & Howlett, 2003). The pH of the intracellular solution was 7.2, and KOH was used to alter the pH to achieve that level. The intracellular solution was nominally Na\(^+\) free.

Voltage clamp methods used with patch pipettes were the same as with high resistance microelectrodes, including the headstage and amplifier circuitry. The process of “zeroing the electrode” was the same, however, once the electrode was “zeroed” a value corresponding to the liquid junction potential between the intracellular solution in the pipette and the extracellular superfusion buffer had to be subtracted from the input offset of the amplifier. The liquid junction potential was calculated using Clampex 8.2 software. The liquid junction potential was determined to be approximately 12.8 mV.
Once the input offset had been set at -12.8 mV, the myocyte was voltage clamped. However, rather than impalement of the myocyte membrane with the electrode, the patch pipette was pressed gently against the membrane of the myocyte, and gentle suction was applied to rupture the membrane. A suction system consisting of a syringe and tubing was attached to a side port on the electrode holder so that suction could be applied.

5. Simultaneous Recording of Fluorescence and Contraction

Recordings of fluorescence and contraction were made simultaneously. Contraction amplitude was measured with a closed circuit television camera (custom designed CCD camera, Crescent Electronics) attached to the microscope. The closed circuit television camera collected the image of the cell produced by red (>600 nm) light. The red light was generated by placing a red filter (Nikon Canada) in the light path (Figure 6) of the microscope illumination system. The cell image was collected by the 40x oil immersion objective and transmitted to the camera attached to the side port of the microscope. A dichroic mirror (Figure 6) separated the long wavelength, red, light from the fluorescence emission light, discussed later, so that only the red light image of the cell was directed to the camera and edge detector. The camera image was transmitted to a Crescent Electronics (Sandy UT, USA) video edge detector at 120 Hz and a closed circuit television monitor (Electrohome, Kitchener ON). Raster lines were used to track the movement of the edge (or edges) of the cell. With the video edge detector, it was possible to position and focus these lines to ensure accurate recording of the movement of the edge of the cell. In most experiments, contraction amplitudes were recorded by
Figure 6: Schematic illustration of the light paths for fluorescence excitation, fluorescence recording and contraction recording. The violet line represents the light path for excitatory (340 and 380 nM) light. The green line represents the light path for emitted (510 nM) light. The dashed red line represents the light path for the red light used to illuminate the cell for measurement of contraction. The dichroic cube selects and separates wavelengths of light to ensure that only certain wavelengths reach certain components of the light path, and that each wavelength travels in a specific direction. The adjustable aperture enables collection of emitted light from a single myocyte. Further details can be found in the text.
tracking one edge of the cell. In some experiments cell length and whole cell shortening were determined by tracking both edges of the cell.

Fluorescence was recorded at the same time as contraction amplitudes. The fluorescent Ca$^{2+}$ indicator Fura-2 was used to determine intracellular Ca$^{2+}$ concentrations. Fura-2 is available as an acetoxy methyl ester derivative (fura-2 AM, Invitrogen, Burlington ON). This form of fura-2 is cell permeant (Tsien, 1981). Once in the cytosol the esterified group, which confers the membrane permeability to the molecule, is cleaved by non-specific esterases. Once the esterified group is cleaved, fura-2 is no longer lipophilic, and is confined to the cytosol (Tsien, 1981). Loaded cells were superfused with one of the three buffers described in tables 1 to 3 for at least 10 minutes prior to fluorescence recording. This washed out any ester linked fura-2 not taken up into cells. Also the ester linked form of fura-2 is not active (Tsien, 1981), thus there was little potential for background fluorescence from free fura-2 in the bath.

Fura-2 fluorescence was recorded with Photon Technologies International (PTI, Brunswick, NJ, USA) equipment. A schematic of the fluorescence excitation and detection system and the light paths for excitation light, emitted light and red light is shown in figure 6. To record fluorescence from the myocyte, excitation light was generated by a xenon arc lamp (Ushio Inc., Japan). The arc lamp was powered with a 75 watt LPS-220B lamp power supply (PTI). A DeltaRAM high speed multiwavelength illuminator (PTI) acted to select and switch between the appropriate excitation wavelengths. For excitation of fura-2, the illuminator was capable of cycling between 340 and 380 nm excitation wavelengths every 2 msec. Emissions corresponding to each wavelength were collected at a sampling rate of 100 Hz. Fura-2, when not bound to Ca$^{2+}$,
is excited by the 380 nm wavelength, while Ca\textsuperscript{2+} bound fura-2 is excited by 340 nm (Gryniewicz et al., 1985). The excitation light was further controlled by a computer driven shutter device (SC-500 shutter controller, PTI), which allowed remote control of excitation events. The excitation light path is shown by the violet lines in figure 6. Excitation light was directed from the DeltaRAM to the microscope by a fiber optic coupler (PTI), and directed further to the 40x oil immersion lens by a dichroic mirror (400 nm, Chroma Technology, Brattleboro VT, USA). The excitation wavelengths of 340 and 380 nm led to the emission of photons from Ca\textsuperscript{2+} bound and Ca\textsuperscript{2+} unbound fura-2, respectively.

The path of the emitted light is shown by green lines in figure 6. Briefly, emitted light was collected by the objective, and directed to the photomultiplier tube (Model 810/814 PMT detection system, PTI, Brunswick NJ, USA) by the same dichroic cube that also directed excitation light to the objective, and red light to the edge detection camera. An adjustable aperture, which excluded emitted light from cells other than the cell of interest, was placed in the light path so that emitted light was collected from the cell of interest alone. The aperture could be adjusted to mask the cell of interest when in the “view” position, and then turned to the “measure” position so that emitted light from that cell could reach the photomultiplier tube. Between the aperture and the photomultiplier tube there was also an 80 nm barrier filter to ensure that only 470 to 550 nm light reached the photomultiplier tube. Fluorescence emission at each wavelength was recorded at 510 nm (80 nm bandwidth). The shutter, excitation light, and emission recording were controlled by FeliX software (PTI, Brunswick NJ, USA). The emission at each wavelength (340 and 380 nm) was recorded and the ratio of emissions (e340/e380 nm)
was generated and recorded, all at a rate of 100 samples/sec.

Once fluorescence recording from a myocyte was complete, a 30 second recording of the background fluorescence specific to that myocyte was made. This background value accounted for auto-fluorescence in any of the components of the light path, including the experimental bath chamber, the immersion oil, and the bathing solutions themselves. The background recording was made in an area near the myocyte from which recordings were made, but that was devoid of living cells or cell debris. The size of the aperture window was not adjusted. The means of the background values for emission in response to each excitation wavelength (340 and 380 nm) were determined for the 30 second recording. These mean background values were subtracted from their respective experimental recordings (Figure 7A & B). A new, background subtracted, ratio of emission was then determined (Figure 7C), and the calibration curve (Figure 8, generated as described below) used to convert the ratios to intracellular Ca²⁺ concentration (Figure 7D).

An in vitro calibration curve was used to convert the background subtracted fura-2 fluorescence to an estimate of intracellular Ca²⁺ concentration (Figure 8). The calibration curve was generated using a method adapted from Grynkiewicz et al. (1985). Serial additions of a Ca²⁺ containing, EGTA-buffered solution were made to a Ca²⁺-free EGTA-buffered solution to generate different Ca²⁺ concentrations. Cell impermeant (active) fura-2 (1 μM) was added to both buffers. The ratio of emission for fura-2 excited at 340 and 380 nm (e340/e380 nm) was recorded following each serial addition of EGTA buffered Ca²⁺. The fluorescence ratio was recorded for the different Ca²⁺ concentrations in the experimental bath chamber on the stage of the inverted microscope
Figure 7: Background subtraction of fluorescence signals and conversion of the fluorescence ratio to Ca\(^{2+}\) concentration.  

A. Initial raw recording of emitted light from a fura-2 loaded myocyte. Fura-2 was excited with light of 340 and 380 nm wavelengths and the 510 nm emitted light corresponding to each of the two excitation wavelengths was recorded. The signal corresponding to fura-2 when excited by 340 nm light represents the Ca\(^{2+}\) bound form of fura-2, while that corresponding to emission excited by 380 nm light represents the Ca\(^{2+}\) unbound form.  

B. The background recordings corresponding to this cell were subtracted from the signals recorded following excitation at 340 and 380 nm, respectively.  

C. The ratio (e340/e380 nm) of the background subtracted signals was determined.  

D. Using a lookup table containing the calibration curve data shown in figure 8, the intracellular [Ca\(^{2+}\)] corresponding to the background subtracted ratio was determined. This trace can now be measured to determine diastolic [Ca\(^{2+}\)], systolic [Ca\(^{2+}\)] and Ca\(^{2+}\) transient amplitude.
Figure 8: Calibration curve for determining intracellular $\text{Ca}^{2+}$ concentration from a fluorescence ratio. The first part of the curve is linear (first order polynomial regression, $r^2 = 0.993$), and makes it possible to determine the $\text{Ca}^{2+}$ concentration as a function of a linear relationship with the ratio of emission in response to excitation by 340 and 380 nm light. Inset: The complete calibration curve for fura-2. The complete relationship is non-linear. The relationship, however, is linear for the range of $[\text{Ca}^{2+}]$ expected in an intact ventricular myocyte.
so that the light path for recording of the calibration would be consistent with the light path for experimental recordings. EGTA was used to buffer Ca\(^{2+}\) during the calibration to ensure that a known free Ca\(^{2+}\) could be determined, as fura-2 itself also can act as a weak Ca\(^{2+}\) buffer (Gryniewicz et al., 1985). The free Ca\(^{2+}\) concentration was estimated from the EGTA buffered Ca\(^{2+}\) concentration. A curve showing the relationship between the fluorescence ratio (e340/e380) and the free Ca\(^{2+}\) concentration was generated (Figure 8) from the data collected in vitro. Figure 7 shows the data as they are transformed from raw data to the ratio of emission elicited by excitation with 340 and 380 nm light, and then to intracellular Ca\(^{2+}\) concentration. The ratio of emissions was converted to Ca\(^{2+}\) concentration using the FeliX software ratio to concentration look-up table. The look-up table values were the values generated in the calibration curve (Figure 8, inset). Since the range of ratios recorded experimentally falls on the linear part of the curve (Figure 8), a simple linear function also could be used to determine intracellular Ca\(^{2+}\) concentration.

6. Experimental Protocols

In some protocols extracellular [Ca\(^{2+}\)] was rapidly changed with a rapid solution switching device. The rapid solution switching device consisted of a series of 5 tubes which all joined into one perfusion line, and was encased in a heat exchanger that warmed the solutions to 37°C. When the flow from the rapid solution switcher was directed at a specific cell, it rapidly altered the solution bathing a cell within 300 msec (Levi et al., 1993; Spitzer, 1994; Hobai et al., 1997) of application. The solutions were delivered by gravity feed, and flow was turned on by solenoid valves (LFAA12017110H,
The Lee Co., Westbrook CT, USA). Solenoid valves were controlled either manually or by computer with a custom built switching device (Spitzer, 1994; Levi et al., 1993) connected to an output signal from the Digidata 1322A digitizer. The solution bathing the cell could be rapidly changed from the control solution to one of four test solutions by closing the control solenoid valve and opening one of the test valves.

The control extracellular Ca\(^{2+}\) concentration was 2.0 mM, and was used to superfuse the cells throughout the preceding CP trains. Extracellular Ca\(^{2+}\) concentration was rapidly changed from 2.0 mM to 0.1, 0.5 or 5.0 mM by bathing the cell in buffers of different Ca\(^{2+}\) concentrations. The rapid solution switching device also was used in some experiments to rapidly expose the cell to a reduced Na\(^{+}\) buffer (Table 2) to which 100 \(\mu\)M cadmium had been added. 100 \(\mu\)M cadmium blocks cadmium sensitive Ca\(^{2+}\) currents, and can be used to isolate Ca\(^{2+}\) currents from other currents (Talo et al., 1990), as will be discussed in "Measurements and Analyses". When altering extracellular Ca\(^{2+}\), or applying cadmium, the duration of application prior to a test step or caffeine application was always a minimum of three seconds to allow for exchange of the extracellular [Ca\(^{2+}\)] at the t-tubules (Yao et al., 1997).

In some experiments, rather than recording responses to a test step, the response to a rapid application of 10 mM caffeine was recorded. In these experiments the holding potential remained constant and 10 mM caffeine in a Na\(^{+}\) and Ca\(^{2+}\) free solution was applied for one second at the time point at which the test step would have occurred. The caffeine-induced Ca\(^{2+}\) transient was used as an estimate of the magnitude of SR Ca\(^{2+}\) stores. Caffeine has been shown to release Ca\(^{2+}\) from the SR by dramatically increasing the open probability of the ryanodine receptors (Endo, 1977). The peak of the caffeine
response is a measure of maximum SR Ca\(^{2+}\) release (Bassani et al., 1995b), and so the amplitude of the caffeine-induced Ca\(^{2+}\) transient is a measure of the quantity of Ca\(^{2+}\) available for release from the SR. To inhibit extrusion of Ca\(^{2+}\) released from the SR by the Na\(^{+}/\)Ca\(^{2+}\) exchanger (NCX), caffeine was applied in a nominally Ca\(^{2+}\) and Na\(^{+}\) free solution (Katoh et al., 2000) of the following composition (in mM): 140 LiCl, 4 KCl, 10 glucose, 5 HEPES, 4 MgCl\(_2\), 10 caffeine.

7. Measurements and Analyses

In voltage clamp experiments lidocaine (300 \(\mu\)M, in bulk superfusion solution) was used to block Na\(^{+}\) currents. Blockade of Na\(^{+}\) current by lidocaine is use and voltage dependent (Xiao et al., 2004). These features of lidocaine were used to enhance Na\(^{+}\) channel blockade by lidocaine in this study. Myocytes were repeatedly activated during the CP train to promote use dependent blockade of Na\(^{+}\) channels. Additionally holding potentials positive to -70 mV prior to test steps also were used to improve Na\(^{+}\) channel blockade.

In most situations peak amplitudes of \(I_{\text{Ca-L}}\) were measured as the peak inward current with respect to net current at the end of the 250 msec test step (Figure 9B). This method of current measurement has previously been confirmed as a good estimate of the amplitude of \(I_{\text{Ca-L}}\) (Grandy et al., 2004), and has been commonly employed in the past by other researchers (Janczewski et al., 2000; Xiong et al., 2001). In some experiments where slowly activating outward current made measurement of inward Ca\(^{2+}\) current with reference to the end of the test step difficult, a separate method to determine peak inward
Figure 9: Representative examples of measurement of transmembrane currents elicited by a 250 msec step from -60 mV to 20 mV. A. Schematic illustration of the activation protocol used to elicit the current responses shown in panels B & C. Amplitudes of currents were measured as the peak amplitude of the current with respect to the net current at the end of the 250 msec voltage step (B), or as the peak amplitude of current with respect to the time point when current, measured as in B, on a step to 0 mV was 90% inactivated (C). The reference line noted on all current traces represents 0 nA current.
A. 

-60 mV  

20 mV  

-70 mV 

B. 

Current Amplitude 

1.0 nA 

100 msec 

C. 

Current Amplitude 

1.0 nA 

100 msec
Ca\(^{2+}\) current was employed. Ca\(^{2+}\) current was measured as the peak current with reference to the point at which the current on a step to 0 mV was 90% inactivated (Figure 9C). An example of mean current measured at both the end of the voltage clamp step and at 90% inactivation is shown in figure 10.

To determine if the measurement of current at the 90% inactivation point on a step to 0 mV was a good estimate of peak inward Ca\(^{2+}\) current, the Cd\(^{2+}\)-sensitive (Ca\(^{2+}\)) current was measured by subtraction. Briefly, recordings of total current were made, then 100 µM Cd\(^{2+}\) was applied with the rapid switcher. The difference current, computed by digital subtraction of the Cd\(^{2+}\)-insensitive current from total current, was taken as a measurement of the Cd\(^{2+}\)-sensitive current. These experiments showed that, when the 90% inactivation point was used as a reference point, there was only a minimal (0.2 to 0.3 nA) underestimation of \(I_{Ca-L}\) amplitude. Furthermore, measurement of \(I_{Ca-L}\) amplitude at the 90% inactivation point removed the measurement artifact introduced when \(I_{Ca-L}\) was measured on steps to positive potentials (Figure 11).

In many experiments, contraction amplitudes and intracellular Ca\(^{2+}\) concentrations were measured simultaneously. Contraction amplitudes were measured as the difference between peak contraction and the diastolic value immediately preceding the onset of contraction. Following conversion of the fura-2 fluorescence ratio to Ca\(^{2+}\) concentration, diastolic [Ca\(^{2+}\)] (the concentration immediately preceding the test step), peak systolic [Ca\(^{2+}\)] and Ca\(^{2+}\) transient amplitude (difference between systolic and diastolic intracellular Ca\(^{2+}\) concentrations) were measured.

In some experiments, resting cell length and resting intracellular [Ca\(^{2+}\)] were recorded. Before those parameters were measured, cells were activated with a short
Figure 10: Current-voltage relationship measured either with respect to the end of
the test step, or with respect to the time at which current on a step to 0 mV is 90% inactivated. A. Activation protocol. Each voltage step was preceded by a series of ten CPs from -80 to 0 mV. The voltage steps were made from a holding potential of -60 mV. Voltage steps were made in 20 mV increments from -60 mV to 80 mV. B. Mean data. Measurement at the point where current on a step to 0 mV is 90% inactivated (open circles) more accurately reflects the expected bell-shaped relationship for $I_{Ca,L}$. $n = 14$. 
Figure 11: Measurement of peak current with respect to net current at the end of the test step over-estimates the cadmium-sensitive current. Activation protocol as in figure 9A. Cadmium-sensitive current (red) was determined by subtraction of the cadmium-insensitive current from total current. A slowly activating outward current was seen at the end of the total current record (black). Although measurement at the point where current elicited by a step to 0 mV is 90% inactivated underestimated peak current, the influence of the slowly activating outward current was mitigated.
activation protocol to show that they responded to electrical stimuli with contractions and Ca\(^{2+}\) transients. Cells were then held at a holding potential of -80 mV, and the elevated intracellular Ca\(^{2+}\) concentration caused by the activation protocol was allowed to decay to a steady state in 2.0 mM extracellular Ca\(^{2+}\). The myocyte was then exposed to different extracellular Ca\(^{2+}\) concentrations, applied with the rapid solution switcher. Cells that generated spontaneous Ca\(^{2+}\) waves during the experimental protocol were excluded.

In other experiments recordings of intracellular Ca\(^{2+}\) were made at room temperature. For these experiments *in vitro* fura-2 calibration curves were generated at 22°C and 37°C. The free [Ca\(^{2+}\)] at each temperature was determined using a computer-based free ion concentration calculator, WinMAXC Maxchelator (Chris Patton, Stanford University, Pacific Grove CA, USA), as altering the temperature of EGTA-buffered solutions alters the free [Ca\(^{2+}\)] (Harrison & Bers, 1989). By altering free Ca\(^{2+}\) concentration, temperature also can influence the binding of hydrogen ions by EGTA, and therefore influence pH (Harrison & Bers, 1989). Therefore, when the parallel calibrations at 22°C and 37°C were undertaken, separate adjustments of pH were made. The fura-2 fluorescence ratio at 37°C was shown to be equivalent to intracellular Ca\(^{2+}\) concentrations that were approximately 72% of those for the same ratio at 22°C. This relationship was tested for free Ca\(^{2+}\) concentrations which were representative of the intracellular Ca\(^{2+}\) concentrations that were expected.

Contraction amplitudes, cell length and whole cell currents were digitized by a Digidata 1322A A/D interface (Axon Instruments). Digitization rates varied from 0.7 to 4.7 kHz depending on the duration of the specific voltage clamp protocol. Contraction amplitudes, cell lengths and currents were recorded with ClampEx 8.1 (Axon
Outward current does not influence current when measured at the point where current is 90% inactivated.

Slowly activating outward current influences the measurement of current at the end of the step.

- Raw recorded current
- Cadmium sensitive current

1.0 nA

100 msec
Instruments) software, and analyzed with ClampFit 8.1 (Axon Instruments) software. Sigmaplot 2001 (Jandel Scientific, SPSS Inc.) was used to construct graphs.

8. Statistical Analyses

Statistical analyses were performed with either Sigmaplot 2001 or Sigmastat, version 2.03 (Jandel Scientific, SPSS Inc.). Mean data were expressed ± SEM; * denotes p < 0.05. For bar graphs comparing only two bars Student’s t-tests were performed and, except when comparing responses at 22 and 37°C, all t-test were paired (repeated measures). For contraction-voltage, current-voltage and transient-voltage relationships, two-way repeated measures analysis of variance (RM-ANOVA) was performed. For changes in stimulation rate, positive staircases, and when bar graphs had three or more bars, one-way RM-ANOVA was performed. Following RM-ANOVA, Bonferroni and Tukey post-hoc tests were used to determine which points were statistically significant when making comparisons to control and pair-wise comparisons, respectively.

Regressions were performed using first or second order polynomial functions. The $r^2$ value is the coefficient of determination and is a common measure of the reliability of a regression. Values near 1 indicate that the regression equation is a good description of the data. For the purposes of this thesis, regressions to the mean of the data with coefficients of determination of less than 0.8 were not considered to be good representations of the data.
9. Sources of Chemicals

Lidocaine, choline chloride, HEPES buffer, EGTA, MgCl₂, anhydrous DMSO, and caffeine were purchased from Sigma Aldrich Canada Ltd (Oakville, ON). Invitrogen Inc (Burlington, ON) was the supplier for fura-2 AM. All other chemicals were purchased from BDH Inc. (Toronto, ON). Fura-2 AM stock solution was prepared by dissolving 50 µg of fura-2 AM in 20 µL anhydrous DMSO. CaCl₂, MgCl₂, KCl, lidocaine and cadmium chloride all were dissolved in deionized water and prepared as concentrated stock solutions (0.5, 0.5, 1.0, 1.0 and 0.1 M, respectively) for addition to the superfusion buffer. All components of the buffers were dissolved in purified deionized water (Elix 3 and Milli-Q plus, Millipore Corporation, Cambridge ON).
CHAPTER 3: RESULTS

1. Role of Diastolic [Ca^{2+}] in Increasing Contraction Amplitude During Positive Staircases

Repeated electrical stimulation of cardiac tissue or isolated myocytes following a period of rest is a classical example of a positive inotropic stimulus. However, it is not clear whether diastolic Ca^{2+} contributes to positive staircases. To examine the role of diastolic Ca^{2+} in positive staircases, intracellular Ca^{2+} concentrations and contraction amplitudes were recorded simultaneously. Positive staircases were initiated by regular depolarization following a rest period. Myocytes were activated with trains of ten 200 msec rectangular pulses from -80 to 0 mV, following a three second rest period. Pulses were delivered at a rate of 2 Hz. Figure 12 shows a schematic of the activation train (panel A), as well as representative recordings of contractions and intracellular Ca^{2+} transients (panels B & C respectively). The recording of contractions in panel B shows that the first contraction initiated after a period of rest was small. However, the amplitude of contractions increased stepwise with each sequential activation during the first part of the train. The amplitudes of contractions became constant during the last few beats. In contrast, Ca^{2+} transient amplitudes appeared to remain constant throughout the train. Nonetheless, both peak systolic Ca^{2+} and diastolic Ca^{2+} increased over the ten pulse train. Therefore, these contractions appear to exhibit positive staircases, in the absence of changes in amplitudes of Ca^{2+} transients.

Mean amplitudes of contraction and intracellular [Ca^{2+}] during positive staircases
Figure 12: Following a rest period, trains of pulses delivered at a frequency of 2 Hz result in positive staircases. Myocytes were loaded with fura-2, voltage clamped, and superfused with a physiological buffer (Table 2). Contractions and Ca\textsuperscript{2+} transients were recorded simultaneously. A. Schematic representation of the voltage clamp protocol used to activate the cells. Contractions and Ca\textsuperscript{2+} transients were elicited by a series of ten 200 msec square pulses from -80 to 0 mV delivered at a rate of 2 Hz. Stimulation pulses were preceded by a 3 sec rest period. B. Representative example of contractions elicited following a 3 sec rest period. Contraction amplitude was measured as cell shortening, recorded from a single edge of the cell. Contraction amplitude increased over the series of 10 pulses. C. Representative example of the changes in intracellular Ca\textsuperscript{2+} in response to stimulation following a rest period. Ca\textsuperscript{2+} transient amplitudes appeared to remain constant, although diastolic and systolic Ca\textsuperscript{2+} both increased.
are shown in figure 13. Data were fitted with a second order polynomial regression line. Only data from responses to activation pulses two to ten were shown, and fitted as a part of the regression. The first response represented a highly variable rest response which followed a long period of quiescence, and was omitted to facilitate the regression. All responses were normalized to the mean magnitude of the ninth and tenth responses. Contraction amplitudes increased significantly after the third pulse in the pulse train when compared relative to the response to the second pulse (panel A). The solid regression line demonstrated that amplitude of contraction increased over the first half of the train, and then remained constant. Panel B illustrates the relationship between Ca\textsuperscript{2+} transient amplitudes and beat number for the nine measured pulses from two to ten. Amplitudes of Ca\textsuperscript{2+} transients only increased slightly at the end of the train, but did not increase significantly with respect to the second pulse in the train. There was no clear relationship between Ca\textsuperscript{2+} transient amplitude and contraction. However, as shown in panel C, diastolic intracellular [Ca\textsuperscript{2+}] increased significantly after the third pulse. The regression line for diastolic [Ca\textsuperscript{2+}] appeared to increase in parallel with the regression for mean amplitudes of contractions. Peak systolic Ca\textsuperscript{2+} also appeared to increase in parallel with increasing contraction amplitude (data not shown).

These data suggest that changes in contraction amplitudes are more closely related to changes in diastolic [Ca\textsuperscript{2+}] than amplitudes of Ca\textsuperscript{2+} transients. To examine this, the data from figure 13 were re-plotted as shown in figure 14. Panel A of figure 14 shows the relationship between the amplitude of contraction and the amplitude of the Ca\textsuperscript{2+} transient. Panel B shows the relationship between the amplitude of contraction and diastolic [Ca\textsuperscript{2+}]. Amplitudes of contractions showed no relationship to amplitudes of
Figure 13: Positive staircases of contraction occurred in parallel with increasing diastolic [Ca$^{2+}$], not increasing Ca$^{2+}$ transient amplitude. Protocol as in 12 A. Data were normalized to the means of the ninth and tenth responses. The first response was omitted. A. Mean contraction amplitudes increased with increasing pulse number, when compared to the amplitude of the contraction elicited by the second pulse. B. Mean Ca$^{2+}$ transient amplitudes did not differ significantly throughout the train of pulses. C. Mean diastolic [Ca$^{2+}$] increased significantly from the second pulse to the ninth pulse. n = 9 cells, * indicates p < 0.05 as tested by one-way RM-ANOVA, and Bonferroni post-hoc test when the response to pulse 2 was used as control.
Figure 14: Amplitudes of contraction showed no relationship to Ca\(^{2+}\) transient amplitude, but showed a direct linear correlation to diastolic Ca\(^{2+}\). A. The mean contraction-Ca\(^{2+}\) transient amplitude relationship was determined from the data shown in Figure 13, and a linear first order polynomial regression line was fitted to the data. The standard errors of the means of contraction and Ca\(^{2+}\) transient amplitude are expressed as the bi-directional error bars. Increasing contraction amplitude showed no relationship to Ca\(^{2+}\) transient amplitude. B. The mean contraction-diastolic Ca\(^{2+}\) relationship was determined from the data presented in Figure 13. Data were fitted with a linear polynomial regression line. Contraction amplitude was directly related to diastolic Ca\(^{2+}\) \((r^2 = 0.920)\). n = 9 cells, all data are paired.
Ca\textsuperscript{2+} transients (panel A). In contrast, amplitude of contraction showed a strong direct linear relationship to diastolic [Ca\textsuperscript{2+}], as illustrated by the excellent fit of the linear regression line to the data ($r^2 = 0.92$) in panel B. These results indicate that the amplitude of contraction during positive staircases increased in parallel with increasing diastolic [Ca\textsuperscript{2+}], but not with amplitude of Ca\textsuperscript{2+} transients.

2. Role of Peak Systolic [Ca\textsuperscript{2+}], Diastolic [Ca\textsuperscript{2+}] and Ca\textsuperscript{2+} Transient Amplitude in Increasing Contraction Amplitude at Different Rates of Stimulation

In the next set of experiments the role of diastolic Ca\textsuperscript{2+} in producing positive inotropy associated with positive force-frequency relationships was examined. Myocytes were field stimulated with pulses (3-5 msec duration) delivered by silver wire electrodes and a Grass SD9 Stimulator (Grass Medical Instruments, Quincy Massachusetts USA) at rates of 0.5 to 2.0 Hz. Intracellular [Ca\textsuperscript{2+}] and cell length were recorded simultaneously. Figure 15 shows a representative example of Ca\textsuperscript{2+} transients (panel A) and cell shortening (panel B) elicited by stimulation pulses delivered at 0.5, 1.0, 1.5 and 2.0 Hz. As shown in panel A, Ca\textsuperscript{2+} transients elicited at 0.5 Hz were relatively small. When the stimulation rate was increased, Ca\textsuperscript{2+} transient amplitude also increased. Diastolic Ca\textsuperscript{2+} increased with increasing stimulation rate, as denoted by the dotted line which represents diastolic [Ca\textsuperscript{2+}] at 0.5 Hz stimulation rate. Peak systolic [Ca\textsuperscript{2+}] also increased with increased stimulation frequency. Contraction amplitude (panel B) was relatively small at 0.5 Hz stimulation rate, however, it increased as the rate of stimulation was increased, in parallel with the increase in Ca\textsuperscript{2+} transient amplitude. Panel B also shows that, as stimulation rate
Figure 15: $\text{Ca}^{2+}$ transient and contraction amplitudes in field stimulated myocytes increased with increasing rates of stimulation. Fura-2 loaded myocytes were superfused with 37°C physiological buffer (Table 1) containing 2.0 mM $\text{Ca}^{2+}$. A. Representative records of $\text{Ca}^{2+}$ transients elicited by 3-5 msec field stimulation pulses at rates from 0.5 to 2.0 Hz (left to right) in a single cell. The dashed line denotes diastolic $\text{Ca}^{2+}$ at 0.5 Hz. $\text{Ca}^{2+}$ transient amplitude, peak systolic $\text{Ca}^{2+}$ and diastolic $\text{Ca}^{2+}$ increased with increasing stimulation rate. B. Representative cell-shortening records recorded simultaneously with fluorescence records shown in part A. The myocyte was stimulated at rates from 0.5 to 2.0 Hz. The dashed line denotes diastolic cell length during stimulation at 0.5 Hz. Contraction amplitude increased with increasing rate of stimulation, while diastolic cell length decreased.
increased, there was a marked decrease in diastolic cell length (dotted line denotes
diastolic cell length at 0.5 Hz).

Mean amplitudes of contraction and mean intracellular [Ca$^{2+}$] during the positive
force-frequency relationship are shown in figure 16. Responses were measured once
contraction and intracellular [Ca$^{2+}$] had reached steady state for each stimulation rate.
Amplitude of contraction increased significantly when stimulation rate was increased
(panel A). The dashed line is a second order polynomial regression to the data, with an $r^2$
value of 0.998, indicating that the function describes the data well. Contraction
amplitude increased with increased stimulation rate. Panel B illustrates the relationship
between Ca$^{2+}$ transient amplitudes and stimulation rate. Amplitudes of Ca$^{2+}$ transients
increased when the rate of stimulation was increased. The second order polynomial
regression to the data shows that Ca$^{2+}$ transient amplitude increased in parallel with
increasing contraction amplitude. As shown in panel C, systolic Ca$^{2+}$ also increased with
increasing rate of stimulation and the peak systolic [Ca$^{2+}$] shows a direct relationship to
the rate of stimulation. The increase in peak systolic [Ca$^{2+}$] nearly was linear, although
the dotted line and $r^2$ value shown in panel C of figure 16 indicate the second order
polynomial regression to the data. Panel D shows the relationship between diastolic Ca$^{2+}$
and increasing stimulation rate. As stimulation rate increased, diastolic [Ca$^{2+}$] increased
however, the increase in diastolic Ca$^{2+}$ appeared to occur at lower stimulation frequencies
and then to approach a maximum at 2.0 Hz stimulation rate. In contrast, peak systolic
Ca$^{2+}$, amplitudes of Ca$^{2+}$ transients and amplitudes of contractions all continued to
increase with increasing rates of stimulation. Although Ca$^{2+}$ transient amplitude, systolic
[Ca$^{2+}$] and diastolic [Ca$^{2+}$] all increased in response to increases in stimulation frequency,
Figure 16: Amplitudes of contractions and Ca\textsuperscript{2+} transients increased in parallel as stimulation rate was increased. Contraction and Ca\textsuperscript{2+} transient amplitudes were measured once they had reached steady state following a change in rate. Mean data expressed as stimulation rate-response curves. Second order polynomial regression lines were fitted to the data and are represented by dashed lines. A. Mean cell shortening, expressed as a percentage of cell length. Contraction amplitudes increased as stimulation rate was increased. \( r^2 \) value for regression is 0.998. B. Mean Ca\textsuperscript{2+} transient amplitude increased exponentially with increasing stimulation rate, and in parallel with increasing amplitudes of contraction (\( r^2 = 0.995 \)). C. Mean peak systolic Ca\textsuperscript{2+} increased with increasing extracellular Ca\textsuperscript{2+} (\( r^2 = 0.997 \)). D. Mean diastolic Ca\textsuperscript{2+} increased with increasing stimulation rate, however it did not increase in parallel with contraction or transient amplitudes, and showed a trend towards saturation (\( r^2 = 0.999 \)). \( n = 8 \), * denotes \( p < 0.05 \) when responses at other simulation rates were compared to the response at the 1.0 Hz stimulation rate by one-way RM-ANOVA and Bonferroni post-hoc test.
the changes in contraction amplitudes appear to be most closely related to changes in Ca\textsuperscript{2+} transient amplitudes.

These data suggest that the changes in contraction amplitudes that occur in response to increases in rates of stimulation are closely related to increases in Ca\textsuperscript{2+} transient amplitude which also occur in response to increases in rates of stimulation. To further examine the relationships between contraction amplitude and Ca\textsuperscript{2+} transient amplitude, systolic [Ca\textsuperscript{2+}] and diastolic [Ca\textsuperscript{2+}], the mean data were re-plotted as shown in figure 17. Panel A shows that there is a direct relationship between the amplitude of contraction and Ca\textsuperscript{2+} transient amplitude. The dashed line indicates the linear regression to the data, which showed that there was a good linear correlation between increasing contraction amplitude and Ca\textsuperscript{2+} transient amplitude during positive force-frequency relationships. Panel B also shows a direct linear relationship between amplitude of contraction and peak systolic [Ca\textsuperscript{2+}], with a similarly strong correlation. Panel C shows that, although a linear relationship exists between increasing contraction amplitude and diastolic [Ca\textsuperscript{2+}] when stimulation rate is increased, the correlation does not appear to be as strong as that for either Ca\textsuperscript{2+} transient amplitude or peak systolic [Ca\textsuperscript{2+}]. These results indicate that amplitude of contraction during positive force-frequency relationships shows a close relationship to Ca\textsuperscript{2+} transient amplitude and peak systolic [Ca\textsuperscript{2+}]. At low stimulation frequencies diastolic Ca\textsuperscript{2+} also may contribute to the positive inotropy associated with positive force-frequency relationships, although, as shown in figure 16, diastolic Ca\textsuperscript{2+} appeared to approach a maximum as stimulation rate was increased. Amplitudes of contraction, Ca\textsuperscript{2+} transients and systolic Ca\textsuperscript{2+} all continued to increase as stimulation rate was increased.
Figure 17: As stimulation rate increased, contraction amplitudes showed direct linear correlations to Ca\textsuperscript{2+} transient amplitude and peak systolic Ca\textsuperscript{2+}. The relationships between the mean amplitude of contraction and Ca\textsuperscript{2+} transient amplitude, systolic Ca\textsuperscript{2+} and diastolic Ca\textsuperscript{2+} were determined for the mean data expressed in figure 16 (n = 8, all data are paired). Dashed lines represent first order polynomial regression to the data. A. Cell shortening showed a strong direct relationship to Ca\textsuperscript{2+} transient amplitude ($r^2 = 0.984$). B. Cell shortening also was directly linearly related to peak systolic Ca\textsuperscript{2+} ($r^2 = 0.952$). C. Cell shortening showed a weaker correlation to diastolic Ca\textsuperscript{2+} ($r^2 = 0.824$).
3. Inotropic Effects of Changing Extracellular [Ca\textsuperscript{2+}]

A. Effect of increased extracellular [Ca\textsuperscript{2+}] on resting cell length and diastolic Ca\textsuperscript{2+} concentration.

Elevation of extracellular [Ca\textsuperscript{2+}] is a positively inotropic stimulus. Previously, elevation of extracellular Ca\textsuperscript{2+} has been shown to elevate the diastolic [Ca\textsuperscript{2+}] in myocytes undergoing repetitive stimulation (Frampton et al., 1991b). The influence of elevated extracellular Ca\textsuperscript{2+} on intracellular [Ca\textsuperscript{2+}] and cell length in resting myocytes, however, has not been investigated. Therefore, experiments were undertaken to determine whether extracellular [Ca\textsuperscript{2+}] could alter diastolic intracellular [Ca\textsuperscript{2+}] in resting myocytes, in the absence of electrical stimulation and whether this would affect diastolic cell length. In these experiments, cells were voltage clamped and held at a holding potential of -80 mV. Myocytes were voltage clamped to maintain the membrane potential at a constant, known, voltage, and also to reduce the number of myocytes that became spontaneously active. Myocytes were stimulated with a train of voltage clamp pulses following impalement to ensure viability prior to measurement of resting intracellular [Ca\textsuperscript{2+}].

Initially cells were superfused with 2.0 mM [Ca\textsuperscript{2+}], then extracellular [Ca\textsuperscript{2+}] was increased to 5.0 mM with the rapid solution switcher, and simultaneous recordings of cell length and intracellular [Ca\textsuperscript{2+}] were made for a minimum of five seconds at each extracellular [Ca\textsuperscript{2+}]. Figure 18, panel A, shows a representative recording of intracellular [Ca\textsuperscript{2+}] during a transition in extracellular [Ca\textsuperscript{2+}]. When extracellular [Ca\textsuperscript{2+}] was elevated, intracellular [Ca\textsuperscript{2+}] increased slowly without initiating a rapid Ca\textsuperscript{2+} transient. Panel B shows a representative recording of resting cell length during the transition in
Figure 18: Intracellular [Ca$^{2+}$] increased, and resting cell length decreased, when a voltage clamped myocyte was exposed to 5.0 mM extracellular [Ca$^{2+}$]. Simultaneous recordings of intracellular Ca$^{2+}$ and cell length from a resting myocyte voltage clamped at -80 mV. A. Representative recording of resting intracellular [Ca$^{2+}$] during a rapid increase in extracellular [Ca$^{2+}$] from 2.0 to 5.0 mM. Intracellular [Ca$^{2+}$] increased in response to elevated extracellular [Ca$^{2+}$], without generating a phasic Ca$^{2+}$ transient. B. Representative recording of resting cell length as it changed in response to a rapid increase in extracellular Ca$^{2+}$ from 2.0 to 5.0 mM.
extracellular [Ca\textsuperscript{2+}] from 2.0 to 5.0 mM. When extracellular [Ca\textsuperscript{2+}] was elevated, cell length decreased and this decrease in resting cell length mirrored the increase in intracellular [Ca\textsuperscript{2+}]. Figure 19 shows the comparison between mean resting intracellular [Ca\textsuperscript{2+}] (panel A) and resting cell length (panel B) recorded in 2.0 and 5.0 mM extracellular [Ca\textsuperscript{2+}]. Measurements of intracellular [Ca\textsuperscript{2+}] and cell length were made after they had reached a steady state following the change in extracellular [Ca\textsuperscript{2+}]. As shown in panel A, mean intracellular [Ca\textsuperscript{2+}] increased significantly in 5.0 mM extracellular [Ca\textsuperscript{2+}]. Panel B shows the mean resting cell length in 2.0 and 5.0 mM extracellular [Ca\textsuperscript{2+}]. Mean resting cell length decreased significantly when extracellular [Ca\textsuperscript{2+}] was elevated. Thus, when extracellular [Ca\textsuperscript{2+}] was elevated from 2.0 to 5.0 mM, diastolic [Ca\textsuperscript{2+}] increased significantly, and this increase in diastolic [Ca\textsuperscript{2+}] was sufficient to decrease cell length, presumably by activating the contractile myofilaments.

Elevation of extracellular Ca\textsuperscript{2+} in voltage clamped resting myocytes held at -80 mV led to a significant elevation of intracellular [Ca\textsuperscript{2+}] sufficient to produce cell shortening. To further investigate the influence of extracellular [Ca\textsuperscript{2+}] on resting intracellular [Ca\textsuperscript{2+}], the extracellular [Ca\textsuperscript{2+}] also was decreased. As described above, cells initially were superfused with 2.0 mM [Ca\textsuperscript{2+}], then extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to either 0.5 or 0.1 mM, and simultaneous recordings of cell length and intracellular [Ca\textsuperscript{2+}] were made for a minimum of five seconds at each extracellular [Ca\textsuperscript{2+}]. All myocytes were exposed to each of the three different extracellular [Ca\textsuperscript{2+}], although the order of application was varied, and the cell always was returned to control (2.0 mM) extracellular Ca\textsuperscript{2+} between applications of other extracellular [Ca\textsuperscript{2+}]. Figure 20 panel A shows the mean resting intracellular [Ca\textsuperscript{2+}] at each extracellular [Ca\textsuperscript{2+}].
Figure 19: Elevated extracellular [Ca$^{2+}$] caused an elevation in resting intracellular Ca$^{2+}$ and a decrease in resting cell length. Mean responses to elevated extracellular [Ca$^{2+}$]. n=8 cells, * denotes p< 0.05 when tested by paired Student’s t-test. A. Mean resting intracellular [Ca$^{2+}$] was significantly elevated in response to 5.0 mM extracellular [Ca$^{2+}$]. B. Mean resting cell length decreased significantly in 5.0 mM extracellular [Ca$^{2+}$].
Figure 20: When extracellular [Ca$^{2+}$] was changed from 2.0 to 0.1 and 0.5 mM in a resting myocyte, intracellular Ca$^{2+}$ decreased and cell length increased. Voltage clamped myocytes (-80 mV) were exposed to different extracellular Ca$^{2+}$ concentrations by superfusion with the rapid solution switcher (as in figures 18 & 19). A. Mean resting intracellular [Ca$^{2+}$] response to decreased extracellular [Ca$^{2+}$]. Resting intracellular [Ca$^{2+}$] decreased significantly in 0.1 and 0.5 mM extracellular [Ca$^{2+}$], when compared to the 2.0 mM control extracellular [Ca$^{2+}$]. B. Concentration-response curve for resting cell length. Resting cell length was measured following application of 0.1, 0.5 and 2.0 mM extracellular [Ca$^{2+}$]. Resting cell length increased significantly in low extracellular [Ca$^{2+}$] and when compared to 2.0 mM extracellular [Ca$^{2+}$]. n = 8, * denotes p < 0.05 when compared to 2.0 mM, as determined by one-way RM-ANOVA and Bonferroni post-hoc test.
Decreasing extracellular [Ca\textsuperscript{2+}] from 2.0 to 0.1 or 0.5 mM decreased intracellular Ca\textsuperscript{2+} significantly (one way RM-ANOVA). Panel B of figure 20 shows the mean resting cell length at each extracellular [Ca\textsuperscript{2+}]. When extracellular [Ca\textsuperscript{2+}] was decreased, resting cell length increased slightly and significantly. These responses were similar to those seen when extracellular [Ca\textsuperscript{2+}] was elevated, and resting intracellular Ca\textsuperscript{2+} increased while resting cell length decreased. Thus, decreasing extracellular [Ca\textsuperscript{2+}] can decrease intracellular [Ca\textsuperscript{2+}], and this decrease appeared to be sufficient to produce relaxation of the resting myocyte. Therefore, cardiac myocytes can generate active resting tension which is dependent on the resting intracellular [Ca\textsuperscript{2+}].

**B. Effect of changing extracellular [Ca\textsuperscript{2+}] on amplitudes of I\textsubscript{Ca-L}, contractions and Ca\textsuperscript{2+} transients.**

In the next set of experiments the role of diastolic Ca\textsuperscript{2+} in determining amplitudes of contraction when myocytes were electrically stimulated while being superfused with different concentrations of extracellular Ca\textsuperscript{2+} was examined. Panel A of figure 21 shows a schematic of the activation train, test step and test solution application. Amplitudes of contractions, I\textsubscript{Ca-L} and Ca\textsuperscript{2+} transients were recorded simultaneously in voltage clamped myocytes activated by test steps from -50 to 0 mV. Extracellular [Ca\textsuperscript{2+}] was changed from 2.0 to 0.1, 0.5 and 5.0 mM only following the CP train and throughout the test step used to elicit responses. Thus, inotropic effects may reflect changes in both diastolic [Ca\textsuperscript{2+}] prior to the test step and the driving force for I\textsubscript{Ca-L} triggered during the test step. Figure 21 also shows representative recordings of I\textsubscript{Ca-L} (panel B), Ca\textsuperscript{2+} transients (panel
Figure 21: Amplitudes of contractions and $I_{Ca,L}$ increased markedly with increasing extracellular $Ca^{2+}$, but $Ca^{2+}$ transients increased only slightly. A. Schematic representation of activation protocol. Different extracellular [Ca$^{2+}$] (0.1, 0.5, 2.0 and 5.0 mM) were applied with a computer controlled rapid solution changer for 3 seconds prior to and throughout a step from -50 to 0 mV. Solution application and test steps were preceded by ten CPs delivered at a rate of 2 Hz. Amplitudes of contractions, currents and $Ca^{2+}$ transients were recorded simultaneously. B. Representative contractions recorded in 0.1 (top), 0.5, 2.0 and 5.0 (bottom) mM extracellular [Ca$^{2+}$]. C. Representative transmembrane currents recorded in increasing extracellular [Ca$^{2+}$]. D. Representative recordings of intracellular [Ca$^{2+}$], showing the Ca$^{2+}$ transient responses to 0.1, 0.5, 2.0 and 5.0 mM extracellular [Ca$^{2+}$]. The reference line noted on all current traces represents 0 nA current.
C) and contractions (panel D) from a single cell exposed to 0.1, 0.5, 2.0 and 5.0 mM extracellular [Ca\(^{2+}\)]. When the extracellular [Ca\(^{2+}\)] was decreased from 2.0 to 0.1 and 0.5 mM, there was a decrease in the amplitudes of inward current, Ca\(^{2+}\) transients and contractions. When extracellular [Ca\(^{2+}\)] was increased from 2.0 to 5.0 mM, there was a marked increase in the peak amplitude of inward current (panel B) and there was a corresponding large increase in the amplitude of contraction (panel D). In contrast, Ca\(^{2+}\) transient amplitude increased only slightly (panel C).

Figure 22 shows mean responses to test steps during superfusion with 0.1, 0.5, 2.0 and 5.0 mM extracellular [Ca\(^{2+}\)], expressed as concentration-response curves. As shown in figure 22, panel A, contraction amplitude increased with increasing extracellular [Ca\(^{2+}\)]. Amplitude of I\(_{\text{Ca-L}}\) (panel B) also increased with increasing extracellular [Ca\(^{2+}\)], in parallel with the increase in the amplitude of contraction. The parallel increases in contraction amplitude (panel A) and I\(_{\text{Ca-L}}\) amplitude (panel B) were highlighted by the parallel second order polynomial regression lines, which are represented by dashed lines. Diastolic Ca\(^{2+}\) (panel C) and peak systolic Ca\(^{2+}\) (panel D) also increased markedly with increasing extracellular [Ca\(^{2+}\)]. Amplitudes of Ca\(^{2+}\) transients increased only slightly as extracellular [Ca\(^{2+}\)] was elevated, and this increase did not parallel the increases in the amplitudes of contraction and peak I\(_{\text{Ca-L}}\). Rather, Ca\(^{2+}\) transient amplitude appeared to saturate between 0.5 and 5.0 mM extracellular [Ca\(^{2+}\)]. In contrast, the amplitudes of contraction and I\(_{\text{Ca-L}}\) (panels A & B) continued to increase with increasing extracellular [Ca\(^{2+}\)].

To further examine the relationship between increasing contraction amplitude and I\(_{\text{Ca-L}}\), Ca\(^{2+}\) transient amplitude, diastolic Ca\(^{2+}\) and peak systolic Ca\(^{2+}\), direct relationships
Figure 22: Contraction, I_{Ca-L}, diastolic Ca^{2+} and systolic Ca^{2+} increased in parallel as extracellular Ca^{2+} was elevated, but Ca^{2+} transient amplitude did not. Activation protocol as in 21 A. Concentration-response curves for contraction amplitude (A), peak I_{Ca-L} (B), diastolic Ca^{2+} (C), peak systolic Ca^{2+} (D) and Ca^{2+} transient amplitude (E). Dotted lines represent second order regression to the mean data. Amplitudes of contraction, I_{Ca-L} and peak systolic and diastolic [Ca^{2+}] increased markedly in elevated extracellular [Ca^{2+}], and showed similar relationships to extracellular [Ca^{2+}]. Ca^{2+} transient amplitude (E) increased with increasing extracellular [Ca^{2+}], however it appeared to saturate, and did not increase in parallel with amplitudes of I_{Ca-L} or contraction. n = 5-14 cells.
between these parameters were examined. The mean data shown in panels A & B of figure 22 were used to determine the contraction-I_{Ca-L} relationship shown in panel A of figure 23. As described by the linear first order polynomial regression, contraction amplitude showed a direct relationship to increasing I_{Ca-L} when extracellular [Ca^{2+}] was elevated. Contraction amplitude also showed a similar relationship to peak systolic Ca^{2+}, as shown in panel B. The relationship between increasing contraction amplitude and increasing diastolic Ca^{2+} also was linear (panel C). The contraction amplitude-Ca^{2+} transient amplitude relationship is shown in panel D. Contraction amplitude showed no simple direct relationship to the amplitude of the Ca^{2+} transient. The r^2 value for the regression to the mean data was less than 0.8 (r^2 = 0.661), and thus, the regression line has been omitted from the figure. Therefore, the positive inotropy arising from elevation of extracellular [Ca^{2+}] showed no direct relationship to the amplitudes of Ca^{2+} transients. Increased contraction amplitude in response to increasing extracellular [Ca^{2+}], however, showed a direct relationship to increases in peak systolic Ca^{2+}, diastolic Ca^{2+} and I_{Ca-L}.

C. Effect of increasing extracellular [Ca^{2+}] on amplitudes of I_{Ca-L}, contractions and Ca^{2+} transients.

When extracellular [Ca^{2+}] was elevated from 2.0 to 5.0 mM contraction amplitude and I_{Ca-L} amplitude increased, however, Ca^{2+} transient amplitude remained constant. Thus the roles of diastolic Ca^{2+}, I_{Ca-L} and Ca^{2+} transient amplitude in the regulation of positive inotropy arising from exposure to 5.0 mM extracellular [Ca^{2+}] were examined in detail. First, the mean amplitudes of contractions and I_{Ca-L} were compared directly when
Figure 23: When extracellular [Ca$^{2+}$] was elevated, I$_{Ca-L}$ amplitude, systolic Ca$^{2+}$ and diastolic Ca$^{2+}$ showed direct relationships to increasing amplitudes of contraction. Activation protocol as in 21 A. The mean contraction-current (A), contraction-systolic Ca$^{2+}$ (B), contraction-diastolic Ca$^{2+}$ (C) and contraction-Ca$^{2+}$ transient (D) relationships were determined from the contraction-response curves in figure 10. Contraction amplitude showed direct linear correlations to current amplitude ($r^2 = 0.998$) peak systolic Ca$^{2+}$ ($r^2 = 0.997$) and diastolic Ca$^{2+}$ ($r^2 = 0.964$). Increasing contraction amplitude showed a poor correlation to Ca$^{2+}$ transient amplitude, with an $r^2$ value of less than 0.8 (panel D; $r^2 = 0.661$). n = 5-14 cells, all data are paired.
extracellular [Ca\(^{2+}\)] was elevated from 2.0 to 5.0 mM in figure 24. Panel A shows a schematic illustration of the activation protocol used to elicit responses from the myocyte. In order to elicit the responses in 2.0 and 5.0 mM extracellular [Ca\(^{2+}\)], myocytes were stimulated with a train of ten 200 msec CPs from -80 to 0 mV. The CPs were delivered at a rate of 2 Hz, and the post conditioning potential was -50 mV. Following the CP train, the extracellular [Ca\(^{2+}\)] was elevated from 2.0 to 5.0 mM for three seconds. A 250 msec test step from -50 to 0 mV was then made; the extracellular [Ca\(^{2+}\)] was elevated throughout the test step. Contraction and current were then measured simultaneously. Panel B shows representative recordings of contraction (upper) and current (lower) in a single myocyte exposed to 2.0 mM (left) and 5.0 mM (right) extracellular [Ca\(^{2+}\)]. Contraction amplitude increased when extracellular [Ca\(^{2+}\)] was increased from 2.0 to 5.0 mM for three seconds prior to and throughout the test step. Representative current amplitude also increased in 5.0 mM extracellular [Ca\(^{2+}\)]. Panels C & D show the mean contraction and current responses of 13 cells, respectively. Mean amplitude of contraction increased significantly in 5.0 mM extracellular [Ca\(^{2+}\)] when compared to 2.0 mM [Ca\(^{2+}\)]. Elevation of extracellular [Ca\(^{2+}\)] from 2.0 to 5.0 mM also increased the amplitude of I\(_{Ca-L}\) significantly. Thus, increasing extracellular Ca\(^{2+}\) was positively inotropic.

Next the role of the Ca\(^{2+}\) transient, peak systolic Ca\(^{2+}\) and diastolic Ca\(^{2+}\) in positive inotropy arising from elevated extracellular Ca\(^{2+}\) was investigated. Panel A of figure 25 shows a schematic representation of the activation protocol with which the responses were elicited. Following the CP train, extracellular Ca\(^{2+}\) was elevated for three seconds prior to and throughout the test step from -50 to 0 mV. Panel B shows
Figure 24: Elevated extracellular Ca\(^{2+}\) is positively inotropic. A. Activation protocol. Extracellular [Ca\(^{2+}\)] was increased from 2.0 to 5.0 mM for 3 seconds following the conditioning pulse train and throughout a test step from -50 to 0 mV.

B. Representative contraction and current recordings from the same cell. Responses to a step from -50 to 0 mV in 2.0 and 5.0 mM extracellular Ca\(^{2+}\) are shown. Amplitudes of contraction and current increased in elevated extracellular [Ca\(^{2+}\)]. C. Mean contraction amplitude increased significantly following a 3 sec application of elevated extracellular [Ca\(^{2+}\)]. n = 13 cells, all data are paired. D. Mean amplitude of I\(_{Ca-L}\) increased significantly in 5.0 mM extracellular [Ca\(^{2+}\)]. n = 10 cells, * denotes p<0.05 when tested by paired Student’s t-test.
A.

2.0 Hz  -50 mV  Illustrated
-80     Test Solution

B.

2.0 mM Ca^{2+}  5.0 mM Ca^{2+}

2.0 mM Ca^{2+}  5.0 mM Ca^{2+}

C.

Contraction (μm)

D.

Current (nA)

-2.0
-1.5
-1.0
-0.5
0.0

2.0 mM Ca^{2+}  5.0 mM Ca^{2+}
Figure 25: Although diastolic and systolic Ca\(^{2+}\) increase when extracellular Ca\(^{2+}\) was elevated, Ca\(^{2+}\) transient amplitude appeared to be saturated. A. Activation protocol. Extracellular [Ca\(^{2+}\)] was increased from 2.0 to 5.0 mM. B. Representative recordings of intracellular [Ca\(^{2+}\)] in a single cell on a step from -50 to 0 mV in 2.0 and 5.0 mM extracellular [Ca\(^{2+}\)]. C. Diastolic and peak systolic intracellular [Ca\(^{2+}\)] increased significantly when extracellular [Ca\(^{2+}\)] was elevated. Ca\(^{2+}\) transient amplitude did not increase when extracellular [Ca\(^{2+}\)] was elevated from 2.0 to 5.0 mM. \(n = 14\), * denotes \(p < 0.05\) when tested by paired Student's t-test.
representative Ca\(^{2+}\) transients in 2.0 and 5.0 mM extracellular [Ca\(^{2+}\)]. Intracellular [Ca\(^{2+}\)] was recorded simultaneously with the current and contraction recordings in figure 24. Although the amplitude of the representative Ca\(^{2+}\) transient increased slightly in 5.0 mM extracellular Ca\(^{2+}\), it did not increase as markedly as contraction or current amplitude did in figure 24. Mean intracellular [Ca\(^{2+}\)] is expressed in panel C of figure 25. Diastolic and peak systolic intracellular [Ca\(^{2+}\)] both increased with increasing extracellular [Ca\(^{2+}\)]. In contrast, elevation of the extracellular [Ca\(^{2+}\)] from 2.0 to 5.0 mM did not significantly affect the mean amplitudes of Ca\(^{2+}\) transients. In these experiments, increasing extracellular [Ca\(^{2+}\)] from 2.0 to 5.0 mM led to positive inotropy. Interestingly, although the amplitudes of both I\(_{\text{Ca-L}}\) and contraction were increased, the positive inotropic effect of elevated extracellular [Ca\(^{2+}\)] occurred without a corresponding increase in the amplitude of the Ca\(^{2+}\) transient.

**D. Voltage dependence of amplitudes of I\(_{\text{Ca-L}}\), contractions and Ca\(^{2+}\) transients in elevated extracellular [Ca\(^{2+}\)].**

As amplitudes of contractions, I\(_{\text{Ca-L}}\) and Ca\(^{2+}\) transients vary with the voltage of test steps to different membrane potentials, it was important to examine responses to a wide range of test step potentials. It was important to determine whether the relationship between these variables remained similar to that seen with test steps to only one potential. To that end, test steps were made from a holding potential of -60 mV, following CP trains. This activation protocol, shown as a schematic in panel A of figure 26, was repeated eight times. The test step potential was increased by 20 mV following each repetition of the protocol to initiate responses by test steps from -60 to +80 mV. This
Figure 26: Saturation of Ca\(^{2+}\) transient amplitude in 5.0 mM extracellular Ca\(^{2+}\) was not voltage dependent. A. Representative activation protocol. Extracellular [Ca\(^{2+}\)] was increased for 3 seconds prior to, and throughout the test pulse. Ten CPs delivered at a rate of 2 Hz were followed by repolarization to a holding potential of -60 mV. B. The amplitude of the mean contraction-voltage relationship was significantly increased by 5.0 mM extracellular [Ca\(^{2+}\)]. C. The mean amplitude of the current-voltage relationship was significantly elevated in elevated extracellular [Ca\(^{2+}\)]. D. Ca\(^{2+}\) transient amplitude did not increase in response to elevated extracellular [Ca\(^{2+}\)] over the range of voltages tested. E. Diastolic [Ca\(^{2+}\)] measured prior to each of the test steps increased significantly in 5.0 mM extracellular [Ca\(^{2+}\)]. n = 14 all data are paired. * indicates p < 0.05 when significance was tested with two-way RM-ANOVA and Tukey post-hoc test.
whole sequence was then repeated with the extracellular $[\text{Ca}^{2+}]$ increased to 5.0 mM for three seconds prior to and throughout the test step. Contraction, current and $\text{Ca}^{2+}$ transient amplitudes were plotted as functions of test step amplitudes to generate contraction-voltage, current-voltage, $\text{Ca}^{2+}$ transient-voltage, and diastolic $\text{Ca}^{2+}$-voltage relationships as shown in panels B to E.

Panel B of figure 26 shows that elevated extracellular $[\text{Ca}^{2+}]$ caused a significant increase in the amplitude of contraction, and that this increase was observed over a wide range of membrane potentials. Panel C shows that when extracellular $[\text{Ca}^{2+}]$ was elevated from 2.0 to 5.0 mM, peak $I_{\text{Ca-L}}$ amplitude was significantly increased at potentials from -20 to +60 mV. These contraction-voltage and current-voltage relationships show that the positive inotropy and increase in peak $I_{\text{Ca-L}}$ amplitude associated with elevated extracellular $[\text{Ca}^{2+}]$ were not voltage dependent, but rather occurred at a wide range of membrane potentials. The $\text{Ca}^{2+}$ transient-voltage relationship shown in panel D shows that, under these conditions, elevation of extracellular $[\text{Ca}^{2+}]$ did not increase $\text{Ca}^{2+}$ transient amplitude at any of the voltages tested. Thus, $\text{Ca}^{2+}$ transient amplitude appeared to saturate and did not increase in 5.0 mM extracellular $[\text{Ca}^{2+}]$. This saturation was independent of both voltage and contraction amplitude. This indicates that the saturation of $\text{Ca}^{2+}$ transient amplitude shown in figure 25 was not caused by an alteration in the voltage dependence of $\text{Ca}^{2+}$ release in response to elevated extracellular $[\text{Ca}^{2+}]$. Mean diastolic $[\text{Ca}^{2+}]$ was measured directly preceding each of the eight test steps. The diastolic $[\text{Ca}^{2+}]$ was recorded at the post CP train holding potential of -60 mV, immediately before each voltage step. For the diastolic $\text{Ca}^{2+}$-voltage relationship shown in panel E of figure 26, diastolic $[\text{Ca}^{2+}]$ was expressed as the diastolic
[Ca$^{2+}$] corresponding to the voltage to which the test step was made, although diastolic Ca$^{2+}$ was recorded prior to the test step. Diastolic [Ca$^{2+}$] increased significantly when the extracellular [Ca$^{2+}$] was elevated from 2.0 to 5.0 mM. The elevation of diastolic Ca$^{2+}$ was maintained throughout all eight steps of the protocol, and showed little rundown or variability. Peak systolic Ca$^{2+}$ also was significantly elevated when extracellular Ca$^{2+}$ was elevated (data not shown). The absence of changes in Ca$^{2+}$ transient amplitude suggests that diastolic [Ca$^{2+}$], and possibly peak systolic [Ca$^{2+}$], were important contributors to increased contraction amplitude in 5.0 mM extracellular [Ca$^{2+}$].

E. Effect of increasing extracellular [Ca$^{2+}$] on SR Ca$^{2+}$ load.

The next series of experiments was designed to determine whether a brief, three second, elevation of extracellular [Ca$^{2+}$], and the subsequent elevation in diastolic Ca$^{2+}$ levels had an effect on SR Ca$^{2+}$ content. To that end, SR Ca$^{2+}$ content was estimated by rapid application of caffeine. As in previous protocols, extracellular [Ca$^{2+}$] was increased for three seconds following the conditioning pulse train. However, rather than a test step, a one second rapid application of 10 mM caffeine was used to induce a Ca$^{2+}$ transient. Caffeine (10 mM) was applied in a nominally Na$^+$ and Ca$^{2+}$ free solution (described in METHODS), to induce release of SR contents into the cytosol. A schematic representation of this protocol is shown in panel A of figure 27. Panel B shows representative caffeine induced Ca$^{2+}$ transients. Although diastolic Ca$^{2+}$ directly preceding the caffeine application was elevated in 5.0 mM extracellular [Ca$^{2+}$], the amplitude of the caffeine-induced Ca$^{2+}$ transient was not elevated when extracellular [Ca$^{2+}$] was increased in this example. The
Figure 27: When extracellular Ca\(^{2+}\) was elevated briefly for 3 seconds following the CP train, SR Ca\(^{2+}\) load remained constant. A. Activation protocol. Extracellular [Ca\(^{2+}\)] was elevated from 2.0 to 5.0 mM for three seconds following the CP train. 10 mM caffeine was then applied in a nominally Ca\(^{2+}\) and Na\(^{+}\) free buffer for one second to assess SR Ca\(^{2+}\) load. B. Recordings of intracellular [Ca\(^{2+}\)] in the same myocyte. Representative caffeine transients were elicited following 3 sec application of either 2.0 (left) or 5.0 mM (right) extracellular [Ca\(^{2+}\)]. C. Mean data show that caffeine releasable SR stores in 5.0 mM extracellular [Ca\(^{2+}\)] did not differ significantly from stores in 2.0 mM extracellular [Ca\(^{2+}\)]. Diastolic Ca\(^{2+}\) measured directly preceding caffeine application, and peak systolic Ca\(^{2+}\) both increased significantly following application of 5.0 mM extracellular [Ca\(^{2+}\)]. n = 16, * denotes p < 0.05 when tested by paired Student’s t-test.
mean data are shown in panel C. Diastolic [Ca\textsuperscript{2+}] directly preceding the caffeine application, and the peak systolic caffeine response achieved both were significantly increased in 5.0 mM extracellular Ca\textsuperscript{2+}. The mean caffeine induced Ca\textsuperscript{2+} transient, however, was not elevated when extracellular [Ca\textsuperscript{2+}] was elevated. These data suggest that SR Ca\textsuperscript{2+} load was not significantly increased by elevation of extracellular [Ca\textsuperscript{2+}] from 2.0 to 5.0 mM for three seconds following the CP train. Thus, the positive inotropy seen under these experimental conditions occurred in the absence of changes in SR load. Also, the saturation of mean transient amplitude seen in figure 25 and figure 26 occurred in the absence of increased SR Ca\textsuperscript{2+} load.

F. Negative inotropic effect of decreasing extracellular [Ca\textsuperscript{2+}].

Increasing extracellular [Ca\textsuperscript{2+}] can lead to positive inotropy, even in the absence of changes in Ca\textsuperscript{2+} transient amplitude. To further investigate the role of extracellular [Ca\textsuperscript{2+}] in inotropic responses, contraction, $I_{C_{a-L}}$ and Ca\textsuperscript{2+} transient amplitude were recorded simultaneously in myocytes exposed to 2.0 and 0.5 mM extracellular [Ca\textsuperscript{2+}]. Decreased extracellular [Ca\textsuperscript{2+}] should be negatively inotropic. Panel A of figure 28 shows a schematic of the activation protocol used to elicit the contraction and current responses in 2.0 and 0.5 mM extracellular [Ca\textsuperscript{2+}]. Extracellular Ca\textsuperscript{2+} was decreased for three seconds following the CP train, and then throughout a test step from -50 to 0 mV. The representative example shown in panel B was recorded from a single myocyte. The representative contraction recordings show that a decrease in extracellular [Ca\textsuperscript{2+}], from 2.0 to 0.5 mM, is negatively inotropic. The amplitude of the current elicited by a step from -50 to 0 mV also was decreased in 0.5 mM extracellular [Ca\textsuperscript{2+}]. The mean data are
Figure 28: When extracellular Ca\textsuperscript{2+} was decreased from 2.0 to 0.5 mM, contraction and current amplitudes decreased. A. Activation protocol. Extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to 0.5 mM for 3 seconds following conditioning pulse train and throughout a test step from -50 to 0 mV. B. Representative contraction and current recordings from the same cell. Amplitudes of contraction and current decreased in 0.5 mM extracellular [Ca\textsuperscript{2+}] (right) when compared to responses elicited in 2.0 mM extracellular [Ca\textsuperscript{2+}]. C. Mean contraction amplitude decreased significantly following a 3 sec application of 0.5 mM extracellular [Ca\textsuperscript{2+}]. n = 5, all data are paired. D. Mean amplitude of I_{Ca-L} decreased significantly when extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to 0.5 mM. n = 5, * denotes p < 0.05 when tested by paired Student's t-test.
plotted in panel C of figure 28. In 0.5 mM extracellular [Ca\(^{2+}\)], mean amplitudes of contraction were significantly reduced. This reduction was paralleled by a similar significant reduction in the mean of the peak amplitude of I_{Ca-L}. Thus, reduction of extracellular Ca\(^{2+}\) was negatively inotropic, and this negative inotropy may have been due to a reduction in I_{Ca-L} trigger amplitude.

To determine if the decrease in contraction amplitude was due to a decrease in Ca\(^{2+}\) transient amplitude in conjunction with the decrease in I_{Ca-L} amplitude, intracellular [Ca\(^{2+}\)] and Ca\(^{2+}\) transient amplitudes were measured in conjunction with contraction and I_{Ca-L}. Panel A of figure 29 shows a schematic representation of the activation protocol used to elicit the Ca\(^{2+}\) transients, contractions and I_{Ca-L}. Representative Ca\(^{2+}\) transient recordings, made in 2.0 mM (left) and 0.5 mM (right) extracellular [Ca\(^{2+}\)] are shown in panel B. The amplitude of the Ca\(^{2+}\) transient does not appear to be different in 0.5 mM extracellular [Ca\(^{2+}\)], although peak systolic [Ca\(^{2+}\)] and diastolic [Ca\(^{2+}\)] appear to be slightly decreased. The mean intracellular [Ca\(^{2+}\)] data are shown in panel C. Mean diastolic Ca\(^{2+}\) and mean peak systolic Ca\(^{2+}\) both were significantly decreased by a 3 sec application of 0.5 mM extracellular [Ca\(^{2+}\)]. Mean Ca\(^{2+}\) transient amplitude on a step from -50 to 0 mV, however, was not significantly different in 0.5 mM extracellular [Ca\(^{2+}\)] when compared to 2.0 mM. Thus, despite a negative inotropic influence of decreased extracellular [Ca\(^{2+}\)], Ca\(^{2+}\) transient amplitude remained constant when 0.5 mM extracellular [Ca\(^{2+}\)] was applied for three seconds prior to and throughout a test step. Thus, negative inotropy occurred in the absence of changes in Ca\(^{2+}\) transient amplitude, although peak systolic Ca\(^{2+}\) and diastolic Ca\(^{2+}\) were reduced, when extracellular was decreased from 2.0 to 0.5 mM. Also, in these experiments, Ca\(^{2+}\) transient amplitude
Figure 29: Although diastolic and peak systolic Ca\textsuperscript{2+} decreased significantly in 0.5 mM Ca\textsuperscript{2+}, Ca\textsuperscript{2+} transient amplitude remained constant. A. Activation protocol. Extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to 0.5 mM. B. Representative recordings of intracellular [Ca\textsuperscript{2+}] from a single myocyte in 2.0 (left) and 0.5 (right) mM extracellular [Ca\textsuperscript{2+}]. C. Diastolic and peak systolic intracellular [Ca\textsuperscript{2+}] decreased significantly in 0.5 mM extracellular [Ca\textsuperscript{2+}]. There was no significant difference in amplitudes of Ca\textsuperscript{2+} transients elicited in 0.5 mM extracellular [Ca\textsuperscript{2+}] when compared to those elicited in 2.0 mM extracellular [Ca\textsuperscript{2+}]. n = 8, * denotes p < 0.05 when tested by paired Student's t-test.
remained constant despite a significant reduction in $I_{Ca-L}$ amplitude.

G. Effect of decreasing extracellular [Ca$^{2+}$] on SR Ca$^{2+}$ load.

In order to further examine the negative inotropic role of decreasing extracellular Ca$^{2+}$, and to determine if decreased extracellular Ca$^{2+}$ could significantly decrease SR Ca$^{2+}$ load, the SR stores were assessed by a rapid application of 10 mM caffeine. As in previous protocols, myocytes were stimulated by a series of 10 CPs at a rate of 2 Hz. The myocyte was then held at a post conditioning potential of -65 mV, and extracellular [Ca$^{2+}$] was decreased from 2.0 to 0.5 mM for 3 sec following the CP train. This was followed by a 1.0 sec rapid application of 10 mM caffeine. A schematic representation of the activation protocol is shown in panel A of figure 30. Representative responses to rapid application of 10 mM caffeine, elicited from the same myocyte, following application of either 2.0 mM (left) or 0.5 mM (right) extracellular [Ca$^{2+}$] are shown in panel B. The representative caffeine transients elicited in 2.0 and 0.5 mM extracellular [Ca$^{2+}$] appeared to be of similar amplitude, although the diastolic baseline prior to caffeine application appeared to be lower in 0.5 mM extracellular [Ca$^{2+}$]. The mean responses are shown in panel C of figure 30. Diastolic Ca$^{2+}$ preceding caffeine application, and the peak systolic Ca$^{2+}$ in response to caffeine application both were significantly decreased in 0.5 mM extracellular [Ca$^{2+}$]. The amplitude of the caffeine induced Ca$^{2+}$ transient, which is an estimate of SR Ca$^{2+}$ content, was not significantly altered in 0.5 mM extracellular Ca$^{2+}$, indicating that SR Ca$^{2+}$ load was not altered when extracellular [Ca$^{2+}$] was decreased briefly from 2.0 to 0.5 mM. Thus, in these experiments, decreased extracellular [Ca$^{2+}$] had a negatively inotropic effect in the
Figure 30: SR Ca\(^{2+}\) load remained constant when extracellular Ca\(^{2+}\) was decreased briefly from 2.0 to 0.5 mM Ca\(^{2+}\). A. Extracellular [Ca\(^{2+}\)] was decreased briefly for three seconds from 2.0 to 0.5 mM following the CP train. Following the three second application of either 2 or 0.5 mM extracellular [Ca\(^{2+}\)], 10 mM caffeine was applied for one second. B. Recordings of intracellular [Ca\(^{2+}\)] in the same myocyte. Representative caffeine transients were elicited following 3 sec application of either 2.0 (left) or 0.5 mM (right) extracellular [Ca\(^{2+}\)]. C. Diastolic Ca\(^{2+}\) measured directly preceding caffeine application, and peak systolic Ca\(^{2+}\) both decreased significantly following application of 0.5 mM extracellular [Ca\(^{2+}\)]. However, caffeine releasable SR stores in 0.5 mM extracellular [Ca\(^{2+}\)] did not differ significantly from stores in 2.0 mM extracellular [Ca\(^{2+}\)]. n = 9, * denotes p < 0.05 when tested by paired Student’s t-test.
absence of changes in SR Ca\textsuperscript{2+} load and Ca\textsuperscript{2+} transient amplitude. Interestingly, under these experimental conditions, when SR Ca\textsuperscript{2+} load remained constant Ca\textsuperscript{2+} transient amplitude appeared to be saturated despite increased (in 5.0 mM extracellular [Ca\textsuperscript{2+}]) and decreased (in 0.5 mM extracellular [Ca\textsuperscript{2+}]) amplitude of I\textsubscript{Ca-L}. In both cases, however, peak systolic Ca\textsuperscript{2+} and diastolic Ca\textsuperscript{2+} changed with changing extracellular [Ca\textsuperscript{2+}].

In previous experiments it was determined that Ca\textsuperscript{2+} transient amplitude remained constant, despite changes in I\textsubscript{Ca-L}, when extracellular [Ca\textsuperscript{2+}] was either increased or decreased briefly following a CP train. However, in these experiments SR Ca\textsuperscript{2+} load also remained constant. When SR Ca\textsuperscript{2+} load remains constant, the quantity of Ca\textsuperscript{2+} available for release from the SR is constant, and therefore a larger trigger I\textsubscript{Ca-L} does not guarantee a larger Ca\textsuperscript{2+} release event from the SR, and similarly a smaller I\textsubscript{Ca-L} trigger does not necessarily induce a smaller Ca\textsuperscript{2+} transient. Thus, the constant SR Ca\textsuperscript{2+} load when extracellular [Ca\textsuperscript{2+}] was increased briefly for 3 sec to 5.0 mM, or decreased to 0.5 mM, could have contributed to the lack of significant variation in the amplitudes of Ca\textsuperscript{2+} transients when compared to 2.0 mM extracellular [Ca\textsuperscript{2+}]. The following set of experiments was designed to determine if SR Ca\textsuperscript{2+} content could be decreased by further reducing extracellular [Ca\textsuperscript{2+}]. To that end, the same protocol as described in panel A of figure 30 was repeated. However, extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to 0.1 mM for three seconds following the CP train. Caffeine (10 mM) then was rapidly applied for 1 sec in order to assess SR Ca\textsuperscript{2+} load.

Figure 31, panel A, shows a schematic illustration of the activation protocol used to elicit caffeine-induced Ca\textsuperscript{2+} transients. Representative recordings of caffeine transients (panel B) from a single cell were made following a 3 sec application of 2.0 and 0.1 mM
Figure 31: Decreasing the extracellular Ca\(^{2+}\) concentration to 0.1 mM led to a decrease in caffeine releasable SR Ca\(^{2+}\) stores. A. Extracellular [Ca\(^{2+}\)] was decreased from 2.0 to 0.1 mM for three seconds following the CP train. Caffeine (10 mM) was then applied for one second in a nominally Na\(^{+}\) and Ca\(^{2+}\) free solution. B. Representative caffeine transients were elicited by a one second caffeine application following application of either 2.0 (left) or 0.1 mM (right) extracellular [Ca\(^{2+}\)]. C. Mean caffeine releasable SR stores were significantly reduced following exposure to 0.1 mM extracellular [Ca\(^{2+}\)]. Diastolic Ca\(^{2+}\) and peak systolic Ca\(^{2+}\) also were significantly decreased following application of 0.1 mM extracellular [Ca\(^{2+}\)]. n = 9, * denotes p < 0.05 when tested by paired Student’s t-test.
A. 

\[ \text{-65 mV} \]

\[ \text{2.0 or 0.1 mM Ca}^{2+} \quad \text{Caffeine} \]

B. 

\[ \text{2.0 mM Ca}^{2+} \]

\[ \text{0.1 mM Ca}^{2+} \]

\[ \text{Intracellular Ca}^{2+} \text{ (nM)} \]

\[ \text{2.0 sec} \]

C. 

\[ \text{Diastolic Ca}^{2+} \quad \text{Systolic Ca}^{2+} \quad \text{Caffeine-Induced Ca}^{2+} \text{ Transient} \]

\[ \text{2.0 mM Ca}^{2+} \]

\[ \text{0.1 mM Ca}^{2+} \]

\[ \text{Intracellular [Ca] (nM)} \]

\[ \text{*} \]
extracellular [Ca\textsuperscript{2+}]. The caffeine transient elicited following application of 0.1 mM extracellular [Ca\textsuperscript{2+}] appeared to be smaller than that elicited following application of 2.0 mM extracellular [Ca\textsuperscript{2+}]. Diastolic [Ca\textsuperscript{2+}] also appeared to decrease following application of 0.1 mM extracellular [Ca\textsuperscript{2+}]. The mean diastolic Ca\textsuperscript{2+}, peak systolic Ca\textsuperscript{2+} and caffeine induced Ca\textsuperscript{2+} transient amplitude are shown in panel C. Diastolic Ca\textsuperscript{2+} and peak systolic Ca\textsuperscript{2+} were significantly decreased when extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to 0.1 mM. The mean amplitude of the caffeine induced Ca\textsuperscript{2+} transient also was significantly decreased in 0.1 mM extracellular [Ca\textsuperscript{2+}]. Thus, by decreasing the extracellular [Ca\textsuperscript{2+}] to 0.1 mM, SR Ca\textsuperscript{2+} load could be decreased by a 3 sec reduction in the extracellular [Ca\textsuperscript{2+}]. Therefore, SR Ca\textsuperscript{2+} stores were not saturated under these conditions. The gain of SR Ca\textsuperscript{2+} release should decrease when SR Ca\textsuperscript{2+} load is reduced, and therefore, the gain of SR Ca\textsuperscript{2+} should be reduced when compared to responses elicited in 2.0 and 0.5 mM extracellular [Ca\textsuperscript{2+}].

H. Effect of decreasing extracellular [Ca\textsuperscript{2+}] and SR Ca\textsuperscript{2+} content on amplitudes of contractions, I\textsubscript{Ca-L} and Ca\textsuperscript{2+} transients.

When extracellular [Ca\textsuperscript{2+}] was decreased from 2.0 to 0.1 mM for three seconds following a CP train, SR load was significantly reduced. To investigate the influence of this reduction on contraction, I\textsubscript{Ca-L}, and Ca\textsuperscript{2+} transient amplitudes, myocytes were exposed to 0.1 mM extracellular [Ca\textsuperscript{2+}] for three seconds prior to and throughout a test step from -50 to 0 mV. Panel A of figure 32 shows a schematic illustration of the activation protocol used to elicit the contraction and I\textsubscript{Ca-L} responses. Representative examples of contraction and I\textsubscript{Ca-L} in 2.0 and 0.1 mM extracellular [Ca\textsuperscript{2+}] are shown in
Figure 32: Amplitudes of contractions and peak $I_{Ca-L}$ decreased markedly when extracellular $Ca^{2+}$ was decreased to 0.1 mM.  

A. Activation protocol. Extracellular $[Ca^{2+}]$ was reduced from 2.0 to 0.1 mM for three seconds prior to and throughout a test step from -50 to 0 mV.  

B. Representative examples of contractions (top) and $I_{Ca-L}$ (bottom) from a single myocyte. Contraction and current amplitudes decreased in 0.1 mM extracellular $[Ca^{2+}]$.  

C. Mean contraction amplitude was significantly decreased on a step from -50 to 0 mV when extracellular $[Ca^{2+}]$ was reduced to 0.1 mM, $n = 6$.  

D. Amplitude of mean $I_{Ca-L}$ also was decreased significantly when extracellular $[Ca^{2+}]$ was decreased from 2.0 to 0.1 mM, $n = 5$.  

* denotes $p < 0.05$ when tested by paired Student’s t-test.
panel B. When extracellular [Ca\(^{2+}\)] was decreased from 2.0 to 0.1 mM, amplitudes of contractions and I\(_{Ca-L}\) decreased markedly. Mean cell shortening and current responses to a step from -50 to 0 mV in 2.0 or 0.1 mM extracellular Ca\(^{2+}\) are shown in panel C. Mean amplitudes of contractions and I\(_{Ca-L}\) decreased significantly in 0.1 mM extracellular [Ca\(^{2+}\)]. Thus, decreasing extracellular Ca\(^{2+}\) briefly, from 2.0 to 0.1 mM for 3 sec following the CP train reduced the amplitudes of I\(_{Ca-L}\) and contractions, as well as SR Ca\(^{2+}\) load.

The next set of experiments was designed to determine whether decreasing extracellular [Ca\(^{2+}\)] and SR Ca\(^{2+}\) load in concert would influence Ca\(^{2+}\) transient amplitude. Intracellular [Ca\(^{2+}\)] was recorded simultaneously with contraction and I\(_{Ca-L}\). The effects of decreasing extracellular [Ca\(^{2+}\)] from 2.0 to 0.1 mM for three seconds prior to and throughout a test step on intracellular [Ca\(^{2+}\)] are shown in figure 33. A schematic representation of the protocol used to elicit responses is shown in panel A. The test step from -50 to 0 mV was preceded by a series of 10 CPs at a rate of 2 Hz. Representative Ca\(^{2+}\) transient recordings are shown in panel B. The amplitude of the Ca\(^{2+}\) transient is markedly reduced in 0.1 mM extracellular Ca\(^{2+}\). Mean diastolic and peak systolic Ca\(^{2+}\) decreased significantly when extracellular Ca\(^{2+}\) was decreased (Panel C). Mean Ca\(^{2+}\) transient amplitude also decreased in 0.1 mM extracellular [Ca\(^{2+}\)] when compared to 2.0 mM (Panel C, right). Thus, when both extracellular Ca\(^{2+}\) and SR Ca\(^{2+}\) content are reduced amplitudes of contractions, I\(_{Ca-L}\) and Ca\(^{2+}\) transients all decreased in parallel. The reduced SR Ca\(^{2+}\) load would have reduced the gain of SR Ca\(^{2+}\) release, and paired with the reduction in I\(_{Ca-L}\) amplitude, the reduction in the gain of SR Ca\(^{2+}\) release was sufficient for Ca\(^{2+}\) transient amplitude to decrease. In these experiments a reduction in
Figure 33: Ca²⁺ transient amplitude, diastolic Ca²⁺ and systolic Ca²⁺ decreased in 0.1 mM extracellular Ca²⁺. A. Activation protocol. Extracellular [Ca²⁺] was reduced from 2.0 to 0.1 mM. B. Representative recordings of intracellular [Ca²⁺]. Ca²⁺ transient amplitude decreased significantly in reduced extracellular [Ca²⁺]. C. Mean amplitude of Ca²⁺ transients decreased when extracellular [Ca²⁺] was reduced to 0.1 mM. n = 9, * denotes p < 0.05 when tested by paired Student’s t-test.
SR Ca\(^{2+}\) content appears to have been necessary for the decrease in Ca\(^{2+}\) transient amplitude to occur, as Ca\(^{2+}\) transient amplitude did not decrease when extracellular Ca\(^{2+}\) was reduced from 2.0 to 0.5 mM extracellular [Ca\(^{2+}\)], despite a similar reduction in the amplitude of I\(_{Ca-L}\).

4. Positive Inotropic Effects of Hypothermia

A. Intracellular Ca\(^{2+}\) and SR Ca\(^{2+}\) content at 24\(^{\circ}\)C.

Hypothermia is a positively inotropic stimulus, which is believed to increase SR Ca\(^{2+}\) content (Bers, 2001; Eisner & Lederer, 1980; Puglisi et al., 1996 & 1999). In the previous section of the Results it was shown that increasing extracellular Ca\(^{2+}\) was positively inotropic in the absence of changes in SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude. Hypothermia, therefore, was chosen as a situation where both SR Ca\(^{2+}\) load and Ca\(^{2+}\) transient amplitude were expected to increase, and thereby contribute to positive inotropy. First, the effects of hypothermia on SR Ca\(^{2+}\) content and diastolic [Ca\(^{2+}\)] were examined. Myocytes were superfused either with buffers warmed to 37\(^{\circ}\)C, or with buffers at room temperature, approximately 24\(^{\circ}\)C. The temperature at the position in the bath where recordings were made was measured in several experiments. The mean physiological temperature was determined to be 35 ± 0.13\(^{\circ}\)C, while mean hypothermic temperature was determined to be 24 ± 0.15\(^{\circ}\)C.

First, the effects of hypothermia on caffeine-releasable SR Ca\(^{2+}\) stores were compared to SR Ca\(^{2+}\) stores recorded from myocytes at physiological temperatures. Voltage clamped myocytes were activated with a series of five 200 msec CPs from -80 to
0 mV, delivered at a rate of 2.0 Hz. A 2.0 sec caffeine application followed 250 msec after the last CP. Myocytes were superfused with a buffer containing 145 mM Na\textsuperscript{+} and 1.0 mM Ca\textsuperscript{2+} (Table 3). Caffeine (10 mM) was applied in a nominally Na\textsuperscript{+} and Ca\textsuperscript{2+} free solution. The activation protocol for assessment of caffeine releasable SR Ca\textsuperscript{2+} stores is shown in panel A of figure 34. Figure 34, panel B, shows representative caffeine induced Ca\textsuperscript{2+} transients. Raw data were corrected for the difference in fura-2 calibration at 35 and 24\textdegree C (in detail in METHODS section). The representative caffeine induced Ca\textsuperscript{2+} transient at 24\textdegree C was much larger than that elicited from a different cell at 35\textdegree C. The mean data (panel C) show that hypothermia elevated SR stores and that peak intracellular [Ca\textsuperscript{2+}] also was significantly elevated in response to hypothermia. Diastolic Ca\textsuperscript{2+}, however, remained constant at 24\textdegree C when compared to 35\textdegree C. Thus, although diastolic [Ca\textsuperscript{2+}] remained unchanged, hypothermia increased SR Ca\textsuperscript{2+} content.

B. Contractions and Ca\textsuperscript{2+} transients at 35 and 24\textdegree C.

To further investigate the role of hypothermia in positive inotropy, guinea pig ventricular myocytes were field stimulated at a rate of 1.0 Hz. Myocytes were superfused with HEPES buffered solution with 145 mM [Na\textsuperscript{+}] and 1.0 mM [Ca\textsuperscript{2+}]. The complete composition of the buffer is described in table 1. Figure 35 shows representative Ca\textsuperscript{2+} transients (upper traces) and contractions (lower traces) elicited by field stimulation pulses at a rate of 1.0 Hz at 35 and 24 \textdegree C. The intracellular [Ca\textsuperscript{2+}] was adjusted for the difference in Fura-2 calibration between the two temperatures, and the scale bars shown in panel A apply to both panels. At physiological temperature (panel A) Ca\textsuperscript{2+} transients and contractions were small, and contraction amplitude appeared to be
Figure 34: Hypothermia increased caffeine-releasable SR Ca\(^{2+}\) stores without changing diastolic Ca\(^{2+}\). Myocytes were voltage clamped, and superfused with the buffer containing 1.0 mM Ca\(^{2+}\) and 300 \(\mu\)M lidocaine described in table 3.  

A. A two second caffeine application was preceded by a series of five 200 msec CPs at a rate of 2 Hz.  

B. Representative caffeine-induced Ca\(^{2+}\) transients in guinea pig myocytes at 35\(^\circ\)C (left) and 24\(^\circ\)C (right). Caffeine releasable Ca\(^{2+}\) stores were increased at 24\(^\circ\)C.  

C. Mean data show that caffeine-releasable Ca\(^{2+}\) stores in guinea pig myocytes were significantly elevated at 24\(^\circ\)C. Hypothermia led to an elevated peak systolic Ca\(^{2+}\) in response to caffeine application. Diastolic Ca\(^{2+}\) did not differ between the two temperatures.  

\(n = 6\), * denotes \(p < 0.05\) when tested by Student's t-test (unpaired data).
Figure 35: Hypothermia was positively inotropic. Representative Ca\(^{2+}\) transients (top) and cell shortening (bottom) recorded simultaneously from guinea pig ventricular myocytes field stimulated at a rate of 1 Hz. Myocytes were superfused with a buffer containing 1.0 mM Ca\(^{2+}\) (Table 1), and warmed either to 35°C (A.) or maintained at room temperature, approximately 24°C (B.). Hypothermia produced marked increases in amplitudes of both Ca\(^{2+}\) transients and contractions when compared to responses from myocytes at physiological temperatures.
proportional to Ca\(^{2+}\) transient amplitude. Hypothermia (panel B) produced large Ca\(^{2+}\) transients, and large contractions. The amplitudes of contractions appeared to be proportional to the amplitudes of the Ca\(^{2+}\) transients. The mean intracellular [Ca\(^{2+}\)], amplitudes of Ca\(^{2+}\) transients and contractions at 35 and 24°C are shown in figure 36. Hypothermia increased peak systolic Ca\(^{2+}\) and Ca\(^{2+}\) transient amplitudes significantly (panel A). In contrast, diastolic Ca\(^{2+}\) was not altered at 24°C when compared to 35°C. Mean contraction amplitude (panel B) was converted to fractional cell shortening, expressed as the percent change in cell length. Contraction amplitude was significantly increased in hypothermic field stimulated myocytes. Similar results were seen when myocytes were stimulated at a rate of 2.0 Hz (data not shown). Thus, hypothermia was a positively inotropic stimulus. Ca\(^{2+}\) transient amplitude, peak systolic Ca\(^{2+}\) and SR Ca\(^{2+}\) load all were elevated in response to hypothermia. Contraction amplitudes appeared to be proportional to amplitudes of Ca\(^{2+}\) transients. The positive inotropy associated with hypothermia occurred in the absence of changes in diastolic [Ca\(^{2+}\)]. Diastolic Ca\(^{2+}\) was an important contributor to positive inotropy in some situations, and appeared to contribute, at least in part, to positive inotropy arising from changes in stimulation rate, stimulation from rest, and increases in extracellular [Ca\(^{2+}\)]. However, elevation of diastolic Ca\(^{2+}\) did not occur during hypothermia-induced positive inotropy. Therefore, although elevated diastolic Ca\(^{2+}\) can contribute to positive inotropy, elevation of diastolic Ca\(^{2+}\) is not necessary for positive inotropy to occur.
Figure 36: Peak systolic Ca\(^{2+}\), and amplitudes of Ca\(^{2+}\) transients and contractions were elevated by hypothermia. A. Mean intracellular [Ca\(^{2+}\)] at 35°C and 24°C. Ca\(^{2+}\) transient amplitude and peak systolic [Ca\(^{2+}\)] show a marked and statistically significant increase in response to hypothermia. Diastolic [Ca\(^{2+}\)], however, did not differ significantly between 35°C and 24°C. B. Contraction amplitude, expressed as percentage change in cell length, was significantly increased in hypothermia. n = 10 at 24°C and 7 at 35°C, * denotes p < 0.05 when tested by Student's t-test (unpaired data).
5. Role of Reverse Mode NCX in the Increase in Diastolic Ca$^{2+}$ in Response to Elevated Extracellular [Ca$^{2+}$]

As shown in figures 19, 22, 25, 26 and 27 elevation of extracellular [Ca$^{2+}$] produces marked increases in resting and diastolic intracellular Ca$^{2+}$ concentrations. These responses occurred rapidly, in the absence of a phasic Ca$^{2+}$ transient. One potential mechanism for Ca$^{2+}$ entry in a resting myocyte would be reverse mode NCX. In order to determine if the changes in diastolic Ca$^{2+}$ which followed a 3 sec application of elevated extracellular Ca$^{2+}$ were due to NCX-induced Ca$^{2+}$ influx, reverse mode NCX was inhibited. NCX-induced Ca$^{2+}$ influx into the myocyte can be inhibited by using voltage clamp techniques with patch pipettes which do not contain Na$^+$ in the intracellular dialysis solution. When intracellular Na$^+$ is removed from the cytosol by dialysis with a Na$^+$ free intracellular solution, then exchange of extracellular Ca$^{2+}$ for intracellular Na$^+$ by NCX is inhibited. Therefore, if the protocol shown in figure 25 were repeated with patch pipette techniques to dialyze intracellular Na$^+$ then any contribution of NCX to the increase in diastolic Ca$^{2+}$ reported in figure 25 panel C could be examined. To that end, myocytes were voltage clamped with patch pipettes containing a nominally Na$^+$ free intracellular solution (full composition described in METHODS). As in figure 25, myocytes were stimulated with a series of ten 200 msec CPs delivered at a rate of 2 Hz, followed by application of either 2.0 or 5.0 mM extracellular [Ca$^{2+}$] for three seconds prior to, and throughout a test step from -50 to 0 mV. A schematic representation of the activation protocol is shown in panel A of figure 37. Representative examples of fura-2 fluorescence recordings of the responses elicited on a step from -50 to 0 mV in 2.0 and
Figure 37: Diastolic and peak systolic $\text{Ca}^{2+}$ increase with increasing extracellular $\text{Ca}^{2+}$ when reverse mode NCX is inhibited. A. Representative activation protocol. Myocytes were voltage clamped with patch pipettes containing a nominally $\text{Na}^+$ free intracellular solution. Myocytes then were stimulated with a series of 10 CPs from -80 to 0 mV at a rate of 2 Hz. Extracellular [Ca$^{2+}$] was elevated from 2.0 to 5.0 mM for three seconds prior to and throughout a test step from -50 to 0 mV. B. Representative intracellular Ca$^{2+}$ recordings from a single myocyte. Diastolic and peak systolic Ca$^{2+}$ appeared to increase with increasing extracellular [Ca$^{2+}$]. C. Mean intracellular [Ca$^{2+}$] recorded during the step from -50 to 0 mV. Diastolic and peak systolic Ca$^{2+}$ were significantly elevated in 5.0 mM extracellular [Ca$^{2+}$]. Ca$^{2+}$ transient amplitude was not significantly increased in 5.0 mM extracellular [Ca$^{2+}$] when compared to 2.0 mM. n = 5, * denotes $p < 0.05$ when tested by paired Student’s t-test.
5.0 mM extracellular $[Ca^{2+}]$ are shown in panel B of figure 37. Diastolic $Ca^{2+}$, peak systolic $Ca^{2+}$ and $Ca^{2+}$ transient amplitude all appeared to be slightly elevated in 5.0 mM extracellular $[Ca^{2+}]$. The mean data (panel C) indicate that, when the responses of five cells were examined, only diastolic and peak systolic $Ca^{2+}$ were significantly elevated, and $Ca^{2+}$ transient amplitude appeared to remain constant. Diastolic $Ca^{2+}$ was significantly elevated by a three second application of 5.0 mM extracellular $[Ca^{2+}]$, even when myocytes were voltage clamped with patch pipettes containing a nominally Na$^+$ free intracellular solution. Elevation of diastolic $Ca^{2+}$ by reverse mode NCX, therefore, is not likely to be responsible for the elevated diastolic $Ca^{2+}$ that preceded test steps made from -50 to 0 mV in 5.0 mM extracellular $[Ca^{2+}]$. It is likely that the increase in diastolic $Ca^{2+}$ caused by an increase in the extracellular $[Ca^{2+}]$ is attributable to some mechanism other than reverse mode NCX, such as a $Ca^{2+}$ window current, enhanced SR $Ca^{2+}$ leak or reduced extrusion of cytosolic $Ca^{2+}$. 
CHAPTER 4: DISCUSSION

1. Overview

Changes in the amplitude of $I_{Ca,L}$ and SR Ca$^{2+}$ load are important mediators of positive inotropy in the heart (Eisner et al., 2000; Janczewski et al., 2000; Trafford et al., 2001; reviewed in Bers, 2001). These effects are mediated through an influence on the amplitude of the Ca$^{2+}$ transient (Eisner et al., 2000; Janczewski et al., 2000; Trafford et al., 2001; reviewed in Bers, 2001), which in turn is believed to be the primary determinant of contraction amplitude (Bers, 2001). Diastolic intracellular [Ca$^{2+}$] also has been shown to contribute to positive inotropy during staircases (Hattori et al., 1991). In addition, diastolic Ca$^{2+}$ has been reported to be elevated in other situations where positive inotropy arises, such as positive-force frequency relationships (Frampton et al., 1991a; 1991b) and in elevated extracellular [Ca$^{2+}$] (Frampton et al., 1991b). However, other studies have shown that positive inotropy also can arise in the absence of changes in diastolic Ca$^{2+}$ (duBell & Houser, 1989; Janczewski et al., 2000). Therefore, the contribution of diastolic Ca$^{2+}$ to positive inotropy remains unclear.

The overall objective of this thesis, therefore, was to examine factors that influence inotropy in isolated cardiac ventricular myocytes. It was hypothesized that diastolic Ca$^{2+}$ would make an important contribution to positive inotropy in ventricular myocytes isolated from guinea pig hearts. Specifically, this thesis examined the role of diastolic Ca$^{2+}$ in determining the amplitudes of contractions during four different interventions which are understood to produce positive inotropy. First, the role of
diastolic Ca\(^{2+}\) in positive inotropy was examined when myocytes were stimulated following a period of rest; second, when the stimulation rate was altered; third, in hypothermic myocytes; and finally, when extracellular [Ca\(^{2+}\)] was elevated. The role of diastolic Ca\(^{2+}\) in the negative inotropic response associated with reduced extracellular [Ca\(^{2+}\)] also was investigated.

The results of this study demonstrate that diastolic Ca\(^{2+}\) can play an important role in the determination of contraction amplitude. Previous studies which have demonstrated similar findings have often done so with hypothermic myocyte preparations. The results of this thesis showed that, at physiological temperatures, diastolic Ca\(^{2+}\) influenced contraction amplitude in the absence changes in Ca\(^{2+}\) transient amplitude. Specifically, elevated diastolic Ca\(^{2+}\) contributed significantly to positive inotropy in myocytes that were stimulated following a rest period. Diastolic Ca\(^{2+}\) also contributed to positive inotropy when extracellular [Ca\(^{2+}\)] was elevated, and to negative inotropy when extracellular [Ca\(^{2+}\)] was decreased. Elevated diastolic Ca\(^{2+}\) also elevated peak systolic Ca\(^{2+}\), and may have contributed to positive inotropy, during force-frequency relationships. In contrast, diastolic Ca\(^{2+}\) did not play a role in hypothermia-induced positive inotropy. Interestingly, when extracellular [Ca\(^{2+}\)] was elevated, Ca\(^{2+}\) transient amplitude appeared to saturate despite increased amplitude of I\(_{\text{Ca-L}}\). Although Ca\(^{2+}\) transient amplitude was saturated, the amplitude of contraction increased in increasing extracellular [Ca\(^{2+}\)]. This positive inotropic effect of elevated extracellular [Ca\(^{2+}\)] appeared to be due to an influence of extracellular [Ca\(^{2+}\)] on diastolic Ca\(^{2+}\) and thereby contraction amplitude. Therefore, the results of this study demonstrate that diastolic Ca\(^{2+}\) can play an important role in positive inotropy.
Other researchers have suggested that diastolic Ca\textsuperscript{2+} may play a role in positive inotropy. Bers (Bers, 1987; Bers, 2001) has hypothesized that elevated diastolic [Ca\textsuperscript{2+}] should be inherently positively inotropic because it necessarily increases the peak systolic [Ca\textsuperscript{2+}] and decreases the intracellular Ca\textsuperscript{2+} buffering capacity. Little experimental evidence, however, for a role of diastolic Ca\textsuperscript{2+} in positive inotropy has been reported. The data presented in the RESULTS section of this thesis demonstrate positive inotropy arising from elevation of diastolic [Ca\textsuperscript{2+}]. As discussed in later sections of this discussion, the role of diastolic Ca\textsuperscript{2+} in positive inotropy may be mediated by the effects of diastolic Ca\textsuperscript{2+} on the peak systolic Ca\textsuperscript{2+} level achieved.

2. Positive Inotropy in Response to Classical Stimuli

The contribution of diastolic Ca\textsuperscript{2+} to increasing amplitudes of contraction was examined in three different situations where positive inotropy had been reported in other studies. The three classically positively inotropic stimuli investigated were: positive inotropic staircases of contraction from rest (Hattori et al., 1991; duBell & Houser, 1989), the positive force-frequency relationship (Frampton et al., 1991a; 1991b), and hypothermia induced positive inotropy (Puglisi et al., 1996; 1999). Initially, it was established that these stimuli were indeed positively inotropic. Second, the contributions of diastolic Ca\textsuperscript{2+} and Ca\textsuperscript{2+} transient amplitude to increasing contraction amplitude were investigated in each of these situations.
A. Positive inotropic staircases of contraction.

When myocytes were stimulated by a series of rectangular depolarization pulses following a four second rest period, contraction amplitudes increased with increasing pulse number. When the amplitudes of Ca$^{2+}$ transients and diastolic Ca$^{2+}$ were measured, it was determined that contraction amplitude depended on diastolic Ca$^{2+}$ concentration in positive inotropic staircases of contraction. Ca$^{2+}$ transient amplitudes remained constant, while diastolic [Ca$^{2+}$] and contraction amplitudes increased in parallel over a series of pulses. These findings are in agreement with results from previous studies, which had shown positive inotropy in response to increasing diastolic [Ca$^{2+}$] during positive staircases in guinea pig myocytes (Hattori et al., 1991). In contrast, duBell and Houser (1989) reported that progressive increases in Ca$^{2+}$ transient amplitudes throughout positive staircases were the primary mediator of increasing contraction amplitude, and hypothesized that elevated SR Ca$^{2+}$ load was contributing to increased Ca$^{2+}$ transient amplitudes. In the experiments conducted as a part of this thesis, Ca$^{2+}$ transient amplitudes did not increase significantly throughout the train of stimulated pulses. Therefore, positive inotropy must have arisen due to the influence of another variable, such as diastolic Ca$^{2+}$. duBell and Houser (1989), however, did not report changes in diastolic [Ca$^{2+}$]. One explanation for the disparities between the results of previous studies and this study would be differences in the species of animal used in the experiments. duBell and Houser (1989), who did not report a role for increasing diastolic Ca$^{2+}$ in positive staircases, used feline myocytes, while all experiments conducted for this thesis used myocytes isolated from guinea pig hearts. Hattori et al. (1991) also used guinea pig myocytes, and reported that they exhibit positive staircases. Under the same
conditions, however, Hattori et al. (1991) determined that rat myocytes do not exhibit positive staircases, supporting a species-specific difference between guinea pig and other species in the mechanisms that produce positive staircases. Interestingly, both duBell and Houser (1989) and Hattori et al. (1991) elicited positive staircases from myocytes under hypothermic conditions (room temperature), and at slower stimulation frequencies (0.5 and 1.0 Hz, respectively) than those used to elicit positive staircases in this thesis. The slow stimulation rate, 0.5 Hz, used by duBell and Houser (1989) may have contributed significantly to the differences between the results shown in this thesis and the results of their study, as will be discussed later.

Therefore, this thesis shows, for the first time, that positive staircases occur in myocytes isolated from guinea pig ventricle, stimulated at 2 Hz, and superfused with buffers warmed to 37°C. During positive staircases elicited under these conditions, diastolic Ca$^{2+}$, rather than Ca$^{2+}$ transient amplitude, increased in parallel with increasing contraction amplitude. Although SR Ca$^{2+}$ load and the amplitude of I_{Ca-L} may have been changing with increasing pulse number, Ca$^{2+}$ transient amplitude did not change, and therefore, SR Ca$^{2+}$ load and I_{Ca-L} amplitude did not appear to have a direct influence on contraction amplitude. Thus, diastolic Ca$^{2+}$ appears to be a primary contributor to positive inotropy arising from staircases in guinea pig ventricular myocytes at physiological temperatures.

**B. Positive force-frequency relationship.**

When guinea pig ventricular myocytes were field stimulated at stimulation rates from 0.5 to 2.0 Hz, steady state amplitudes of contractions increased with increasing rates
of stimulation. Ca$^{2+}$ transient amplitudes, diastolic Ca$^{2+}$ and peak systolic Ca$^{2+}$ also all increased with increasing rates of stimulation. When the direct relationship between contraction amplitude and Ca$^{2+}$ transient amplitude was examined, contraction amplitudes appeared to be directly correlated to Ca$^{2+}$ transient amplitude. Contraction amplitude also was strongly correlated with increasing peak systolic Ca$^{2+}$. Diastolic Ca$^{2+}$ also increased with increasing rates of stimulation, and may have contributed to increasing amplitudes of contraction, especially at low stimulation frequencies.

Previously, increased force of contraction with increasing rates of stimulation was reported in guinea pig ventricular muscle stimulated at rates between 0.25 and 3 Hz at physiological temperature (Kurihara & Sakai, 1985; Bers, 2001). Increasing Ca$^{2+}$ transient amplitudes and elevated diastolic Ca$^{2+}$ during positive force-frequency relationships also have been reported previously in hypothermic isolated rat ventricular myocytes (Frampton et al., 1991a; 1991b). When investigating the elevation of diastolic Ca$^{2+}$ associated with the positive force-frequency relationship, Frampton et al. (1991a) determined that the elevated diastolic Ca$^{2+}$ was associated with elevation of SR Ca$^{2+}$ content. However, Frampton et al. (1991a) did not consider a direct influence of diastolic Ca$^{2+}$ on contraction amplitude. As suggested by Frampton et al. (1991a), elevation of SR load by increased stimulation frequency may be the only mechanism by which elevated diastolic Ca$^{2+}$ influences contraction amplitude during positive force-frequency relationships. However, as determined in the first part of this thesis, and supported by Hattori et al. (1991), elevated diastolic Ca$^{2+}$ can play an important role in increasing contraction amplitude when myocytes are stimulated following a period of rest. There may, therefore, also be a direct role for diastolic Ca$^{2+}$ in increasing contraction amplitude.
when rates of stimulation are increased. Increased in SR Ca\textsuperscript{2+} load may not be the only determinant of positive force-frequency relationships, but increasing diastolic Ca\textsuperscript{2+} also may contribute directly.

The results described in this thesis showed that the increase in contraction amplitude arising from increases in rate of stimulation, in guinea pig ventricular myocytes at 37°C, was directly related to an increase in the amplitudes of Ca\textsuperscript{2+} transients. Peak systolic Ca\textsuperscript{2+} increased linearly with increasing rate of stimulation. It appeared that diastolic Ca\textsuperscript{2+} contributed to increasing peak systolic Ca\textsuperscript{2+} at low stimulation frequencies, where Ca\textsuperscript{2+} transient amplitude was small. At higher stimulation frequencies, where Ca\textsuperscript{2+} transient amplitude was increasing dramatically, diastolic Ca\textsuperscript{2+} appeared to approach a maximum. Diastolic Ca\textsuperscript{2+}, therefore, did contribute to increasing peak systolic Ca\textsuperscript{2+} at low (0.5 and 1.0 Hz) stimulation frequencies, where Ca\textsuperscript{2+} transient amplitude was not changing, and could have contributed to increasing contraction amplitudes at slower stimulation frequencies as well.

C. Hypothermia-induced positive inotropy.

Guinea pig ventricular myocytes were field-stimulated at a rate of 1.0 Hz, and superfused with buffers either warmed to physiological temperatures or maintained at hypothermic (24°C) temperatures. Hypothermia induced significant positive inotropy. Amplitudes of Ca\textsuperscript{2+} transients, and peak systolic Ca\textsuperscript{2+} also were significantly increased when elicited at 24°C. SR Ca\textsuperscript{2+} stores in hypothermic myocytes were significantly elevated. In contrast, diastolic Ca\textsuperscript{2+} was not altered by hypothermia. Hypothermia is a well known positively inotropic stimulus (reviewed in Bers, 2001) which increases SR
Ca\textsuperscript{2+} content (Puglisi et al., 1996) and also increases the duration of RyR openings (Sitsapesan et al., 1991). Conversely, hypothermia has been shown to decrease I_{Ca-L} amplitude (Puglisi et al., 1999; Cavalie et al., 1985), although it also decreases the rate of inactivation of I_{Ca-L}, which may increase net Ca\textsuperscript{2+} influx (Puglisi et al., 1999).

Hypothermia also may alter myofilament Ca\textsuperscript{2+} sensitivity, although the direction and influence of that alteration is disputed (Bers, 2001; Mikane et al., 1997). Increased SR Ca\textsuperscript{2+} load and increased open time of the RyR increase the gain of SR Ca\textsuperscript{2+} release (Bers, 2001). Therefore, even in the face of reduced amplitude of I_{Ca-L}, positive inotropy in response to hypothermia would be expected. In this thesis, both contraction and Ca\textsuperscript{2+} transient amplitudes were increased by hypothermia and these increases appeared to be proportional to one another. These results suggest that there was no marked evidence of changes in myofilament Ca\textsuperscript{2+} sensitivity, although determination of myofilament Ca\textsuperscript{2+} sensitivity was not a specific objective of this study. Also, in accord with a previous report by Puglisi et al. (1996), in this thesis hypothermia increased the caffeine-releasable SR Ca\textsuperscript{2+} content. This significant increase in SR Ca\textsuperscript{2+} stores would be expected to have contributed to the increase in Ca\textsuperscript{2+} transient amplitude and to hypothermia-induced positive inotropy. Interestingly, diastolic Ca\textsuperscript{2+} was not elevated in hypothermic myocytes. Thus, elevated diastolic Ca\textsuperscript{2+} does not contribute to hypothermia induced positive inotropy.

Hypothermia was used in this thesis as a tool to investigate positive inotropy in a situation where SR Ca\textsuperscript{2+} load and the gain of SR Ca\textsuperscript{2+} release were expected to be elevated (Eisner & Lederer, 1980; Puglisi et al., 1996). As hypothesized, hypothermia caused dramatic changes in SR Ca\textsuperscript{2+} load and release when the responses of hypothermic
myocytes were compared to those of myocytes superfused with buffers at physiological temperatures. Ca$^{2+}$ transient amplitudes, peak systolic Ca$^{2+}$ and SR Ca$^{2+}$ load all were significantly elevated under hypothermic conditions. The times to peak contraction and half-relaxation (data not shown) also were significantly prolonged by hypothermia. Hypothermia is a potent positively inotropic stimulus which alters Ca$^{2+}$ handling dramatically (Eisner & Lederer, 1980; Puglisi et al., 1996; Ferrier, 1996; Hobai & Levi, 1999). The use, therefore, of hypothermic conditions to investigate other forms of inotropy may fail to show differences in SR Ca$^{2+}$ loading or in the gain of SR Ca$^{2+}$ release, as both are artificially elevated under hypothermic conditions. Further increases in SR Ca$^{2+}$ load or the gain of SR Ca$^{2+}$ release may be restricted due to the elevations already induced by hypothermia. Finally, some reports indicate that there may be a temperature-sensitive component of EC-coupling (Ferrier, 1996; Hobai & Levi, 1999), which can influence SR Ca$^{2+}$ release and contraction at physiological temperature, but that is inhibited by hypothermia. Therefore, several potential mediators of positive inotropy, including SR Ca$^{2+}$ load and the gain of SR Ca$^{2+}$ release may not be available to change in response to the intervention of interest when myocytes are studied at hypothermic temperatures.

In this thesis, when myocytes were stimulated from rest, or when rates of stimulation were elevated, positive inotropy occurred. In both situations, diastolic Ca$^{2+}$ was elevated when positive inotropy occurred, even when Ca$^{2+}$ transient amplitude was not changing. Interestingly, when myocytes were field stimulated at a rate of 1 Hz at 35 and 24°C there was no significant difference in the diastolic Ca$^{2+}$ levels. Amplitudes of Ca$^{2+}$ transients and the influence of the amplitude of the Ca$^{2+}$ transient on peak systolic
Ca\textsuperscript{2+}, therefore, were the primary determinants of contraction amplitude during hypothermia-induced positive inotropy. Thus, although elevated diastolic Ca\textsuperscript{2+} can contribute to positive inotropy during positively inotropic stimuli, and appears to be the primary contributor to increasing contraction amplitudes during positive staircases, elevated diastolic Ca\textsuperscript{2+} is not necessary for positive inotropy to occur.

3. Inotropic Effects of Changing Extracellular [Ca\textsuperscript{2+}]

A. Changing extracellular [Ca\textsuperscript{2+}] in a resting myocyte.

When extracellular Ca\textsuperscript{2+} was elevated, resting intracellular [Ca\textsuperscript{2+}] levels increased in guinea pig myocytes voltage clamped at -80 mV. These observations agree with the findings of Frampton et al. (1991b), who showed that diastolic [Ca\textsuperscript{2+}] increased in response to elevated extracellular [Ca\textsuperscript{2+}] in field stimulated rat myocytes. In contrast, Janczewski et al. (2000) did not report changes in diastolic [Ca\textsuperscript{2+}] in response to elevated extracellular [Ca\textsuperscript{2+}] in rat myocytes voltage clamped with patch pipettes, and dialyzed with EGTA. However, changes in intracellular [Ca\textsuperscript{2+}] may have been masked in these latter experiments because EGTA would be expected to buffer intracellular Ca\textsuperscript{2+}. In the experiments described in this thesis, intracellular dialysis was minimized and intracellular [Ca\textsuperscript{2+}] was not buffered by EGTA. In this thesis, the increase in resting intracellular Ca\textsuperscript{2+} which occurred in response to elevated extracellular [Ca\textsuperscript{2+}] also was accompanied by a decrease in resting cell length.

In this study, reduction of extracellular [Ca\textsuperscript{2+}] in resting ventricular myocytes voltage clamped at -80 mV caused the myocytes to lengthen significantly. The increase
in resting myocyte length induced by reduction of extracellular [Ca\(^{2+}\)] was accompanied by a significant decrease in resting intracellular [Ca\(^{2+}\)]. This finding is supported by the work of other researchers, who have shown that cardiac myocytes exhibit active resting tension (Sollott et al., 1996; Clusin, 1980). Sollott et al. (1996) demonstrated that active actin-myosin dependent resting tension exists in intact rat and guinea pig cardiac myocytes by applying 2,3-butanedione monoxime, which reduces actin-myosin interactions, to resting myocytes. Sollott et al. (1996) showed that application of 2,3-butanedione monoxime increased resting cell length in the absence of changes in intracellular [Ca\(^{2+}\)]. They also showed that cell length could be increased through application of Ca\(^{2+}\) free extracellular solution, or by chelating intracellular Ca\(^{2+}\) (Sollott et al., 1996). Therefore, as shown in this thesis, and by Sollott et al. (1996), resting myocytes exhibit active, Ca\(^{2+}\)-dependent, resting tension and resting [Ca\(_i\)] is sufficient to generate resting tension in intact myocytes.

In this thesis, active Ca\(^{2+}\) dependent resting tension was shown in ventricular myocytes superfused with buffers containing 2.0 mM extracellular [Ca\(^{2+}\)] warmed to 37\(^\circ\)C. Cells lengthened when diastolic Ca\(^{2+}\) was reduced by reducing the extracellular [Ca\(^{2+}\)]. Interestingly, resting myocyte length decreased when diastolic Ca\(^{2+}\) increased in response to superfusion with elevated extracellular [Ca\(^{2+}\)]. This increase in tension was not the result of a phasic Ca\(^{2+}\) transient. By showing that elevated extracellular [Ca\(^{2+}\)] elevated intracellular Ca\(^{2+}\) and caused resting cell length to decrease, this thesis extends beyond the scope of the findings of Sollott et al. (1996), who determined that reduced extracellular [Ca\(^{2+}\)] could cause myocytes to lengthen. Thus the findings of this study represent a novel finding of increased active resting tension in response to elevated
diastolic Ca\(^{2+}\) in a resting myocyte. Therefore, changing extracellular [Ca\(^{2+}\)] can influence diastolic [Ca\(^{2+}\)], and thereby influence myofilament activation. The mechanism, however, by which elevation of extracellular Ca\(^{2+}\) increases resting intracellular Ca\(^{2+}\) remains unclear. Some potential explanations will be discussed later in this thesis.

**B. Effects of changing extracellular Ca\(^{2+}\) on SR load.**

When extracellular [Ca\(^{2+}\)] was increased from 2.0 to 5.0 mM, or reduced to 0.5 mM, briefly for three seconds following a CP train, SR load remained constant. Interestingly, when extracellular Ca\(^{2+}\) was reduced briefly from 2.0 to 0.1 mM following a train of CPs, caffeine-releasable SR Ca\(^{2+}\) content decreased. Other researchers have shown that reduction of extracellular [Ca\(^{2+}\)] throughout a series of stimulated pulses will increase SR Ca\(^{2+}\) load (Trafford et al., 2001). The difference between the results of this study and the observations of Trafford et al. (2001) may reflect a difference in the point at which extracellular [Ca\(^{2+}\)] was reduced, and also may reflect species-specific or temperature-dependent differences in SR Ca\(^{2+}\) loading.

Trafford et al. (2001) reduced the extracellular [Ca\(^{2+}\)] (from 1.0 to 0.2 mM) throughout a series of stimulated pulses prior to caffeine application. Trafford et al. (2001) attributed the elevated SR Ca\(^{2+}\) load to an increase in the fraction of SR Ca\(^{2+}\) taken back up into the SR from the cytosol. Trafford et al. (2001) also hypothesized that the fraction of SR Ca\(^{2+}\) exchanged and extruded from the cytosol through NCX and the SL Ca\(^{2+}\) ATPase pump on a beat-to-beat basis would be reduced in response to reduced extracellular [Ca\(^{2+}\)]. However, Trafford et al. (2001) examined SR Ca\(^{2+}\) load in rat
myocytes at room temperature. SR $\text{Ca}^{2+}$ load is elevated at room temperature (Puglisi et al., 1996), and SR $\text{Ca}^{2+}$ loading appears to be favoured due to hypothermia-induced inhibition of the $\text{Na}^+$ pump (Eisner & Lederer, 1980). The hypothermic rat myocytes investigated by Trafford et al. (2001), therefore, would be expected to have elevated SR $\text{Ca}^{2+}$ content, and might maintain SR $\text{Ca}^{2+}$ load despite a reduction in extracellular $[\text{Ca}^{2+}]$.

In this thesis, extracellular $[\text{Ca}^{2+}]$ was not reduced throughout a stimulation train, but only during the post-conditioning interval following the CP train when no stimulation was occurring. Since beats were not being triggered during the 3 sec when extracellular $[\text{Ca}^{2+}]$ was reduced, SR $\text{Ca}^{2+}$ load may have decreased due to $\text{Ca}^{2+}$ leak from the SR, which has been shown to be prominent in guinea pig myocytes (Terracciano et al., 1995). SR $\text{Ca}^{2+}$ leak may have been further enhanced by the reduced diastolic $\text{Ca}^{2+}$ levels. Furthermore, reduced extracellular $[\text{Ca}^{2+}]$ could increase the pumping efficiency for $\text{Ca}^{2+}$ out of the cytosol, by reducing the gradient against which the SL $\text{Ca}^{2+}$ ATPase must pump (Horton et al., 2002). Also, hypothermia-induced SR $\text{Ca}^{2+}$ loading (Eisner & Lederer, 1980; Puglisi et al., 1996) would not have occurred in the studies described as a part of this thesis, as myocytes were superfused with buffers warmed to physiological temperatures. Thus, Trafford et al. (2001) showed that SR $\text{Ca}^{2+}$ load was elevated in hypothermic rat myocytes continuously stimulated throughout superfusion with a reduced extracellular $[\text{Ca}^{2+}]$ (0.2 mM). In this thesis, however, superfusion of a myocyte with 0.1 mM extracellular $\text{Ca}^{2+}$ for three seconds following a CP train resulted in reduced SR $\text{Ca}^{2+}$ load. Given that guinea pig ventricular myocytes have a high rate of $\text{Ca}^{2+}$ leak from the SR (Terracciano et al., 1995), and that myocytes were not stimulated during the application of the reduced extracellular $[\text{Ca}^{2+}]$, it may not be surprising that SR $\text{Ca}^{2+}$ load
decreased when extracellular [Ca$^{2+}$] was reduced. However, the finding that reduced extracellular [Ca$^{2+}$] could reduce SR Ca$^{2+}$ load when applied for three seconds following a CP train, was a novel finding.

C. Elevation of extracellular [Ca$^{2+}$] as an inotropic intervention.

When extracellular [Ca$^{2+}$] was elevated for three seconds prior to and throughout a test step, diastolic Ca$^{2+}$ increased. Also, $I_{\text{Ca-L}}$ amplitude, contraction amplitude and systolic [Ca$^{2+}$], recorded on a test step from -50 to 0 mV, increased in 5.0 mM extracellular [Ca$^{2+}$]. However, Ca$^{2+}$ transient amplitude did not increase significantly when extracellular [Ca$^{2+}$] was elevated, and appeared to be saturated in 5.0 mM extracellular [Ca$^{2+}$]. Janczewski et al. (2000) showed that, when extracellular Ca$^{2+}$ was elevated for two seconds prior to a test step, Ca$^{2+}$ transient amplitude did not increase, despite increased amplitude of $I_{\text{Ca-L}}$. In their study, Janczewski et al. (2000) determined that the saturation of Ca$^{2+}$ transient amplitude occurred because the maximal fraction of SR Ca$^{2+}$ load available for release was already being released in control extracellular [Ca$^{2+}$]. When the amount of Ca$^{2+}$ in the SR was elevated, Ca$^{2+}$ transient amplitude did increase with increased extracellular [Ca$^{2+}$] and amplitude of $I_{\text{Ca-L}}$ (Janczewski et al., 2000). The increase in amplitudes of contractions despite apparent saturation of the amplitudes of Ca$^{2+}$ transients seen in this thesis, however, was unanticipated. Interestingly, as discussed earlier, Janczewski et al. (2000) did not report changes in diastolic [Ca$^{2+}$] in response to elevated extracellular Ca$^{2+}$ in patch pipette dialyzed rat myocytes and therefore might not have seen positive inotropy, had they measured contraction amplitudes. Thus, in intact guinea pig myocytes when intracellular dialysis is
minimized, elevated extracellular $[Ca^{2+}]$ increased the amplitude of $I_{Ca-L}$, increased peak systolic $Ca^{2+}$, and increased contraction amplitude. Contraction amplitude and peak systolic $Ca^{2+}$ increased in the absence of changes in $Ca^{2+}$ transient amplitude. Diastolic $Ca^{2+}$, however, was increased in elevated extracellular $[Ca^{2+}]$, pointing to a role for diastolic $Ca^{2+}$ in the positive inotropic effects of elevated extracellular $[Ca^{2+}]$.

The mechanism responsible for the elevation in diastolic $Ca^{2+}$ following an increase in the extracellular $[Ca^{2+}]$ was not clear. To further examine the role of elevated $Ca^{2+}$ in increasing diastolic $Ca^{2+}$, myocytes were voltage clamped with patch pipettes containing a sodium free intracellular solution to exclude the contribution of reverse mode NCX (Ferrier & Howlett, 2003) to the increase in diastolic $Ca^{2+}$ following application of elevated extracellular $[Ca^{2+}]$. Diastolic $Ca^{2+}$ increased significantly following a 3 sec application of 5.0 mM extracellular $[Ca^{2+}]$. Thus, intracellular $Ca^{2+}$ increased in response to increased extracellular $[Ca^{2+}]$ when reverse mode NCX was inhibited by the absence of intracellular Na$^+$. Therefore, although NCX-induced $Ca^{2+}$ entry can contribute to changes in intracellular $Ca^{2+}$ in some situations (Levesque et al., 1994; Blaustein & Lederer, 1999; Weisser-Thomas et al., 2003), NCX induced $Ca^{2+}$ entry is not responsible for increases in diastolic $Ca^{2+}$ in voltage clamped myocytes held at -50 mV. The increased diastolic $Ca^{2+}$, which occurs in response to elevated extracellular $[Ca^{2+}]$ must arise through a mechanism other than reverse mode NCX. Other possible mechanisms for which might lead to the elevation of diastolic $Ca^{2+}$ include L-type $Ca^{2+}$ channel window currents (Talo et al., 1990) and retention of SR leak $Ca^{2+}$ in the cytosol, and will be discussed further below.

Window currents are sustained channel openings which can occur at voltages that
fall between the steady state activation and inactivation (or availability) of the L-type Ca\(^{2+}\) channel (McDonald et al., 1994; Bers, 2001). When guinea pig ventricular myocytes are held at membrane potentials between -45 and 0 mV a subset of L-type Ca\(^{2+}\) channels will be available for activation but will not inactivate due to voltage (McDonald et al., 1994; Bers, 2001). L-type Ca\(^{2+}\) channel window current may be evident at potentials as polarized as -60 mV, and even at that voltage could be responsible for a significant Ca\(^{2+}\) entry (McDonald et al., 1994). Previously, L-type Ca\(^{2+}\) channel window current has been shown to be responsible for a sustained elevation of diastolic Ca\(^{2+}\) and sustained cell shortening in rat myocytes when small depolarizations were made from holding potentials of -45 and -50 mV (Talo et al., 1990). Ca\(^{2+}\) also may be able to enter the myocyte at less depolarized holding potentials (-60 to -40 mV) in the form of T-type Ca\(^{2+}\) channel window currents (Hirano et al., 1989). Therefore, in the experiments described in this thesis, when myocytes were held at holding potentials between -50 and -60 mV, sustained Ca\(^{2+}\) entry through T- or L-type Ca\(^{2+}\) channels is a possible mechanism by which diastolic Ca\(^{2+}\) increased.

The elevated extracellular [Ca\(^{2+}\)] also may have increased diastolic Ca\(^{2+}\) through reduced extrusion of SR Ca\(^{2+}\) leak from the cytosol. As discussed earlier, Ca\(^{2+}\) leaks from the SR of guinea pig ventricular myocytes at rest (Terracciano et al., 1995). If, however, extracellular Ca\(^{2+}\) is elevated, then a larger proportion of the Ca\(^{2+}\) lost from the SR through leak may remain in the cytosol, increasing diastolic Ca\(^{2+}\). This could occur, as elevation of extracellular [Ca\(^{2+}\)] would increase the gradient against which SL Ca\(^{2+}\) ATPase pump must work to remove Ca\(^{2+}\) from the cytosol. Also, the driving force for Ca\(^{2+}\) extrusion by NCX would be reduced in elevated extracellular [Ca\(^{2+}\)], when
compared to control extracellular $[\text{Ca}^{2+}]$ (Blaustein & Lederer, 1999). Therefore, the increase in diastolic $\text{Ca}^{2+}$ in response to elevated extracellular $\text{Ca}^{2+}$ may reflect a build up of SR leak $\text{Ca}^{2+}$ in the cytosol.

D. Negative inotropic effects of reduction of extracellular $[\text{Ca}^{2+}]$.

Extracellular $[\text{Ca}^{2+}]$ was reduced from 2.0 to 0.5 mM for 3 sec following the CP train and throughout the test step. Reduction of extracellular $[\text{Ca}^{2+}]$ decreased the amplitude of contractions. Amplitude of $I_{\text{Ca-L}}$, diastolic $\text{Ca}^{2+}$ and systolic $\text{Ca}^{2+}$ also were significantly reduced by exposure to 0.5 mM extracellular $\text{Ca}^{2+}$. The amplitude of the $\text{Ca}^{2+}$ transient, however, did not decrease in 0.5 mM extracellular $\text{Ca}^{2+}$ when compared to 2.0 mM. Other researchers have reported that decreases in extracellular $[\text{Ca}^{2+}]$ decrease amplitudes of $I_{\text{Ca-L}}$ (Trafford et al., 2001), amplitudes of $\text{Ca}^{2+}$ transients (Trafford et al., 2001; Frampton et al., 1991b), as well as contraction amplitude and diastolic $[\text{Ca}^{2+}]$ (Frampton et al., 199b). Therefore, the saturation of the $\text{Ca}^{2+}$ transient in 0.5 mM extracellular $[\text{Ca}^{2+}]$ when compared to 2.0 mM in this thesis was not expected. Rather, as SR load was unchanged, and $I_{\text{Ca-L}}$ amplitude was reduced, a reduction in $\text{Ca}^{2+}$ transient amplitude was expected. However, in this thesis the $\text{Ca}^{2+}$ transient appears to saturate between 0.5 and 5.0 mM extracellular $[\text{Ca}^{2+}]$, and amplitude does not change despite changes in the amplitude of $I_{\text{Ca-L}}$. The inotropic effects associated with the changes in extracellular $\text{Ca}^{2+}$ appear to be related to the influence of changing extracellular $\text{Ca}^{2+}$ on diastolic and peak systolic intracellular $[\text{Ca}^{2+}]$, rather than an influence on $\text{Ca}^{2+}$ transient amplitude. Where SR $\text{Ca}^{2+}$ load remains constant, $\text{Ca}^{2+}$ transient amplitude does not appear to change in the experiments performed as a part of this thesis.
As already discussed, when the extracellular [Ca\(^{2+}\)] was further reduced to 0.1 mM, SR Ca\(^{2+}\) load decreased significantly. In 0.1 mM extracellular [Ca\(^{2+}\)], amplitudes of contractions, I\(_{\text{Ca-L}}\) and Ca\(^{2+}\) transients all decreased, as well as diastolic Ca\(^{2+}\) and systolic Ca\(^{2+}\) levels. Other researchers had reported similar findings when extracellular [Ca\(^{2+}\)] was reduced (Frampton et al., 1991b; Trafford et al., 2001). In the experiments performed as a part of this thesis, however, when 0.5 mM extracellular [Ca\(^{2+}\)] was applied SR Ca\(^{2+}\) load remained constant and Ca\(^{2+}\) transient amplitude also remained constant, despite reduced I\(_{\text{Ca-L}}\) amplitude. Thus, when SR Ca\(^{2+}\) load decreased, Ca\(^{2+}\) transient amplitude also was decreased. When SR Ca\(^{2+}\) load was constant, however, Ca\(^{2+}\) transient amplitude remained constant.

These findings, together with the finding that diastolic Ca\(^{2+}\) appears to be the primary contributor to the positive inotropy arising in response to elevation of extracellular [Ca\(^{2+}\)], suggest that when the extracellular [Ca\(^{2+}\)] is altered in guinea pig ventricular myocytes the amplitudes of Ca\(^{2+}\) transients remain constant unless SR Ca\(^{2+}\) load changes. As discussed above, a reduction in SR Ca\(^{2+}\) load appears to have been necessary for Ca\(^{2+}\) transient amplitude to decrease when extracellular [Ca\(^{2+}\)] was reduced. Interestingly, other researchers have shown previously that changes in stimulation rate produce changes in SR Ca\(^{2+}\) load (Frampton et al., 1991a; 1991b), which may explain why changes in Ca\(^{2+}\) transient amplitude occurred during positive force-frequency relationships in this thesis. SR Ca\(^{2+}\) load, therefore, is not only an important determinant of Ca\(^{2+}\) transient amplitude, but also appears to be an important determinant of whether or not Ca\(^{2+}\) transient amplitude can change in response to changes in I\(_{\text{Ca-L}}\) amplitude.
4. The Shortening-Intracellular $[\text{Ca}^{2+}]$ Relationship: How diastolic $\text{Ca}^{2+}$ can contribute to positive inotropy

The relationship between the amplitude of a contraction and the intracellular $\text{Ca}^{2+}$ concentration achieved can be described by a sigmoidal relationship, similar to the force-p$\text{Ca}^{2+}$ relationship described by Bers (1987, 2001). As peak systolic intracellular $\text{Ca}^{2+}$ concentration increases, so does contraction amplitude. As indicated previously, $\text{Ca}^{2+}$ transient amplitude is generally considered to be the primary determinant of contraction amplitude (reviewed in Bers, 2001). The $\text{Ca}^{2+}$ transient amplitude is conventionally measured as the difference between the peak systolic $\text{Ca}^{2+}$ concentration and the diastolic $\text{Ca}^{2+}$ concentration. A positive diastolic $\text{Ca}^{2+}$ staircase, such as that seen during positive staircases recorded as a part of this thesis and those reported by Hattori et al. (1991), causes progressive increases in diastolic $\text{Ca}^{2+}$, which produce progressive increases in peak systolic $\text{Ca}^{2+}$. These progressive increases in peak systolic $\text{Ca}^{2+}$ can occur in the absence of changes in the $\text{Ca}^{2+}$ transient amplitude, as shown in this thesis and as indicated by Hattori et al. (1991). The findings of this thesis suggest that the peak systolic $[\text{Ca}^{2+}]$ achieved, and not the $\text{Ca}^{2+}$ transient amplitude alone, determines amplitude of contraction.

Some researchers have reported that elevation of diastolic $\text{Ca}^{2+}$ promotes $\text{Ca}^{2+}$ uptake into the SR and elevates SR $\text{Ca}^{2+}$ load (Frampton et al., 1991a; 1991b). When SR $\text{Ca}^{2+}$ load is elevated, the gain of SR $\text{Ca}^{2+}$ release should be elevated (ZhuGe et al., 1999; Sitsapesan & Williams, 1994; Gyorke & Gyorke, 1998; Xu & Meissner, 1998; Bers, 2001), and therefore $\text{Ca}^{2+}$ transient amplitude should increase. However, as shown in this
thesis, amplitudes of Ca\textsuperscript{2+} transients did not increase significantly during positive staircases. Hattori et al. (1991) also reported that increases in Ca\textsuperscript{2+} transient amplitude did not correspond to the increase in contraction amplitude during positive staircases. Therefore, diastolic Ca\textsuperscript{2+} must contribute directly to positive inotropy, rather than by indirectly influencing SR Ca\textsuperscript{2+} uptake and load. Other researchers have not discussed diastolic staircases (duBell & Houser, 1989), or attribute their positive inotropic influence exclusively to an elevation of SR Ca\textsuperscript{2+} load (Frampton et al., 1991a; 1991b).

Diastolic Ca\textsuperscript{2+} may contribute to positive inotropy by reducing Ca\textsuperscript{2+} buffering within the cell. Ca\textsuperscript{2+} can be buffered by many intracellular proteins, and simply by the volume of cytosol in the myocyte (Bers, 2001). To overcome the loss of I\textsubscript{Ca-L} to Ca\textsuperscript{2+} buffering before it can reach the RyRs, the RyRs and the L-type Ca\textsuperscript{2+} channel are closely associated in the diadic cleft (Langer & Peskoff, 1997; Scriven et al., 2000). The Ca\textsuperscript{2+} transient, however, must be propagated the length and breadth of the cell to activate the myofilaments (Bers, 2001). Elevated diastolic Ca\textsuperscript{2+} may reduce the Ca\textsuperscript{2+} buffering capacity of the cell, thus allowing more of the Ca\textsuperscript{2+} released as a part of the Ca\textsuperscript{2+} transient to reach the myofilaments themselves (Bers, 2001; 1987).

In order to further investigate the relationship between cell shortening and intracellular [Ca\textsuperscript{2+}], the cell shortening-intracellular [Ca\textsuperscript{2+}] relationship in undialyzed guinea pig ventricular myocytes superfused with buffers at physiological temperatures was predicted from experimental results expressed in this thesis. The resting cell length and resting intracellular [Ca\textsuperscript{2+}] data presented in figures 19 & 20 were used to determine the initial portion of a cell shortening-intracellular [Ca\textsuperscript{2+}] relationship. The experimental cell shortening was expressed as percent shortening with respect to maximum cell length,
where maximum cell length was assumed to be the cell length in 0.1 mM extracellular [Ca$^{2+}$]. The initial portion of the relationship is shown in panel A of figure 38. The normalized cell shortening was expressed as a function of intracellular [Ca$^{2+}$], and a four parameter logistic regression was fitted to the data. The four parameter logistic regression was then extended to a hypothetical maximum of 100% cell shortening in 1 μM intracellular [Ca$^{2+}$]. This complete hypothetical relationship, based on the experimental data in panel A, is shown in panel B of figure 38. Using this relationship the roles of diastolic Ca$^{2+}$, Ca$^{2+}$ transient amplitude and peak systolic Ca$^{2+}$ in cell shortening can be examined. This curve is similar to the force-[Ca$^{2+}$] relationship described by Bers (2001; 1987).

The effects of increasing diastolic Ca$^{2+}$ on contraction amplitude are shown in figure 39. Panel A of figure 39 shows the relationship between cell shortening and intracellular [Ca$^{2+}$] when a 50 nM Ca$^{2+}$ transient is elicited. In this example, the diastolic Ca$^{2+}$ was at a “normal” 100 nM level (blue line), and peak systolic Ca$^{2+}$ was 150 nM (diastolic + transient; red line). A rightward shift in the starting point on the cell shortening - intracellular [Ca$^{2+}$] relationship occurs when diastolic [Ca$^{2+}$] is elevated (Figure 39 panel B; blue line). When the starting point on the curve is shifted right-ward, the change in intracellular Ca$^{2+}$ associated with the Ca$^{2+}$ transient is more likely to occur on the steep part of the relationship. The degree of cell shortening should, therefore, be greater in higher diastolic [Ca$^{2+}$] for the same Ca$^{2+}$ transient amplitude (50 nM), as is illustrated in figure 39. Conversely, if diastolic Ca$^{2+}$ is reduced (Panel B of figure 40), then the degree of cell shortening for the same 50 nM Ca$^{2+}$ transient will be reduced when compared to cell shortening initiated from the “normal” diastolic Ca$^{2+}$ of 100 nM.
Figure 38: As intracellular Ca\textsuperscript{2+} concentration increased cell shortening increased. The direct relationship between intracellular Ca\textsuperscript{2+} concentration and cell shortening was compared for the data shown in figure 19 & 20. A. Mean data. As intracellular Ca\textsuperscript{2+} concentration increased in response to increasing extracellular Ca\textsuperscript{2+}, cell shortening increased as well. The mean data agree with the initial portion of the theoretical curve described by Bers (1987, 2001) and can be fit with a four parameter logistic curve to give an indication of sigmoid shape. B. A point assuming that at 1000 nM intracellular Ca\textsuperscript{2+}, cell shortening will be equivalent to 100\% of maximum cell shortening was added to the mean data to extend the four parameter logistic curve. This theoretical curve shows a complete classical sigmoid concentration-response curve shape. From the theoretical curve it can be determined that cell shortening equivalent to 50\% of maximum will occur at an intracellular Ca\textsuperscript{2+} of 211 nM, and that this theoretical curve has a Hill slope of -4.38. This slope is similar to those determined in rat ventricular muscle by Gao et al. (1994).
Figure 39: Elevated diastolic Ca\textsuperscript{2+} shifted the starting point on the cell shortening - intracellular [Ca\textsuperscript{2+}] relationship rightward. A. Cell shortening response is estimated based on the theoretical curve determined from the data shown in figure 38. A. Ca\textsuperscript{2+} transient amplitude was assumed to be 50 nM (parenthesis), and diastolic Ca\textsuperscript{2+} (blue lines) 100 nM (peak systolic Ca\textsuperscript{2+} 150 nM; red lines). The degree of cell shortening is shown by the parenthesis beside the y-axis. B. Cell shortening response for the same Ca\textsuperscript{2+} transient amplitude (50 nM), but with elevated diastolic Ca\textsuperscript{2+} (approx. 130 nM). The estimated relationship suggests that the degree of cell shortening will be much greater when diastolic Ca\textsuperscript{2+} is elevated.
Figure 40: Reduced diastolic Ca\textsuperscript{2+} decreases the degree of cell shortening. A. As in figure 39, panel A. B. Reduction of diastolic Ca\textsuperscript{2+} (blue line) from 100 (Panel A) to 75 nM while Ca\textsuperscript{2+} transient amplitude remains constant (50 nM) markedly reduces the amplitude of contraction.
This study has shown that the contribution of diastolic Ca\textsuperscript{2+} to peak systolic Ca\textsuperscript{2+} was an important determinant of contraction amplitude in increased extracellular [Ca\textsuperscript{2+}], where Ca\textsuperscript{2+} transient amplitude appeared to saturate. Also, a similar dependence of increasing contraction amplitude on diastolic Ca\textsuperscript{2+} concentration exists for positive staircases of contraction. Other researchers also have shown positive inotropy in response to increasing diastolic [Ca\textsuperscript{2+}]. For example, Periyasamy et al. (2001) showed that, when rat ventricular myocytes were exposed to uremic serum, diastolic [Ca\textsuperscript{2+}], systolic [Ca\textsuperscript{2+}] and percent shortening were significantly increased. However, although Periyasamy et al. (2001) did not measure it directly, there was no apparent effect of uremic serum on Ca\textsuperscript{2+} transient amplitude. Similar effects to those seen with uremic serum were seen with the Na\textsuperscript{+} pump inhibitor ouabain (Periyasamy et al., 2001). Under the experimental conditions used by Periyasamy et al. (2001), SR Ca\textsuperscript{2+} load was not elevated by application of either uremic serum or the Na\textsuperscript{+} pump inhibitor ouabain. Inhibition of the Na\textsuperscript{+} pump leads to accumulation of intracellular Na\textsuperscript{+} and promotes elevation of cytosolic Ca\textsuperscript{2+} through reverse mode NCX. Interestingly, Bers (1987) hypothesized that blockade of the Na\textsuperscript{+} pump could lead to positive inotropy through elevation of diastolic [Ca\textsuperscript{2+}]. Diastolic Ca\textsuperscript{2+} therefore, by influencing the peak systolic [Ca\textsuperscript{2+}] achieved, can play an important role in determining contraction amplitude, and this influence of diastolic Ca\textsuperscript{2+} on inotropy is especially evident when Ca\textsuperscript{2+} transient amplitude is not changing.
5. Possible Mechanisms of Saturation of Ca\textsuperscript{2+} Transient Amplitude

In this thesis, Ca\textsuperscript{2+} transient amplitude saturated between 0.5 and 5.0 mM extracellular [Ca\textsuperscript{2+}]. There was no increase in transient amplitudes in 5.0 mM extracellular [Ca\textsuperscript{2+}], despite increased I\textsubscript{Ca-L} amplitude. There are several possible explanations that could account for the finding that Ca\textsuperscript{2+} transient amplitudes did not increase, when I\textsubscript{Ca-L} amplitude continued to increase. First, it is possible that the saturation of Ca\textsuperscript{2+} transient amplitude occurred in response to a voltage-dependent effect of elevated extracellular [Ca\textsuperscript{2+}], such as surface charge screening by elevated divalent cation concentration (Hille, 2001). However, if surface charge screening occurred, there would have been an effective hyperpolarization of the membrane at the level of the voltage sensing components of the L-type Ca\textsuperscript{2+} channels. This effective hyperpolarization would have led to a rightward shift in the voltage dependence of I\textsubscript{Ca-L} and Ca\textsuperscript{2+} transient amplitude. However, Ca\textsuperscript{2+} transient amplitudes remained saturated throughout the entire Ca\textsuperscript{2+} transient-voltage relationship, despite elevated I\textsubscript{Ca-L} amplitude. The voltage dependence of I\textsubscript{Ca-L} and Ca\textsuperscript{2+} transient amplitude did not appear to be significantly shifted in 5.0 mM extracellular [Ca\textsuperscript{2+}] and therefore saturation of Ca\textsuperscript{2+} transient amplitude in response to a voltage-dependent effect of elevated extracellular [Ca\textsuperscript{2+}] on I\textsubscript{Ca-L} or Ca\textsuperscript{2+} transient amplitude seems unlikely.

Second, it is possible that saturation arises from a saturation of the amount of SR Ca\textsuperscript{2+} available for release in response to the trigger Ca\textsuperscript{2+} current. Other researchers have shown that, when extracellular Ca\textsuperscript{2+} is elevated, Ca\textsuperscript{2+} transient amplitude does not increase, despite increased amplitude of I\textsubscript{Ca-L}, unless SR Ca\textsuperscript{2+} load also is elevated.
Janczewski et al., 2000). When SR Ca$^{2+}$ load was tested following a 3 second application of 5.0 mM extracellular [Ca$^{2+}$], SR load was not elevated. Therefore, the findings of this thesis are similar to the findings of Janczewski et al. (2000). Ca$^{2+}$ transient amplitude appeared to saturate following a brief application of elevated extracellular [Ca$^{2+}$] in both this thesis, and in the study by Janczewski et al. (2000). However, Janczewski et al. (2000) have shown that Ca$^{2+}$ transient amplitude can increase when SR Ca$^{2+}$ load is elevated. Further investigation is required in order to determine if elevation of SR Ca$^{2+}$ load in ventricular myocytes, maintained at 37°C and impaled with high resistance microelectrodes, would lead to an increase in Ca$^{2+}$ transient amplitude.

Third, it is possible that CICR could be inhibited by high diastolic [Ca$^{2+}$], and that the saturation of transient amplitude reported in this thesis may be a product of the elevated intracellular [Ca$^{2+}$] which developed during staircases and in 5.0 mM extracellular [Ca$^{2+}$]. Xu et al. (1996) have reported that CICR can be inhibited by elevated diastolic [Ca$^{2+}$]. The diastolic Ca$^{2+}$ concentrations described as having an inhibitory effect on CICR (Xu et al., 1996), however, were substantially higher than those seen in the experiments conducted as a part of this thesis. Thus, the saturation of Ca$^{2+}$ transient amplitude in response to diastolic Ca$^{2+}$-induced inhibition of CICR seems unlikely. However, Xu et al. (1996) reported that that millimolar cytosolic [Ca$^{2+}$] could inhibit RyR function in reconstituted bilayers, where recordings were made at room temperature. SR Ca$^{2+}$ handling, EC coupling and RyR properties all are significantly altered in myocytes studied at room temperature when compared to physiological temperature (Puglisi et al., 1996; Sitsapesan et al., 1991; Eisner & Lederer, 1980; Ferrier, 1996; Hobai & Levi, 1999; Puglisi et al., 1999). It follows that diastolic [Ca$^{2+}$]-induced
inhibition of CICR also could be altered by temperature, and that the RyR in intact myocytes at physiological temperature could have a different diastolic [Ca\textsuperscript{2+}] mediated inactivation profile. Therefore, although it is unlikely, elevated diastolic Ca\textsuperscript{2+} could have led to the saturation of Ca\textsuperscript{2+} transient amplitude reported in this thesis in intact guinea pig ventricular myocytes superfused with buffers warmed to 37°C.

Fourth, elevation of extracellular [Ca\textsuperscript{2+}] in either papillary muscle or single myocytes has been shown to increase spontaneous activity in both rabbit and rat cardiac preparations (Capogrossi et al., 1986). Elevation of extracellular [Ca\textsuperscript{2+}] increases the intracellular [Ca\textsuperscript{2+}], and elevated intracellular [Ca\textsuperscript{2+}] may increase spark frequency (Ferrier et al., 2003; Cheng et al., 1993). Increased spark frequency would reflect an increase in spontaneous Ca\textsuperscript{2+} release from the SR (Cheng et al., 1993). Increased spontaneous spark frequency also would lead to an increase in the number of refractory spark units at any one time (Stern & Cheng, 2004). This could decrease the gain of SR Ca\textsuperscript{2+} release. Thus, if RyR inactivation, or refractoriness, due to elevated spark frequency occurred in the experiments conducted as a part of this thesis, then Ca\textsuperscript{2+} transient amplitude may have saturated due to inactivation of a subset of RyRs (Stern & Cheng, 2004).

Finally, it is possible that saturation of local control of CICR at the level of the RyR explains the failure of transient amplitudes to increase when trigger amplitude increased. A finite number of Ca\textsuperscript{2+} release units are coupled to L-type Ca\textsuperscript{2+} channels; therefore, as the amplitude of I_{Ca-L} increases, the number of Ca\textsuperscript{2+} release units available for activation must approach a maximum. Once this maximum is reached, no further increase in Ca\textsuperscript{2+} transient amplitude should occur. This would be especially true if CICR
were an extremely high gain system, as hypothesized by Richard et al. (2003), Xiong et al. (2004) and Sjaastad et al. (2005). If CICR is a very high gain system, then Ca$^{2+}$ transient amplitude will not vary when I$_{Ca,L}$ amplitude alone is altered within a certain range of amplitudes of I$_{Ca,L}$. Diastolic Ca$^{2+}$ however could contribute to contraction amplitude by altering peak systolic Ca$^{2+}$. In this thesis, when SR load decreased, the gain of SR Ca$^{2+}$ release also may have decreased and led to the decrease in Ca$^{2+}$ transient amplitude. Thus, decreases in SR Ca$^{2+}$ load may contribute to changes in the gain of Ca$^{2+}$ release, even if CICR is a very high gain system. If, however, CICR is a sufficiently high gain system, then the gain of SR release may not be able to increase further in response to an elevation in SR Ca$^{2+}$ load, or in response to an increase in amplitude of I$_{Ca,L}$.

6. Implications of These Findings

The results of this study may be relevant to disease states where diastolic Ca$^{2+}$ is elevated. In late reperfusion, following simulated ischemia in an isolated myocyte model of ischemia and reperfusion, transient amplitudes are depressed, however diastolic [Ca$^{2+}$] is elevated (Louch et al., 2002). Elevated diastolic Ca$^{2+}$ in this situation could contribute to larger contraction amplitudes, abrogating the contractile depression associated with myocardial stunning. In general, however, prolonged periods of elevated intracellular [Ca$^{2+}$] are damaging to the heart. Studies have shown that intracellular [Ca$^{2+}$] is increased in heart disease (Yano et al., 2005) and following fetal alcohol exposure (Ren et al., 2002). In these disease states, the increased intracellular [Ca$^{2+}$] levels do not
improve contractile function (Ren et al., 2002; Yano et al., 2005). Based on the results shown in this thesis, and the observations of Periyasamy et al. (2001), we might expect that the increased diastolic $[\text{Ca}^{2+}]$ which accompanies heart failure would improve contractile function. Unfortunately, this is not the case. Rather, prolonged exposure to elevated intracellular $[\text{Ca}^{2+}]$ can activate pathways in which $\text{Ca}^{2+}$ acts as a second messenger to produce deleterious genomic effects (Dolmetsch, 1997; Berridge, 1997; Berridge et al., 2000). $\text{Ca}^{2+}$ mediated second messenger pathways such as $\text{Ca}^{2+}$-calcineurin/NFAT signaling lead to gene expression changes seen in cardiac myocytes undergoing pathological hypertrophy (Molkentin et al., 1998; Nadal-Ginard & Mahdavi, 1989). Thus, the increased diastolic $[\text{Ca}^{2+}]$ level seen in heart failure (Yano et al., 2005) is associated with deficits in contractile force generation, rather than improvements.

The results of this study also are a relevant illustration of the importance of recording not only peak systolic $\text{Ca}^{2+}$ or $\text{Ca}^{2+}$ transient values but also of reporting the changes in diastolic $\text{Ca}^{2+}$ caused by interventions. As shown in this thesis, positive inotropy can arise in the absence of changes in $\text{Ca}^{2+}$ transient amplitude. Positive inotropic effects outside increased $\text{Ca}^{2+}$ transient amplitude may be masked when transient amplitude is measured as a replacement for cell shortening data, especially when experiments are performed with $\text{Ca}^{2+}$ sensitive dyes that do not allow for quantification of diastolic $\text{Ca}^{2+}$ levels. Also, it may be important to consider the possibility that diastolic $\text{Ca}^{2+}$ levels may be artificially lowered by the use of slower, non-physiological, rates of stimulation. As noted in this thesis, diastolic $\text{Ca}^{2+}$ increases with increasing stimulation rate, and diastolic cell length decreases. When rates of 0.2 to 0.5
Hz (duBell & Houser, 1989; Trafford et al., 2001; Janczewski et al., 2000; Frampton et al., 1991a; 1991b) are used to stimulate unloaded myocytes, diastolic Ca\textsuperscript{2+} levels will be artificially low, and contraction amplitudes also may be reduced due to the leftward shift in the cell shortening - intracellular Ca\textsuperscript{2+} relationship, similar to the reduction shown in panel B of figure 40.

The results described in this thesis suggest that in some situations elevation of diastolic Ca\textsuperscript{2+}, through elevation of extracellular [Ca\textsuperscript{2+}] or following rest, can contribute to increases in contraction amplitudes in the absence of increasing Ca\textsuperscript{2+} transient amplitudes. When stimulation rate was changed, which could be considered to be a more physiological stimulus than elevated extracellular [Ca\textsuperscript{2+}] or rest, diastolic Ca\textsuperscript{2+}, peak systolic Ca\textsuperscript{2+}, Ca\textsuperscript{2+} transient and contraction amplitudes all increased in concert. Intuitively it seems more likely that under physiological conditions Ca\textsuperscript{2+} transient amplitude and its determinants (I\textsubscript{Ca-L}, gain and SR Ca\textsuperscript{2+} load) work in concert with diastolic Ca\textsuperscript{2+} to determine peak systolic Ca\textsuperscript{2+}. It would be of interest to examine the role of diastolic Ca\textsuperscript{2+} under other experimental conditions that elevate diastolic Ca\textsuperscript{2+}, catecholamine application or digitalis exposure, which might further our understanding of the physiological and pharmacological roles played by changing diastolic Ca\textsuperscript{2+} levels.

In summary, in addition to changes in SR Ca\textsuperscript{2+} load and amplitude of I\textsubscript{Ca-L}, changes in diastolic [Ca\textsuperscript{2+}] may contribute to the positive inotropic effects that occur following rest or when extracellular [Ca\textsuperscript{2+}] is elevated. Therefore, although Ca\textsuperscript{2+} transient amplitude can be an important determinant of amplitudes of contractions, the amplitude of contraction can be further modulated by the contribution of diastolic [Ca\textsuperscript{2+}] to peak systolic [Ca\textsuperscript{2+}]. By causing a right-ward shift in the cell shortening-intracellular
[Ca\(^{2+}\)] relationship start point, increases in diastolic [Ca\(^{2+}\)] contribute significantly to the positive inotropy associated with increasing extracellular [Ca\(^{2+}\)] and positive staircases. In these situations, diastolic [Ca\(^{2+}\)] was the primary contributor to positive inotropic effects.
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Appendix: PUBLICATIONS

Abstracts (Presented in poster format at the Annual Meeting of the Biophysical Society):

Shutt RH, Howlett SE & Ferrier GR. Increases in diastolic intracellular Ca ([Ca\textsubscript{i}]\textsubscript{d}) can contribute to positive inotropy in guinea pig ventricular myocytes in the absence of changes in amplitudes of Ca transients. Biophys J. 88: 319a, 2005

Ferrier GR, Shutt RH & Howlett SE. Depolarization induces continuous calcium spark activity under conditions that preserve intracellular phosphorylation pathways in mouse ventricular myocytes. Biophys J. 88: 137a, 2005


Ferrier GR, Richard MJP, Shutt RH & Howlett SE. A separate ultra-high gain CICR mechanism accounts for sigmoidal contraction-voltage relations in cardiac ventricular myocytes when phosphorylation pathways are preserved at physiologic temperature. Biophys J. 86: 65a, 2004.
Smith RH, Howlett SE & Ferrier GR. In mouse ventricular myocytes, Ca transients are decreased at room temperature compared to 37°C without significant change in SR Ca stores. Biophys J. 84: 431a, 2003.


Smith RH, Howlett SE & Ferrier GR. Extracellular Ca dependencies of the cardiac voltage sensitive release mechanism (VSRM) and Ca-induced Ca release (CICR) differ. Biophys J. 82: 67a, 2002.

Papers:


Shutt RH, Ferrier GR & Howlett SE. Increases in diastolic Ca^{2+} can contribute to positive inotropy in guinea pig ventricular myocytes in the absence of changes in amplitudes of Ca^{2+} transients (In Revision).