Frequency- and Hypertrophy-Mediated Alterations in Twitch Force and Intracellular Calcium Transients in Rat Cardiac Trabecula

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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0-612-34088-0
The purpose of this study was to investigate the underlying mechanism of the frequency (ν)- and hypertrophy-dependent alterations in the kinetics of twitch force (F) and Ca²⁺ transient. F. [Ca²⁺]ᵢ (by Fura-salt) and pHᵢ (by SNARF-AM) were measured in long and thin rat cardiac trabeculae. We found that in control rats, the rapid (~ 1 sec) ν-dependent TD shortening was not caused by the intracellular Ca²⁺-activated kinases (e.g. CaM/Kinase, PKG and PKA) nor by the rate-dependent decrease in pHᵢ. We demonstrated that the rate of force relaxation is regulated by the contractile filaments and not entirely by the intracellular Ca²⁺ handling, at low and high ν. Therefore, we propose that the mechanism responsible for the rate-dependent TD abbreviation was at the level of the contractile proteins and not the Ca²⁺ handling.

Cardiac hypertrophy caused significant decrease of F, increase of [Ca³⁺]ᵢ, and prolongation of TD, but prolongation of CaD was not statistically significant. The positive phase of the F-ν was not observed, whereas the ν-dependent linear increase in the peak [Ca²⁺]ᵢ and decrease in TD and CaD were not diminished in the hypertrophic compared to the control rats.
ACKNOWLEDGEMENT

First of all, I would like to thank my parents who never hesitated in providing the opportunities for the progress and success of their children. And also many thanks for their never-ending love and support without which I could not have gone nearly as far as I have so far.

I would like to thank my devoted supervisor, Dr. Peter Backx, whose exemplary skills and expertise guided me through the two years of my training. He also made me realize what research is truly about and what it takes to become a good scientist. My committee members, Dr. T. Parker and Dr. U. Ackermann, who were always available for consultations and whose comments and feedbacks I greatly value. thanks for every thing.

I would also like to thank everybody in the lab, Robert, Vito, Roger, Hee cheol and every one else, for making the lab such a fun and friendly working place. I would also like to thank my good friend Gavin, who helped me get over my frustrations when not everything was going my way.

Finally, I would like to thank my dear brother, Kamrouz, who always added lots of fun to my life since our childhood, and my sister and her husband, Sepideh and Payam, for the good times we had together and all the fun.
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CHAPTER 1
INTRODUCTION

1.1 CONTRACTION IN CARDIAC MUSCLE

Cardiac muscle is classified as striated muscle. The striation is due to the regular arrangement of thick (myosin) and thin (actin) contractile filaments. These contractile proteins are surrounded by organelles such as the sarcoplasmic reticulum (SR) and the mitochondria. The cardiac sarcolemma is continuous with membrane T-tubules (the transverse tubules). Electrical excitation of the surface membrane leads to an action potential which propagates as a wave of depolarization along the surface and down the T-tubule. The depolarization of the sarcolemma causes Ca\(^{2+}\) influx via the Ca\(^{2+}\) channels (\(I_{Ca}\)) (in the T-tubules) and the Na\(^{+}/Ca\(^{2+}\) exchanges (Bers. 1991). The Ca\(^{2+}\) entered via the \(I_{Ca}\) (and the Na\(^{+}/Ca\(^{2+}\) exchanger) induces Ca\(^{2+}\) release from the SR (Schulze et al., 1993). The Ca\(^{2+}\) released from the SR (as well as Ca\(^{2+}\) entering via the \(I_{CaL}\)) bind to the Ca\(^{2+}\)-binding subunit of the thin filament protein (troponin-C) thereby activating contraction. Myocyte relaxation occurs by transporting Ca\(^{2+}\) out of the cytosol, allowing dissociation of Ca\(^{2+}\) from Tn-C and relaxation of the contractile proteins.

There are four Ca\(^{2+}\) transport systems which compete for cytosolic Ca\(^{2+}\) in cardiac myocytes: 1) the SR Ca\(^{2+}\)-ATPase, 2) the sarcolemmal Na\(^{+}/Ca\(^{2+}\) exchange system, 3) the sarcolemmal Ca\(^{2+}\)-ATPase, and 4) the mitochondrial Ca\(^{2+}\) uniporter (Bassani RA. et al., 1992; Bassani JMW. et al., 1994, Bassani RA. et al., 1994). The balance of Ca\(^{2+}\) fluxes between the Ca\(^{2+}\) transport systems vary in a species-dependent manner. The SR dominates in rat ventricular myocytes, where about 92% of Ca\(^{2+}\) removal is via SR Ca\(^{2+}\)-ATPase and only 7% via Na\(^{+}/Ca\(^{2+}\) exchange during a twitch (Bers. 1997). In other species (rabbit, ferret, cat and guinea-pig) this balance varies, ranging from 70-75% SR Ca-ATPase and 25-30% Na\(^{+}/Ca\(^{2+}\) exchange (Bers. 1997). The mitochondrial Ca\(^{2+}\) uniporter and the sarcolemmal Ca\(^{2+}\) pump only handle approximately 1% of the intracellular Ca\(^{2+}\) in rat myocardium on a beat-to-beat basis under normal conditions.
1.2 \( \text{Ca}^{2+} \) CURRENT \((I_{Ca})\)

Cardiac muscle contains L and T type \( \text{Ca}^{2+} \) channels. L-type \( \text{Ca}^{2+} \) channels are sensitive to 1,4-dihydropyridines (DHPs) and are activated by large depolarizations. T type channels are insensitive to DHPs, and activation at more negative membrane potential \((E_m)\) (Bers. 1991).

L-type \( \text{Ca}^{2+} \) channels are predominant in ventricular myocytes and are responsible for the vast majority of \( \text{Ca}^{2+} \) entry in ventricular muscle, with the \( \text{Na}^+/	ext{Ca}^{2+} \) exchanger contributing a minor fraction (Bers. 1991). L-type \( \text{Ca}^{2+} \) channels are activated by depolarization, reaching a peak in \( \sim 2-7 \) msec. Inactivation of these \( \text{Ca}^{2+} \) channels is time-dependent and also depends on both \( E_m \) and \([\text{Ca}^{2+}]_i\) (Lee et al.. 1985: Hadley and Hume. 1987). The \( \text{Ca}^{2+} \)-dependence of inactivation provides a sort of feedback control to limit further \( \text{Ca}^{2+} \) entry.

1.3 SARCOPLASMIC RETICULUM \( \text{Ca}^{2+} \) ATPase PUMP

In cardiac myocytes, the SR \( \text{Ca}^{2+} \) uptake is governed by a \( \text{Ca}^{2+} \)-dependent ATPase (Berry and Bridge. 1993). Five distinct \( \text{Ca}^{2+} \) ATPase isoforms have been known among which sarcoplasmic reticulum \( \text{Ca}^{2+} \) ATPase 2a (SERCA2a) has been shown to be the major form expressed in the heart (Lompre et al.. 1994). In a proposed structural model (Taylor and Green. 1989), the SR \( \text{Ca}^{2+} \) ATPase pump is composed of four domains: 1) The transmembrane domain, which consists of 10 helices (M1-M10), four in the N-terminal quarter and six in the C-terminal quarter of the protein. The first five transmembrane helices extend into cytoplasm to form a pentahelical stalk domain. 2) A cytoplasmic \( \beta \)-strand domain between stalk sectors 2 and 3 (M2 and M3). 3) the phosphorylation domain, and 4) the nucleotide binding/hinge domain. The last two domains are between sectors 4 and 5 (M4 and M5). Site-directed mutagenesis studies have shown that the region responsible for \( \text{Ca}^{2+} \) binding and \( \text{Ca}^{2+} \) affinity is located near the center of transmembrane sequences 4, 5, 6 and 8 (M4, M5, M6 and M8) (Clarke et al.. 1989a: Clarke et al.. 1990a). This active center is referred to as the \( \text{Ca}^{2+} \)-translocation site (MacLennan and Toyofuku. 1992). The ATP-binding cleft has been found to be located between the globular-phosphorylation and the nucleotide-binding domains (Clarke et al.. 1990b: Maruyama et al.. 1988: Maruyama et al.. 1990). The cytoplasmic domains forming
the ATP catalytic site can regulate the transmembrane Ca\(^{2+}\)-binding sites (i.e. the Ca\(^{2+}\)-translocation site) and vice versa (MacLennan and Toyofuku, 1992).

In a model for Ca\(^{2+}\) translocation proposed by MacLennan (1990), Ca\(^{2+}\) has access from the cytoplasm to high-affinity Ca\(^{2+}\)-binding sites near the interior of the transmembrane sector, but cannot gain access to the lumen when the pump (also referred to as the enzyme) is in the E\(_1\) conformation. The reaction cycle of the Ca\(^{2+}\) pump begins with the binding of 1 mol of ATP and 2 mol of Ca\(^{2+}\) to form the E\(_1\)-2Ca\(^{2+}\)-ATP conformation (De Meis et al., 1979; Innesi, 1985; Innesi et al., 1990). Phosphorylation of the enzyme by ATP (E\(_1\)-2Ca\(^{2+}\)-P) initiates a process in which Ca\(^{2+}\) becomes occluded, as a result of alteration of the conformation of the protein about the bound Ca\(^{2+}\) (E\(_2\)-2Ca\(^{2+}\)-P), and then is released to the luminal side (E\(_2\)-P). In the E\(_2\) conformation, in which Ca\(^{2+}\) is released, the translocation site has very low affinity for Ca\(^{2+}\) and the site is exposed to the lumen, inaccessible from the cytoplasm. Transition from E\(_2\) back to E\(_1\) is mediated by binding of 1 mol ATP to the protein at the E\(_2\) state. This reaction cycle is reversible. In the absence of Ca\(^{2+}\), but not in its presence, the enzyme can be phosphorylated by P\(_i\) to from E\(_2\)P. The simultaneous addition of ADP and Ca\(^{2+}\) can drive the formation of E\(_1\)-P from E\(_2\)-P and result in ATP synthesis (MacLennan and Toyofuku, 1992). The residues that affect the E\(_1\)P-to-E\(_2\)P conformational change have been found to be located in the hinge domain (Vilson et al., 1991a), the β-strand domain (Anderson et al., 1989; Clarke et al., 1990c), and the stalk sectors (the cytoplasmic extension of the transmembrane domains) (Clarke et al., 1989b; Vilson et al., 1991b).

The functional activity of the cardiac SR Ca\(^{2+}\) ATPase is regulated by phospholamban (PLB). In a dephosphorylated state, PLB inhibits SERCA2a by reducing its affinity for Ca\(^{2+}\) (Edes and Kranias, 1987). It has been suggested that alteration in Ca\(^{2+}\) affinity is most likely due to an alteration in the time that the protein spends in its high-affinity E\(_1\) conformation and the time that it spends in its alternative low-affinity E\(_2\) conformation (Lytton et al., 1992; Toyofuku et al., 1992). Katz and Tada (1982) also suggested a similar mechanism for the effect of PLB on Ca\(^{2+}\) affinity. They believed that the PLB-mediated alteration in Ca\(^{2+}\) affinity of
SERCA2a was due to an alteration in the rate of $E_1P$ to $E_2P$ transition and/or the $E_2$ to $E_1$ transition. Phosphorylation of PLB (by CaM Kinase or cAMP-dependent protein kinase) relieves its inhibitory effect on the SR Ca$^{2+}$ ATPase pump, and accelerates myocardial relaxation. An increase in the rate of SR Ca$^{2+}$ uptake in cardiac myocytes could be mediated by two biochemical mechanisms, i.e. direct stimulation of SERCA2a and interaction with PLB (Berrebi-Bertrand et al., 1997).

1.4 INTERACTION OF SR Ca$^{2+}$ ATPase PUMP AND PHOSPHOLAMBAN

Phospholamban is a homopentamer made up from subunits of 52 amino acids. In a structural model for phospholamban, the monomer is composed of three domains (Simmerman et al., 1986; Fuji et al., 1987). The first 20 residues were proposed to form an amphipathic $\alpha$-helical structure (domain IA). The next 10 residues were proposed to be less structured (domain IB), and the last 22 residues were proposed to form a hydrophobic $\alpha$-helical transmembrane domain (domain II). The phosphorylation sites of PLB, at Ser$^{16}$ by cAMP-dependent protein kinase, and at Thr$^{17}$ by Ca$^{2+}$/calmodulin-dependent protein kinase reside in the cytoplasmic domain IA (Tada and Kadoma, 1989). In earlier studies it was showed that the PLB cytoplasmic interaction site with SR Ca$^{2+}$ ATPase is formed by charged and hydrophobic amino acids 1-20 (Toyofuku et al., 1994b), while the complementary SERCA2a interaction site consists of amino acids Lys$^{297}$-Val$^{402}$ (Toyofuku et al., 1994c). In later evaluation of potential transmembrane interaction sites, it was found that the inhibitory interaction site lies entirely in the transmembrane sequence of PLB and SERCA2a (Kimura et al., 1996).

Phospholamban can exist as a pentomer or a monomer. It has the mobility of a homopentamer in SDS (sodium dodecyl sulfate) gels, but it is not clear that it exists as a pentamer in the membrane (Toyofuku et al., 1994a). It has been suggested (Kimura et al., 1997) that only the PLB monomer is the functional inhibitory form, although depolymerization is not the only requirement for activation of its inhibitory effect on SR Ca$^{2+}$ ATPase pump. The next step must be formation of a PLB monomer-SERCA2a complex. followed by the formation of inhibitory interactions between specific amino acids (Kimura et al., 1997).
model for interaction of PLB with SERCA2a has been suggested by Kimura et al. (1997) based on the deduction that PLB monomers (M) are the functional species and that their dissociation from pentomers (P) is an essential step in SERCA2a (S) inhibition by PLB. The fact that PLB is about 20% depolymerized under normal conditions implies that PLB monomers are normally in relatively abundant supply. The dissociation constants for both PLB pentamer (K_{d1}) and the PLB monomer/SERCA2a heterodimer (K_{d2}) will control both the PLB monomer concentration [M] and the concentration of the monomer-inhibited form of SERCA2a [MS], defined as follows:

\[ P \leftrightarrow 5M \quad (K_{d1} = [P]/[M]^5) \quad (\text{Eq. 1}) \]

\[ M + S \leftrightarrow MS \quad (K_{d2} = [MS]/[M][S]) \quad (\text{Eq. 2}) \]

The baseline of inhibition of wild type SERCA2a by wild type PLB will be proportional to [MS], but [MS] can be altered by mutation or by physiological perturbation. Binding of Ca^{2+} to the transmembrane helices of SERCA2a (Clark et al., 1989) and phosphorylation of the cytoplasmic domain of PLB alter K_{d2}, enhancing PLB/SERCA2a heterodimer dissociation and activating SERCA2a. Kimura et al. predict that any mutation in the PLB transmembrane helix, in SERCA2a transmembrane helices, or elsewhere in PLB or SERCA2a, that would increase the affinity between M and S, would also increase [MS], leading to gain of PLB inhibitory function and to cardiomyopathy (1997).

1.5 SARCOPLASMIC RETICULUM AND INTRACELLULAR Ca^{2+} HANDLING

The role of SR in intracellular Ca^{2+} handling in ventricular myocytes has been best explained by mechanical restitution curves (MRC). The MRC is a plot of the contractile strength of a test beat as a function of the time interval between a steady-state stimulus and a subsequent test stimulus. MRC describes the time course of recovery of ventricular contractile strength following a steady-state beat (Burkhoff et al., 1984). Because contractile strength reflects the amount of calcium supplied to the myofilaments in a beat, the MRC is believed to parallel the time course by which intracellular calcium is made available for release up to the time of excitation of the cell (Edman and Johannsson, 1976; Tritthart et al., 1973). The cardiac
MRC of most mammals (e.g. rabbit and dog) rises monoexponentially to a plateau level from which there is no discernible decay up to test pulse intervals (TPIs) of 10-15 seconds (Burkhoff. 1979: Burkhoff. et al.. 1984). The MRC from rat myocardium, however, is more complex. It rises in an exponential manner with two different time constants (α and β) to the maximum at 100s (TPI). and then declines (Schouten et al., 1987).

The time course of mechanical restitution in rabbit and canine heart is well described by a two-calcium compartment model (Burkhoff et al.. 1984; Fabiato and Fabiato. 1979; Fozzard. 1977; Morad and Goldman. 1973; Tritthart et al.. 1973). In these models. the intracellular Ca\(^{2+}\) store (SR) includes two compartments. Ca\(^{2+}\) is sequestered from the cytoplasm by the uptake compartment in the longitudinal cisternae, which in turn gradually fills the release compartment in the terminal cisternae (Weir and Yue. 1986). On excitation of the cell. the release compartment empties its contents into the cytoplasm. Most of the calcium entering the cell on this same beat is not believed to contribute to its contractile strength (Antoni. 1977; Morad and Goldman. 1973). The MRC as a whole represents the time course of the transfer process of Ca\(^{2+}\) from the uptake compartment to the release compartment. The plateau level of the MRC reflects the maximal amount of activator Ca\(^{2+}\) released by the SR under the chosen experimental conditions (Burkhoff et al.. 1984). In this two-compartment model. the conversion from the uptake to the release compartment is governed by first-order kinetics (Anderson et al.. 1979):

\[
\frac{dR(t)}{dt} = \frac{1}{T} U(t) \quad \text{(Eq. 3)}
\]

where \(R(t)\) and \(U(t)\) are the contents of the release and uptake compartments as functions of time. respectively. and \(T\) is the time constant of the MRC.

The two-calcium compartment model cannot account for the complex MRC of rat myocardium. but rather require a third compartment (an exchange compartment) to account for the relatively strong contractions at TPIs of ~100 seconds (Schouten et al.. 1987). In this three-compartment model. the SR again represents the uptake and release compartments. while the third compartment is the Na\(^+\)/Ca\(^{2+}\)-exchange mechanism combined with the Na\(^+\)/K\(^+\) pump
(Schouten et al., 1987). According to this model, upon membrane depolarization (propagation of action potential) a fixed quantity of Ca$^{2+}$ enters the uptake and the exchange compartments, and a fraction of activator Ca$^{2+}$ (in the release compartment) is recirculated, i.e., it is released from the release compartment, and sequestered by the uptake compartment after interacting with the contractile proteins. A slow transport of Ca$^{2+}$ from the release compartment to the extracellular space has been generally assumed to account for the weak rested state contraction. The exchange compartment controls this slow transport in both directions. The rate constant of Ca$^{2+}$ transfer from the uptake compartment to the release compartment, between the release and the exchange compartment and from the exchange compartment to the extracellular space is $\alpha$, $\beta$, and $\gamma$, respectively. This model suggests that the amount of Ca$^{2+}$ available for release from the release compartment when an interval of $t$ has elapsed since the previous release is given by the following equation (Schouten et al., 1987):

$$R(t) = U, \times \left[ \frac{\alpha}{(\beta - \alpha)} \times (e^{-\beta t} - e^{-\alpha t}) \right] + E_{o}^* \times \left[ \frac{\beta}{(\gamma - \beta)} \times (e^{-\beta t} - e^{-\gamma t}) \right]$$  

(Eq. 4)

where, $U, o$ is the amount of Ca$^{2+}$ in the uptake compartment at $t=0$, $\alpha$ is the rate of Ca$^{2+}$ transfer from the uptake compartment to the release compartment, $\beta$ is the rate of Ca transfer between the Ca$^{2+}$ release and exchange compartments ($= \delta / V_{R}$, where $\delta$ is a constant), $\gamma$ is the rate of Ca$^{2+}$ efflux from the exchange compartment to the extracellular space, and $E_{o}^* = (V_{R} / V_{E}) \times E(t)$  

(Eq. 5)

where, $V_{R}$ is volume of the release compartment, $V_{E}$ is volume of the exchange compartment, and $E(t)$ is the amount of Ca$^{2+}$ in the exchange compartment at time $t$.

1.6 Na$^{+}$/Ca$^{2+}$ EXCHANGER SYSTEM  

Na$^{+}$/Ca$^{2+}$ exchanger system is an important determinant of the intracellular Ca$^{2+}$ handling. This exchange is an electrogenic system that exchanges 3Na$^{+}$ ions for 1Ca$^{2+}$ ion, therefore, transferring one net positive charge across the membrane, at every exchange. This protein is responsible for the removal of only 7% of the cytosolic Ca$^{2+}$ during relaxation in rat myocardium and a greater percentage in other species. The Na$^{+}$/Ca$^{2+}$ exchanger proteins are reported to be localized in the T tubular system (Frank et al., 1992; Kieval et al., 1992).
exterior sarcolemmal surfaces and on the intercalated discs (Kieval et al., 1992) of the heart cells.

\[ \text{Na}^+ / \text{Ca}^{2+} \] exchange system can modify contraction in heart muscle cells in various ways: 1) it can provide \( \text{Ca}^{2+} \) influx that could contribute to either the induction of SR \( \text{Ca}^{2+} \) release or the direct activation of the myofilaments. 2) it may extrude \( \text{Ca}^{2+} \), thereby limiting the rise in \( [\text{Ca}^{2+}]_i \) produced by \( \text{Ca}^{2+} \) current (\( I_{\text{Ca}} \)) or SR \( \text{Ca}^{2+} \) release and contributing to the \( \text{Ca}^{2+} \) transient relaxation. 3) it may also affect the balance of net \( \text{Ca}^{2+} \) fluxes and thereby alter diastolic \( [\text{Ca}^{2+}]_i \), and consequently SR \( \text{Ca}^{2+} \) content (Bers, Lederer and Berlin, 1990). The net fluxes produced by this exchanger depends on the thermodynamic driving force (i.e. \( E_m - E_{\text{Na}^+/\text{Ca}^{2+}} \), where \( E_{\text{Na}^+/\text{Ca}^{2+}} \) is the reversal potential for \( \text{Na}^+ / \text{Ca}^{2+} \) exchange and \( E_m \) is the membrane potential). the kinetic effect of the concentration of transported ions, and any catalytic effect or extrinsic modulators (e.g. phosphorylation).

Under physiological conditions where \([\text{Na}^-]_i = 5 \text{ mM.} \ [\text{Ca}^{2+}]_i = 0.1 \mu \text{M.} \ [\text{Na}^-]_o = 145 \text{ mM.} \ [\text{Ca}^{2+}]_o = 2.5 \text{ mM.} \) at \( 37^\circ \text{C.} \) \( E_{\text{Na}^+/\text{Ca}^{2+}} \) is about +40 mV. At rest, when the membrane potential is more negative than \( E_{\text{Na}^+/\text{Ca}^{2+}} \) (\( E_m = \) about -80 mV), this exchanger will provide a depolarizing current by moving the \( \text{Ca}^{2+} \) out and \( \text{Na}^- \) into the cell (\( \text{Ca}^{2+} \) efflux). When the membrane is depolarized to above this reversal potential, there will be a \( \text{Ca}^{2+} \) influx (hyperpolarizing current) via this exchange system.

1.7 MITOCHONDRIA

\( \text{Ca}^{2+} \) enters the mitochondria via a uniport system down a large electrochemical gradient set by a proton extrusion linked to the passage of electrons down the respiratory chain (Crompton, 1990). The main route of \( \text{Ca}^{2+} \) extrusion from the mitochondria is via an electroneutral (i.e. 2:1) \( \text{Na}^- / \text{Ca}^{2+} \) exchange (Crompton et al., 1976, 1990; Gunter et al., 1990; Cox et al., 1993). This exchanger is particularly active in heart, somewhat less in skeletal muscle and least active in certain non-excitable cells (e.g. liver and renal cortex) (Crompton et al., 1976; Crompton, 1985). A third nonspecific pathway can be induced under pathological
conditions at high $Ca^{2+}$ loading, namely the mitochondrial permeability transition pore. However, the role of this pore, if any, under physiological conditions is not known (Crompton et al., 1993: Bernardi et al., 1994).

Although involvement of the mitochondria in removing $Ca^{2+}$ from the cytoplasm during cardiac relaxation has been suggested (Lehinger, 1974: Carafoli, 1975: Harisson and Miller, 1984), in intact cardiac muscle Bers and Bridge (1989) showed that when both the SR $Ca^{2+}$ uptake and sarcolemmal $Na^{+}/Ca^{2+}$ exchange were inhibited, relaxation was slowed by more than one order of magnitude and was often incomplete. Thus, Bers and Bridge concluded that it is unlikely that mitochondria can compete effectively with the SR $Ca^{2+}$ pump and sarcolemmal $Na^{+}/Ca^{2+}$ exchange under physiological conditions and probably do not contribute quantitatively to normal cardiac relaxation (Bers, 1997).

The role of mitochondrial $Ca^{2+}$ ($[Ca^{2+}]_m$) in heart muscle is thought to be in coordinating ATP supply by the mitochondria with the ATP demand by myocyte contractile and metabolic activities (McCormack et al., 1990: Hansford, 1994). $[Ca^{2+}]_m$ has been shown not to change on a beat-to-beat basis in rat myocardium, but a sustained elevation of cytosolic $Ca^{2+}$ ($[Ca^{2+}]_c$) is required to significantly elevate $[Ca^{2+}]_m$ (Hansford, 1991: Miyata et al., 1991: Griffiths et al., 1997). The rise in mitochondrial $[Ca^{2+}]$ can activate the $Ca^{2+}$-dependent mitochondrial matrix enzymes: pyruvate dehydrogenase, NAD-linked isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase which will increase oxidative metabolism and thereby increase ATP production to meet increased demands (Crompton, 1990: McCormack et al., 1990: Hansford, 1991).

Upon increasing the work of myocardium, energy production by the mitochondria (oxidative phosphorylation) also increases immediately to meet the energy demands of the muscle. Experiments on intact cardiac muscle have shown that when the work was increased by increasing $[Ca^{2+}]_c$ (i.e. by increasing $[Ca^{2+}]_o$ or the pacing frequency), $[NADH]_m$ (carrier of a reducing agent in the mitochondria) rapidly fell and subsequently recovered to a new steady
state level (Brandes and Bers. 1997). The decrease in [NADH]m could be because the increased [Ca^{2+}]c causes increased work and ATP hydrolysis rate (by myofilament and transport ATPases), which in turn would cause increased [ADP] (and/or P_i). That could stimulate the oxidative phosphorylation rate (more than the NADH production rate) and therefore, [NADH]m will fall (Brandes and Bers. 1997). The subsequent recovery of [NADH]m could be because, in a slower time scale, Ca^{2+} could enter the mitochondria and activate pyruvate dehydrogenase, NAD-linked isocitrate dehydrogenase, or 2-oxoglutarate (α-ketoglutarate) dehydrogenase. Activation of the dehydrogenases is expected to increase the NADH production rate and consequently cause [NADH]m to recover. Increased [NADH]m may further increase the rate of oxidative phosphorylation and therefore ATP production. However, increasing the myocardial work without increasing the [Ca^{2+}]c (i.e. by increasing the sarcomere length) also resulted in [NADH]m decline, but it did not recover (Brandes and Bers. 1997). Therefore, recovery of [NADH]m in the presence of elevated [Ca^{2+}]c demonstrates a Ca^{2+}-dependent feed-forward control mechanism of cellular energies in cardiac muscle during increased work (Brandes and Bers. 1997).

1.8 SARCOLEMMLAL Ca^{2+} PUMP

Sarcolemmal Ca^{2+} pumps transport one Ca^{2+} ion per ATP hydrolysis (Dixon and Haynes. 1989). The maximum rate at which Ca^{2+} might be extruded from cardiac myocytes by this pump is slow compared to Ca^{2+} transport by sarcolemmal Ca^{2+} channels and the Na^+-Ca^{2+} exchangers (Dixon and Haynes. 1989). Indeed, if relaxation of cardiac muscle were solely dependent on the sarcolemmal Ca^{2+} pump, it would likely take 20-40 seconds for relaxation even at V_max (the maximum rate of activity of this pump). Thus, while the sarcolemmal Ca^{2+} pump does have a high affinity for [Ca^{2+}], the transport rate is too slow to make important contribution to Ca^{2+} efflux during the cardiac cycle (Bers. 1991. 1997). Inhibition of the SR Ca^{2+} pump and Na^+/Ca^{2+} exchanger system in rabbit ventricular myocytes, slowed the relaxation phase by almost 1100%. This result indicates that sarcolemmal Ca^{2+} pump do not contribute significantly to the relaxation rate.
1.9 **CONTRACTILE PROTEINS (MYOFILAMENTS)**

The contractile proteins occupy about half of the cell volume in mammalian ventricular myocardium (Bers. 1991). The myofilaments are composed of the thick (or myosin) and thin (or actin) filaments as well as associated contractile and cytoskeletal components. The filaments are periodically arranged into sarcomeres. Numerous bundles of these contractile proteins are referred to as myofibrils. Each myosin heavy chain has a long α-helical tail and a globular head. The tails of the myosin heavy chain form the main axis of the thick filament. The heads form the crossbridges and contain the ATP hydrolysis site and interact with the thin filaments. Two myosin light chains are associated with each myosin heavy chain head. The backbone of the thin filament is composed of two chains of the globular protein, G-actin, which form a helical double-stranded F-actin polymer. Tropomyosin (Tm) is a long flexible protein which lays in the groove between the actin strands making contact with seven actin monomers and with a near neighbor Tm by a head to tail overlap between the -COOH and -NH₂ ends of molecules (Soloro and Van Eyke. 1996). Each Tm molecule is associated with a troponin complex. The troponin complex is made up of three subunits: troponin T (Tn-T, the tropomyosin binding subunit), troponin C (Tn-C, the Ca²⁺ binding subunit), and troponin I (Tn-I, the inhibitory subunit). Tm is bound to Tn complex largely through the Tn-T unit. There is also an interaction between Tm and Tn-I, which also binds to actin in the relaxed myofilament. Tn-C is bound to the thin filament through Tn-I and Tn-T. Specific domains of Tn-I interacts with actin-Tm and/or Tn-C (Zot and Potter. 1987; Farah and Reinach. 1995; Soloro and Van Eyk. 1996).

In the diastolic state, intracellular Ca²⁺ concentration (~ 0.1 μM) is below the threshold for binding to the Ca²⁺-specific site of Tn-C. In this condition the interaction between Tn-I and Tn-C, at a critical region of the Tn-I molecule, is weak and Tn-I binds to actin-Tm and inhibits the strong, force generating interaction between actin and myosin. With cellular systolic events, Ca²⁺ concentration increases in the myofilament space and binds to the regulatory site on Tn-C (a single low affinity site at an NH₂ terminal domain of Tn-C). Ca²⁺ binding to Tn-C induces conformational changes in Tn-C, which are transmitted through the regulatory complex
ultimately causing redistribution of the cross-bridge population toward one in which the proportion of cross-bridges in the F-generating states is increased. The end-effect is a transition of the thin filament toward the center of the sarcomere (Solaro and Van Eyk. 1996).

1.10 CROSS-BRIDGE FORMATION

A model for cross-bridge formation was proposed by Hill et al. (1980). In this model, functional units consisting of actin:Tn:Tm in a 7:1:1 ratio exist in two states. "Off" and "On" state, dependent on occupancy of the thin filament with cross-bridges. According to this model the thin filament sites are "off" even in the presence of Ca\(^{2+}\) unless cross-bridges bind. The role of Ca\(^{2+}\) was proposed to shift the equilibrium between the two states such that the activation of thin filaments by bound cross-bridges was eased in the presence of Ca\(^{2+}\). Co-operativity, in this model, is determined by near-neighbor interactions between functional units (Solaro and Van Eyke. 1996).

Recent studies have provided more supporting evidence for the steric hindrance model of myofilament activation. which was originally demonstrated Huxley (1980) who used X-ray diffraction measurements to show that Tm moves on thin filament when Ca\(^{2+}\) is present. Lehman et al. have demonstrated that Ca\(^{2+}\)-binding to thin filaments induces a movement of Tm on the surface of the thin filament (Lehman et al., 1994). According to the steric hindrance model, conformational changes due to binding of Ca\(^{2+}\) to Tn-C will result in movement of Tm on the thin filament, so that myosin head can interact with actin and cross bridges can be formed. Computer modeling, based on structures derived from X-ray crystallographic data of actin and myosin head (myosin-S1), indicate that Tm (and possibly Tn) may block the actin-myosin interaction (Rayment et al., 1993; Holmes. 1995).

Kinetic experiments measuring the onset of the "on" state indicate that. although 95% of the thin filament sites are "off" in the absence of Ca\(^{2+}\), a much smaller percent are "off" in the presence of Ca\(^{2+}\) (Geeves and Lehrer. 1994; McKillop and Geeves. 1995). Therefore, the "off" state could not be simply described by the two-state Hill model. A modified cross-bridge
formation model was proposed by Geeves and Lehrer (1994) that accounted for the steric effect, the kinetic data ("on" and "off" state in the presence and absence of Ca\(^{2+}\)) as well as the co-operative binding of cross-bridges. Geeves and Lehrer modified Hill's model by adding an additional "off" state, termed the "blocked state." According to this model, the thin filament exists in dynamic equilibrium between three states: blocked (off), closed (off) and on (open). In the "blocked" state, Tn does not have bound Ca\(^{2+}\), and cross-bridge binding is physically blocked. In the "closed state", Ca\(^{2+}\) is bound to Tn, but there are no strong cross-bridges. In the "on or open state", cross-bridges are in a strong binding state that splits ATP at relatively high rates and generates force. In this model, Ca\(^{2+}\) has two regulatory roles. It can regulate formation of cross-bridges, as well as isomerization of the cross-bridges to the strong-binding state (Geeves and Lehrer, 1994; Lehrer, 1994).

1.11 FORCE-FREQUENCY RELATIONSHIP IN RAT MYOCARDIUM
The dependency of the contractile force on the stimulation frequency was first discovered by Bowditch (1871) and is known as the force-frequency relation or Bowditch phenomenon. Heart muscles from most mammals show a positive force-frequency relationship, that is, the force of contraction increases as the frequency of stimulation is increased (Koch-Weser, 1963; Morad, 1973; Buckley et al., 1992). In contrast, the force of contraction of rat cardiac muscle generally decreases as stimulation frequency increases (i.e. it exhibits a negative force-frequency relationship) (Hoffman and Kelly, 1959; Bouchard and Bose, 1989). This negative relationship, however, is not universally observed under all experimental conditions. In fact, the relationship in rat becomes positive at low [Ca\(^{2+}\)]\(_o\) (Forester and Mainwood, 1974; Bouchard and Bose, 1992) and at a high stimulus frequency (Forester and Mainwood, 1974; Henry, 1975).

In experiments done on isolated rat ventricular myocytes, Frampton et al. demonstrated that some of the cells showed a positive and some showed a negative force-frequency relationship (1991). In cells that showed a positive relationship, increasing the frequency of stimulation led to increases in the size of the twitch, in intracellular Na\(^+\) activity, in systolic and
diastolic \( [\text{Ca}^{2+}]_i \) and in the \( \text{Ca}^{2+} \) load of the SR. In cells that showed a negative force-frequency relationship, increasing the frequency of stimulation decreased or had little effect on the \( [\text{Ca}^{2+}]_i \). These cells also showed a smaller frequency-dependent change in the intracellular \( \text{Na}^- \) activity than the cells with a positive force-frequency relationship (Frampton et al., 1991). A biphasic F-frequency behavior has also been reported in rat cardiac myocytes. Cardiac trabeculae showed a biphasic staircase effect, negative from 0.1 to 0.5 Hz and positive after 0.5 Hz (Tang et al., 1996). On the other hand, rat ventricular myocytes showed a positive staircase effect from rest to 1 Hz and negative from 1 to 6 Hz (Borzak et al., 1991).

A number of factors have been suggested to play a role in the negative force-frequency relationship in rat cardiac myocytes:

1) **Metabolites.** It has been suggested that, in multicellular preparations, the negative force-frequency relationship occurs because at high stimulation frequencies the muscle is metabolically deprived. In experiments done on isolated rat papillary muscles, increment of the glucose content of the perfusion solution produced a positive force-frequency relationship (Henry, 1975). Furthermore, isolated rat trabeculae of small diameter showed a flat or positive force-frequency relation (Schouten and ter Keurs, 1986; Schouten, 1989). However, this hypothesis can not explain the positive and the negative F-frequency relationships observed in single myocytes in which metabolic deprivation is not an issue (Capogrossi et al., 1986; Frampton et al., 1991).

2) \( I_{\text{Ca,L}} \). The transmembrane \( \text{Ca}^{2+} \) current \( (I_{\text{Ca,L}}) \) that flows during the action potential needs time to recover from inactivation (voltage or \( \text{Ca}^{2+} \)-dependent). After short inter-stimulus intervals, \( I_{\text{Ca}} \) is small and would recover toward control at longer intervals (Frampton et al., 1991). However, it has been shown that, at least in other species, \( I_{\text{Ca}} \) is normally fully restituted within 100 milli-seconds (Tsang, 1988), and a decrease in the strength of contraction has been shown in rat myocardium when the inter-stimulus interval was significantly longer than the time required for \( I_{\text{Ca}} \) restitution (Orchard and Lakatta, 1985; Frampton et al., 1991; Tang et al., 1996). Inhibition of the \( I_{\text{Ca}} \) did not affect the negative staircase effect in the rat ventricular
myocyte which was observed at the lower range of the frequency of stimulation (from rest to 1 Hz) (Borzak et al., 1991). Therefore, \( I_{ca} \) cannot underlie the negative F-frequency relationship (Borzak et al., 1991; Frampton et al., 1991).

3) **Mechanical Restitution.** The \( Ca^{2+} \) that is released to initiate contraction is not immediately available for release after being sequestered by the SR during relaxation. i.e., there appears to be some delay before \( Ca^{2+} \) is again available for activation of the myofilaments. Thus, it was suggested that at short intervals the twitch is small, since little time has been allowed for mechanical restitution (Braveny and Kruta, 1958), whereas at longer intervals the twitch is larger. However, this hypothesis was challenged by the finding that the pattern and time course of the mechanical restitution in rat ventricular myocytes are the same whether they showed a positive or a negative F-frequency relationship (Hoffman and Kelly, 1959: Frampton et al., 1991).

4) **Ca\(^{2+}\) loading and SR Ca\(^{2+}\) load.** Orchard and Frampton observed (unpublished data) that when rat papillary muscles were first mounted, they exhibited a negative F-frequency relation. However, with time there was a decrease in the force development and the muscle started to exhibit a positive F-frequency relation (Allen and Kurihara, 1980). Therefore, they suggested that the change from negative to positive F-frequency relationship could be due to a decrease in the \( Ca^{2+} \) load of the cell (as reflected in the decrease in force). Similarly, isolated myocytes that show some spontaneous activity, presumably because of a high \( Ca^{2+} \) load, show a negative F-frequency relation. whereas those that are normally quiescent may show a positive, or flat, F-frequency relationship (Capogrossi et al., 1986). Bouchard and Bose (1992) also found that the sensitivity of contraction to changes of stimulation frequency was strongly related to the level of cell \( Ca^{2+} \) loading. These investigators observed that positive inotropic response to stimulus frequency was greatest at low levels of SR \( Ca^{2+} \) loading, and maneuvers that further increased the SR \( Ca^{2+} \) content first blunted the frequency response (2.5 mM \([Ca^{2+}]_o\)) and then reversed it (low \([Na^+]_o\)) as the maximal level of SR \( Ca^{2+} \) loading was achieved (Bouchard and Bose, 1992). Borzak et al. demonstrated that ryanodine (an activator of the SR \( Ca^{2+} \) release channel
at nanomolar concentration) abolished the negative F-frequency relation in the lower frequency range, suggesting that accumulated SR Ca\(^{2+}\) stores are involved in the negative staircase effect. Reduction of twitch force under conditions of high cell Ca\(^{2+}\) loading implies that there may be a negative feedback mechanism modulating the release of Ca\(^{2+}\) from the SR, such as that proposed by Fabiato (1985a,b) or Capogrossi et al. (1988).

5) **SR Ca\(^{2+}\) release channel (Ryanodine Receptors)**

In cardiac muscle, Ca\(^{2+}\) release from the SR is mediated by a Ca\(^{2+}\)-activated channel called the SR Ca\(^{2+}\) release channel or ryanodine receptor (RyR) (Smith et al., 1986; Lai et al., 1988). The cardiac RyR is regulated by Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (I\(_{Ca}\)) in the surface membrane. This process, termed Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR), is fundamental to cardiac excitation-contraction coupling. CICR is the mechanism that links surface membrane depolarization to Ca\(^{2+}\) activation of the contractile proteins (Fabiato and Fabiato, 1979; Niggli and Lederer, 1990; Sipido and Wier, 1991). The ryanodine receptor channel in bilayers displays unusual kinetics. The cardiac RyR appears to adapt to the [Ca\(^{2+}\)] to which it is exposed, preserving its capacity to respond to a new higher [Ca\(^{2+}\)] (Gyorke and Fill, 1993). In other words, when Ca\(^{2+}\) is elevated, the channels open and then appear to inactivate. However, in contrast to traditional inactivation such as observed in the acetylcholine receptor, when Ca\(^{2+}\) is further increased the channels reopen and again appear to inactivate. Thus the channel acts as a differentiator, responding primarily to changes in concentration. To avoid confusion with the term inactivation, this reactivatable kind of behavior is termed adaptation (Cheng et al., 1995; Sachs and Qin, 1995).

The adaptation process of this channel has been proposed to include the following events: 1) A change in [Ca\(^{2+}\)] induces channel activity as a consequence of Ca\(^{2+}\) binding to an activation site on the RyR molecule. This step is relatively fast. 2) The RyR molecule undergoes a transformation that induces a slow decrease in the Ca\(^{2+}\) affinity of the activation site. The result is less occupancy of the activation site and decay in channel activity (relaxation phase). 3) The relaxation phase leaves the activation site available to respond to a second Ca\(^{2+}\)
stimulus (Gyorke and Fill, 1993). A model for the adaptation of the RyR has been proposed which is based on the established tetrameric RyR channel structure (Cheng et al., 1995). Biochemical studies indicate that at least three high affinity Ca$^{2+}$-binding sites exist per monomer (Chen et al., 1992). Cheng et al. postulated that one kind of binding site (the O-domain) tends to open the channel when activated by Ca$^{2+}$ and another (the A-domain) tends to close (adapt) it. Thus, the tetramer would then have four O-domains and four A-domains. According to this model, the channel opens when the number of occupied O-domains (O) on the tetramer exceeds or equals the number of active A-domains (A), and remains closed when $A>O$. or $O=0$ (Cheng et al., 1995).

Ryanodine is a highly selective drug for the SR Ca$^{2+}$ release channel and can either cause muscle contracture or a decline in muscle force (Jenden and Fairhurst, 1969). Application of ryanodine at nanomolar concentration can open the SR Ca$^{2+}$ release channel whereas at concentrations exceeding 10 μM, it fully closes the channel (Meissner, 1986; Alderson and Feher, 1987; Lattanzio et al., 1987). Application of 100 nM ryanodine to rat cardiac myocyte (Borzak et al., 1991) and trabeculae (Tang et al., 1996) abolished the negative force-frequency relationship. Therefore, restitution of the SR Ca$^{2+}$ release channels which could cause SR Ca$^{2+}$ overload has been suggested to be the possible cause of the negative force-frequency relationship in rat myocardium (Wier and Yue, 1986; Borzak et al., 1991; Tang et al., 1996). Therefore, development of the force of contraction following altered frequency of stimulation depends on a number of factors.

1.12 FREQUENCY-DEPENDENT ABBREVIATION OF FORCE AND CALCIUM TRANSIENT

Increased frequency of stimulation has been shown to always be accompanied by a decrease in the duration of the twitch and the [Ca$^{2+}$]$_i$ transient regardless the decrease or the increase in their amplitude (Borzak et al., 1991; Frampton et al., 1991; Tang et al., 1996). The mechanism underlying the more rapid relaxation of the [Ca$^{2+}$]$_i$ transient and the twitch at higher frequencies of stimulation is not clear. It has been suggested that the more rapid relaxation of
the \([\text{Ca}^{2+}]_i\) transient is responsible for the more rapid relaxation of the twitch (Frampton et al., 1991). The abbreviation of the \([\text{Ca}^{2+}]_i\) transient has been suggested to be due to an acceleration of the \(\text{Na}^+/\text{Ca}^{2+}\) exchange mechanism or an increase in the rate of \(\text{Ca}^{2+}\) uptake by the SR as a result of \(\text{Ca}^{2+}\)-calmodulin-dependent phosphorylation reaction, which will accelerate removal of \(\text{Ca}^{2+}\) from the cytoplasm (Kirchberger and Antonetz, 1982; Schouten, 1990; Bassani et al., 1995). However, inhibition of CaM kinase II was reported to have no effect on the duration of the twitch or the \([\text{Ca}^{2+}]_i\) transient (Frampton and Orchard, 1992). Contribution of the abbreviated \(\text{Ca}^{2+}\) influx via \(I_{\text{cal}}\) due to the frequency-dependent shortening of the action potential to \([\text{Ca}^{2+}]_i\) transient abbreviation seems unlikely, because the amount of \(\text{Ca}^{2+}\) that enters via \(I_{\text{cal}}\) is small in rat cells (Fabiotto, 1983) and because the action potential in rat cells is short and repolarizes early during the \([\text{Ca}^{2+}]_i\) transient (Frampton et al., 1991). Therefore, the underlying mechanism of the abbreviated \([\text{Ca}^{2+}]_i\) twitch at higher frequency of stimulation is not well understood.

1.13 **SIGNIFICANCE OF THE FREQUENCY-DEPENDENT TWITCH AND \(\text{Ca}^{2+}\) TRANSIENT ABBREVIATION IN DISEASED STATE**

The frequency-dependent abbreviation of the twitch force and the \(\text{Ca}^{2+}\) transient is a very important feature of the cardiac function, and its absence would result in elevation of the diastolic force and \([\text{Ca}^{2+}]_i\) at increased heart rates. A decreased rate of intracellular calcium transient relaxation in papillary muscles (Gwathmey et al., 1987) and isolated ventricular myocytes (Beuckelmann et al., 1992) from patients with terminal heart failure has been reported. This observation has been attributed to differences in \(\text{Ca}^{2+}\) handling between terminally failing and non-failing myocardium (Gwathmey et al., 1990). It has been hypothesized that \(\text{Ca}^{2+}\) uptake (its removal from the cytosol) is diminished in failing myocardium and it is aggravated at higher stimulation rates (Limas et al., 1987; Gwathmey et al., 1990). Twitch duration prolongation is observed in almost all animal models of hypertrophy as well as human heart failure (Jouannot and Hyatt, 1975; Gwathmey et al., 1992; Hajjar et al., 1992; De Tombe et al., 1996).
Twitch duration prolongation can greatly contribute to progression of the cardiac disease. The prolonged twitch duration would result in elevation of the diastolic force at increased heart rate (HR), which could result in decreased end-diastolic volume and therefore decreased SV (Lorell et al., 1990; Piano 1994). When SV decreases sufficiently below that required to meet the body’s needs, compensatory mechanisms (mechanical, neurohumoral, genetic etc.) will be activated. Once these compensatory mechanisms fail to recover the decreased cardiac function, congestive heart failure will ensue. The compensatory mechanisms, which include mechanical (myocyte hypertrophy and non-myocyte hyperplasia), neurohumoral (increased blood concentration secretion of vasopressin, angiotensin II. and ANP), and genetic (recapitulation of the fetal gene program, such as replacement of the α-MHC in rodents with β-MHC) as well as alterations at the level of the ion channels and pumps, will be fully discussed in chapter 4.

Therefore, it is very important to unravel the underlying mechanism of the frequency-dependent twitch abbreviation in the myocardium since the loss of this mechanism could contribute significantly to heart disfunction in disease, particularly at elevated heart rates. I therefore, studied whether this mechanism is altered in diseased hearts, and if it is, at what stage of the disease does it take place?

The purpose of this study was to discover the mechanism underlying the increased rate of relaxation of the force of contraction at increased frequency of stimulation. We studied the factors that are affected when the frequency of stimulation is changed, such Ca2+-activated kinases. We then compared the time-course of the frequency-dependent TD shortening to the time course of the frequency-dependent pHi decline. We also evaluated the contribution of the contractile proteins (relative to intracellular Ca2+ handling) to the kinetics of twitch force.
CHAPTER 2

GENERAL METHOD

2.1 Muscle Preparation and Dissection Procedure

The studies reported in this thesis were performed on right ventricular trabeculae of 220 to 250 g normal male LBN-F1 rats or 350-400 g sham-operated or LAD-ligated hypertrophic rats (more details about the rats with cardiac hypertrophy are given in chapter 4). The rats were anaesthetized with an overdose of sodium pentobarbital. The hearts were excised and immediately (<20 seconds) perfused retrogradely via the proximal aorta with a modified Krebs-Henseleit solution containing an elevated [K+] (about 20 mM) which prevented beating of the heart. Perfusion of the heart prior to dissection is essential in order to remove blood cells from the tissue which would otherwise be trapped in the capillary and act as scattering sources which would interfere with the laser diffraction pattern.

Dissection of the heart was done using a binocular microscope (model SMZ-1, Nikon. 10-30 X’s). The free wall of the right ventricle was separated by cutting from the ventricular septum. The right atrium was removed from the atrio-ventricular ring. Thin, unbranched and uniform trabeculae, running between the free wall of the right ventricle and the valve attached to the atrio-ventricular ring were selected. The trabecula selected for dissection was freed from its atrio-ventricular ring attachment along with a piece of valvular tissue. This end of the trabecula will be referred to as the valvular end. Subsequently a portion of the right ventricular wall attached to the other end of the trabecula was removed in a manner which minimized damage to the free running trabeculae. This end of the preparation will henceforth be referred to as the ventricular end of the trabecula. Trabeculae were selected that were 100-200 μm in width, 60-90 μm in thickness, and 2-5 mm in length. The trabeculae used in this study were selected for uniformity in order to prevent potential artifacts associated with the diffraction and fluorescence measurements. It is important to select a trabecula of small diameter to avoid ischemia in the center of the muscle. Schouten and ter Keurs (1986) demonstrated that the critical diameter for rat myocardium preparation is 0.2 mm at 26 ± 0.3 °C. The critical diameter
for rat myocardium is much smaller than that of other mammals which has been reported to be 0.5-1.2 mm (Paradise et al., 1981). The critical diameter, therefore, may depend on the experimental conditions and species. It is important to note that contractility of isolated myocardium of rat has been reported to be much higher than that of other species (Allen and Kurihara, 1980) and thus the rate of energy metabolism may be much higher (Schouten and ter Keurs, 1986).

After mounting, the muscles were stretched to a sarcomere length of about 2.0 μm and left to equilibrate for 45 minutes to 1 hour at room temperature (22-23°C) in the perfusion medium (1 mM [Ca²⁺]₀) while being stimulated at 0.5 Hz. During this equilibration period sarcomere length, at a given muscle length both at rest and during the twitches decreased, as a result of the changing properties of the series elastic element. If peak twitch force development diminished to less than 70% of control during this equilibration period, the preparation was discarded.

2.2 Experimental Apparatus, Temperature Control and Stimulation

The muscle preparations were positioned in an experimental chamber milled from plastic, mounted on an inverted microscope. The dimensions of the trough were 25 mm long, 2 mm wide and 3 mm deep. The perfusion rate was adjusted to approximately 2-4 mL/min. The bottom of the trough was made from a microscope coverslip which was sufficiently thin to prevent parallax errors and excessive absorption of UV light. After mounting of the preparation, the top of the trough was covered with a piece of a microscope slide. The temperature in the perfusion chamber was maintained at 22-23°C (room temperature) and was controlled by using a glass heat exchanger at the inflow line and a circulating water bath (VWR Scientific, model 1130A).

Electrical stimulation of the preparation was achieved via platinum electrodes (0.4 mm diameter) positioned in the wall of the muscle bath along either side of the muscle preparation. Rectangular stimulating pulses were delivered by an isolated pulse generator (A-M system.
model 2100). Pulse duration was 3 ms. The stimulation frequency was variable. Stimulus strength was adjusted to 20% above the stimulus threshold of the preparation, and usually amounted to about 10 V although some of the thinner preparations required up to 60 volts along with a longer pulse duration.

2.3 Force Measurement

Twitch force was measured with a modified silicon strain gauge (model AE-801, Sensor, Horten, Norway) attached to a micromanipulator. The lightweight basket (0.5 mm wide) fabricated from platinum wire (100 μm diameter) was glued onto the tip of the silicon beam with epoxy glue. Care was taken to use a minimum amount of glue and not to cover the resistors on the silicon beam. The silicon beam with the attached basket was covered with a thin coat of silicon glue mixed with toluene. Once this had hardened a second and third layer silicon glue containing carbon powder and toluene were applied. This procedure ensured both water and light tightness of the transducer with minimal electrical drift (since carbon particles can act as conductors). The basket provided a stable mounting cradle for the ventricular end of the trabecula (See figure 2-1). The remnant of the tricuspid valve was mounted on a stainless steel hook (100 μm diameter. see figure 2-1). The hook was glued onto a custom made light weight titanium motor arm.

2.4 Sarcomere Length Measurement

Sarcomere length was measured by laser diffraction pattern. A mirror located at an angle under the bath, reflected the laser beam (ULWD 0.3, Olympus, Japan), coming from the top of the bath, on a plate located on the side of the set up. The striation of the cardiac muscle diffracted the laser beam. The mirror under the bath reflected the diffraction pattern on the plate beside the set up (See figure 2-2). The diffraction pattern of the laser beam was calibrated by two different gratings, wide (25 μm) and narrow (8.3 μm). The calibration was done by using the following formula: $\sin \theta = n\lambda/d$, where $\theta$ is 1/2 of the angle between the incoming laser light and each diffraction ray. $n$ is the number of the diffraction lines on the plate, and $d$ is the width of every grave on the calibrating gratings (25 μm in wide grating and 8.3 μm in
Figure 2-1: Illustration of the experimental set-up used.

“A” represents the mounting procedure. The trabecula (3) was mounted between a force transducer (1) at the ventricular end and a stationary hook (2) at the valvular end. in a perfusion bath. A mounted trabecula is magnified in “B”. The fluorescent light (for $[\text{Ca}^{2+}]_i$ measurements) was collected by a 10 X’s UV-F objective.

Figure modified from: Backx PH. Force-sarcomere relation in cardiac myocardium. Ph.D. Thesis. 1989. The University of Calgary.
Figure 2-2: Illustration of the arrangement of sarcomere length measurement.

The muscle was illuminated via a laser beam (1). The laser illumination was projected by a mirror located under the bath (2) on a plate next to the set up (3). The plate was calibrated by using grading of different width (see text).

Figure modified from: Backx PH. Force-sarcomere relation in cardiac myocardium. Ph.D. Thesis. 1989. The University of Calgary.
narrow grating). The sarcomere length (d') in this set up is equal to d/n. According to this calibration, the 12th diffraction line of the wide grating and the 4th line of the narrow grating overlap and correspond to sarcomere length 2.1 μm. The 11th line on the wide grating corresponded to sarcomere length of 2.27. All the experiments were done when the muscle was stretched to a sarcomere length of 2.2-2.3 μm.

2.5 [Ca\(^{2+}\)]\(_i\): Measurement

The Ca\(^{2+}\) transient in the trabeculae was measured by the Ca\(^{2+}\) sensitive fluorescent indicator fura-2 pentapotassium salt which was loaded into the trabeculae by the microinjection technique (See Loading Fura-2 salt).

Fura-2 salt loading has many advantages over other fluorescent indicators (e.g. Aequorin) is that it is relatively insensitive to pH in the range 6.8-7.8. is quite selective for calcium versus magnesium and binds calcium with a dissociation constant (K\(_d\)) of 145 nM which is a function of both ionic strength and the concentration of other polyvalent cations (Gryniewicz et al., 1985). The Ca\(^{2+}\) binding kinetics of Fura-2 in cardiac myocytes are fast enough (~ 20 msec) to measure rapid changes in [Ca\(^{2+}\)]\(_i\) (Berlin and Konishi, 1993). Among other advantages of Fura-2 salt is that it binds calcium with a 1:1 stoichiometry. It has an isosbestic point (on the excitation side) which allows the ratio method to be used thereby reducing movement artifacts. The advantage of using Fura-2 salt over Fura-2 AM (which can be loaded chemically into myocardial tissue) is that Fura-2 AM enters into intracellular organelles which interferes with attempts to quantitatively calibrate the fluorescent signals (Spurgeon et al., 1989; Backx and ter Keurs, 1993).

The disadvantages of Fura-2 salt are that it photobleaches with intense light sources and the photolysis products are fluorescent thereby interfering with calibration of fluorescent signals (Cobbold and Rink, 1987). The kinetics (off rate) of the dye have been reported to be slow in vivo which results in the fluorescent signals acting like an integrator when reporting calcium changes (Baylor and Hollingworth, 1988). However an in vitro calibration of the
kinetics of Fura-2 indicates that the first order off rate constant for the decay of fluorescence of these dyes will be about 10 milli-seconds at low [Ca$^{2+}$]'s and will be shorter with increasing calcium. This limitation of this dye is not too serious in cardiac tissue since the decay of the calcium transient has a time constant many times this value (Allen and Kurihara, 1980). The high-affinity binding of Ca$^{2+}$ to Fura-2 results in significant buffering of Ca$^{2+}$ at high [Fura-2] and further limits the use of the dye to conditions where the [Ca$^{2+}$] is less than ~ 2-3 μM (Gryniewicz et al., 1985). Fluorescent measurements with this dye suffer from movement artifacts but this limitation can be minimized by using the ratio method.

2.6 **Loading Fura-2 salt into the cardiac trabeculae**

Fura-2 was dissolved in distilled water to a final concentration of 1 mM. Micropipettes (1mm ID thin wall or 1.2 mm ID thick wall) were pulled and half filled with 1 mM Fura-2 salt and back filled with 140 mM KCl solution. The pipette holder was also back-filled with 140 mM KCl. The micropipette resistance was 150-250 MΩ when placed in the modified Krebs-Henseleit solution. Fura-2 salt was loaded into the trabeculae by iontophoretic injection. This method of loading involves the injection of Fura-2 with the micropipette impaling a single cell (of the trabecula) and allows loading of the entire trabecula. This method is simple and avoids compartmentalization of Fura-2 in the mitochondria (as it happens with Fura-2 AM) (Backx and ter Keurs, 1993). The use of micropipettes with appropriate diameter and wall thickness and especially the proper set-up for pulling the micropipettes is essential for successful loading of the trabeculae. Micropipettes without a small enough tip diameter (< 0.2 μm) caused a significant damage to the trabeculae as evidenced first by local spontaneous activity and later by the development of a contracture in the region of the impalement. After impalement of an unstimulated muscle, the measured membrane potential was between -65 and -95 mV. After achieving a stable impalement, 2-10 nA of hyperpolarizing current was passed for 20-30 minutes. During the injection period, the Fura-2 diffuses from the impalement site into the adjacent cells via gap junctions (Backx and terKeurs, 1993). After the injection period, the preparations were stimulated at 1 Hz for 45 minutes to 1 hour to enhance the rate of spread of
Fura-2 from the site(s) of injection thereby producing a uniform distribution of the dye within the muscle.

2.7 Fluorescent measurements

When the Fura-2 dye was well spread throughout the muscle, excitation ultra-violet (UV) light from a 75-W mercury lamp (Oriel Corp. Stratford, CT) was passed through bandpass filters (Omega Optical. Brattleboro, VT) centered at 340, 357 (the isosbestic point), or 380 nm (band width 10 nm) located in a computer-controlled filter wheel. The filtered light was projected onto the muscle via a 10X objective in the inverted microscope (Olympus CK2, Japan) using a dichroic mirror (400DPLC. Nikon. Japan). The use of a large field of illumination minimizes Ca\(^{2+}\)-independent changes in the fluorescent signal associated with movement of the preparation during a twitch and reduces the amount of excitation light required to measure Ca\(^{2+}\)-dependent changes in fluorescence with a high signal-to-noise ratio.

The emitted fluorescent light was collected by the objective and transmitted through a bandpass filter at 510 nm (10 nm band width) to a photomultiplier (R2368, Hamamatsu. Bridgeport, NJ). The photomultiplier output was filtered at 100 Hz, recorded using an A/D data acquisition board and stored in the computer for later analysis (See Figure 2-3). The preparations were only illuminated with the excitation light for short periods of time with the use of a filter wheel. Brief illumination of the preparation reduced the effects of photo bleaching which could otherwise interfere with the accurate calibration of the fluorescence signals. With this method, Fura-2 fluorescence could be routinely and reliably recorded for periods longer than 5 hours.

2.8 Autofluorescence

Autofluorescence is referred to the Fura-2 independent background fluorescence, i.e. the fluorescence of the muscle and the bath in the absence of Fura-2. In these experiments, the autofluorescence was recorded before loading the muscle and after the experiment. If the difference between these two measurements was more than 5%, then the data from that
Figure 2-3: Illustration of the set up used for fluorescence studies.

The output of the xenon light source passed through two band-pass filters and was then collected by a fiber optic cable. The multiplexed signals illuminated the preparation via dichroic mirror and a 10 X’s UV-F objective. The emitted fluorescent light was collected by the objective and projected onto a low noise photomultiplier tube (PMT). The analogue circuit integrated the current output of the demultiplexed signal and calculated the ratio of the signals. The individual integrated demultiplexed signals were recorded on the PC-AT via an A/D converter.

Figure 2-3
experiment were not used. The autofluorescence was always subtracted from the fluorescent signal recorded from the trabeculae loaded with Fura-2 before the ratio (340/380) was calculated. The autofluorescence at the end of every experiment was measured by application of ionomycin and Mn\(^{2+}\) in a nominally Ca\(^{2+}\)-free K-H solution. The preparation was perfused with modified K-H solution containing 0.5 mM MnCl\(_2\) and zero [Ca\(^{2+}\)] oxygenated with 95% O\(_2\) : 5% CO\(_2\) gas mixture. Ionomycin is an ionophore and is able to transport Mn\(^{2+}\) as well as Ca\(^{2+}\) across the sarcolemma. After 10 minutes of perfusion with MnCl\(_2\)-containing K-H, 4 μL ionomycin from a 10 mM stock solution (in DMSO) was added to the bath (final concentration of 20 -50 μM) for 4-5 minutes while the flow had been stopped. Since ionomycin has fluorescent activities, prolonged exposure of the preparation to this chemical was avoided. After 4-5 minutes, the flow of the modified K-H solution was resumed and after 10-20 minutes fluorescence of the muscle was measured. Complete removal of Fura-2 was confirmed when two consequent fluorescent readings (about 4-5 minutes apart) were identical.

2.9 Calibration Procedure

The in vitro calibration solutions are listed in Table 2.1. These two solutions will be referred to as the EGTA and CaEGTA solutions. 10 nM Fura-2 salt was added to each of these solutions. Different [Ca\(^{2+}\)] were obtained by mixing various proportions of these two solutions. A computer program was used to calculate the [Ca\(^{2+}\)] for each of these solutions. This program took into account both temperature and ionic strength effects on the stability constants. The calcium concentration for various mixtures of the CaEGTA and EGTA solutions are shown in Table 2.2. 150 μL of a solution (with 10 nM Fura-2 of known [Ca\(^{2+}\)] buffered with a 10 mM total [EGTA]) was placed into the perfusion bath and its fluorescence at an emission wavelength of 510 nm after excitation at 340 and 380 was measured.

The Fura-2 fluorescence shows an isosbestic point at about 360 nm excitation when the emission is measured at 510 nm. As a consequence of this, the use of dual excitation allows the determination of the [Ca\(^{2+}\)] by taking the ratio of the intensities of the fluorescence at the two wavelengths provided the Fura-2 independent fluorescence (autofluorescence) is subtracted. As
a result of this property. the estimate for the [Ca\(^{2+}\)] is independent of the amount of dye present in the preparation. Since dye routinely leaks out of the preparation or is photobleached during the course of an experiment, the ratio method allows accurate estimates of the [Ca\(^{2+}\)] to be determined at any point in the experiment. The mathematical relation between the ratio of the intensities of the fluorescence (autofluorescence subtracted) and the [Ca\(^{2+}\)] is given by the following equation (Backx et al., 1995):

\[
[\text{Ca}^{2+}] = K' \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}
\]

(Eqn. 2-1)

where, \(K'\) = apparent dissociation constant of Fura-2 for Ca\(^{2+}\) = \((S^a / S^b) K_d\)

\(S^a / S^b\) = the ratio of the fluorescence at 380 nm of zero calcium to that at a saturation [Ca\(^{2+}\)].

\(K_d\) = the true dissociation constant of Fura-2 for calcium.

\(R_{\text{max}}\) = the ratio of the fluorescence at 340 nm to that at 380 nm at a saturating [Ca\(^{2+}\)].

\(R_{\text{min}}\) = the ratio of the fluorescence at 340 nm to 380 nm at a [Ca\(^{2+}\)] of zero.

The above equation is derived using three assumptions: 1) the only source of the fluorescent signals is Fura-2, and 2) that Fura-2 exists in a calcium bound form and a calcium unbound form and 3) the binding of Fura-2 for calcium has a stoichiometry of 1:1. These assumed properties of Fura-2 have been reported to be well satisfied, as explained earlier (Gryniewicz et al., 1985). The calibration curve is shown in figure 2-3.

2.10 pH$_i$ MEASUREMENT

loading of Carboxy-SNARF-1-AM into trabeculae. Carboxy-SNARF-1 was incorporated into the cardiac trabecula using the acetoxyethyl ester of carboxy-SNARF-1 (SNARF-AM). SNARF-AM was initially dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/mL and stored at -70°C in 50 μl aliquots. It was subsequently diluted in HEPES-buffered Tyrode to give a final concentration of 10-15 μM, and 0.05% weight pluronic acid was added. Trabeculae were incubated in this solution for 1 hour at room temperature. After one hour, the loading solution was thoroughly washed out by oxygenated K-H solution. To measure pH$_i$, the muscle was excited with ultra-violet light passed through a 540 nm filter, and the emitted fluorescent lights (640 and 590 nm) were collected by an
objective and transmitted to two phoromultiplier tubes (PMTs). The changes in the fluorescent ratio (640/590) is a function of the change in pHᵢ. (This technique was adapted from Buckler and Vaughan-Jones, 1990).

2.11 Data acquisition

Force, and Ca²⁺ transient or pHᵢ signals were displayed on pen-recorded chart recorder (model ES 3400, Gould, Cleveland, OH) equipped with universal amplifiers. Force, and [Ca²⁺], or pHᵢ signals were also displayed on a storage oscilloscope and sampled via an analog to digital (A/D) converter installed in a personal computer.

2.12 Solutions

The standard solution used in the experiments described in this thesis was a modified Krebs-Henseleit solution (Table 2.3). The solutions were in equilibrium with a 95% O₂ / 5% CO₂ gas mixture. The solutions for Fura-2 calibration are listed in Table 2.1.

Isoproterenol, a non selective β-adrenoreceptor agonist. (Sigma Chemical Co.) was added to the perfusion solution (K-H) to a final concentration of 100 nM from a 1 mM stock in distilled water. K252-a, a non-selective kinase inhibitor. (Research Biochemical International) was added to a final concentration of 2.5 μM from a stock in dimethylsulfoxide. KN-93, a selective inhibitor of CaM/Kinase II (Research Biochemical International) was added to a range of final concentration (1-5 μM) from a stock in distilled water. Norepinephrine (Sigma Chemical Co.) was added to a final concentration of 100 nM from a 1 mM stock in distilled water. All the stock solutions were kept at -20°C.
Table 2.1- Calibration solutions.

<table>
<thead>
<tr>
<th>EGTA solution</th>
<th>CaEGTA solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td><strong>Concentration</strong></td>
</tr>
<tr>
<td>KCl</td>
<td>100 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.6 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>10 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.2- $[\text{Ca}^{2+}]$ for different mixtures of EGTA and CaEGTA used to calibrate Fura-2 salt.

<table>
<thead>
<tr>
<th>CaEGTA:EGTA ratio</th>
<th>pCa</th>
<th>$[\text{Ca}^{2+}]$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>8.919</td>
<td>1.181 e-03</td>
</tr>
<tr>
<td>1:9</td>
<td>7.174</td>
<td>0.06695</td>
</tr>
<tr>
<td>2:8</td>
<td>6.826</td>
<td>0.1492</td>
</tr>
<tr>
<td>3:7</td>
<td>6.593</td>
<td>0.2551</td>
</tr>
<tr>
<td>4:6</td>
<td>6.402</td>
<td>0.3965</td>
</tr>
<tr>
<td>5:5</td>
<td>6.226</td>
<td>0.5947</td>
</tr>
<tr>
<td>6:4</td>
<td>6.049</td>
<td>0.8925</td>
</tr>
<tr>
<td>7:3</td>
<td>5.857</td>
<td>1.390</td>
</tr>
<tr>
<td>8:2</td>
<td>5.622</td>
<td>2.389</td>
</tr>
<tr>
<td>9:1</td>
<td>5.267</td>
<td>5.405</td>
</tr>
<tr>
<td>10:0</td>
<td>4.059</td>
<td>87.271</td>
</tr>
</tbody>
</table>
Table 2.3- Krebs-Henseleit solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>120 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>4 mM</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>25 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 mM</td>
</tr>
</tbody>
</table>
CHAPTER 3

Section 3.1

FREQUENCY-DEPENDENT CHANGES IN THE FORCE AND THE \([Ca^{2+}]_i\) TRANSIENT AND THEIR KINETICS

3.1.1 SYMPATHETIC STIMULATION

Sympathetic stimulation in vivo results in release of catecholamines (epinephrine and norepinephrine) from the sympathetic nerve endings which activates β-adrenoreceptors (β-AR). Stimulation of the cardiac β-ARs increases heart rate (HR) and contractility of the cardiac myocytes. Also decreases the duration of systole. A reduction in the fractional duration of the systole at increased HR and fractional increase in diastolic duration is an important feature of a normal functioning heart since it ensures an adequate time for diastolic filling of the ventricles (Boyett, 1978). This feature allows for complete relaxation of contraction and hence prevents the development of diastolic force tone. Activation of the β-ARs (by isoproterenol) in isolated rat heart and/or in ventricular myocytes results in elevation of the peak force and the peak Ca, transient and acceleration of their kinetics (Spurgeon, 1990; Ebihara and Karmazyn, 1996). Interestingly, elevation of the heart rate (HR) alone (in the absence of β-AR stimulation) mimics β-AR stimulation in altering the amplitude and the kinetics of the twitch force and the Ca, transient. It also increases the amplitude and accelerates the kinetics of these two parameters. The HR-mediated increased force of contraction, or the force-frequency relationship, has been the subject of many investigations. However, the HR-mediated twitch duration (TD) abbreviation and its underlying mechanism has not yet been identified.

The purpose of this study was to investigate the underlying mechanism of the rate-dependent TD abbreviation in the absence of adrenergic stimulation. Therefore, we started our investigation by studying the pattern of change in the force, the \([Ca^{2+}]_i\) transient and their kinetics over a range of frequencies of stimulation. In the following sections of this chapter further characterization of the frequency-dependent twitch kinetic alterations and the potential underlying mechanism for this effect will be discussed. We also attempted to determine
whether β-AR stimulation and increased frequency of stimulation, that affect the force and the $[Ca^{2+}]_i$ transient similarly, share a common mechanism (Section 3.3).

3.1.2 METHOD

The effect of the frequency of stimulation on the force of contraction and $[Ca^{2+}]_i$ transient was studied by stimulating rat cardiac trabeculae at 0.2, 0.5, 1 and 2 Hz. These trabeculae were stretched to a sarcomere length of 2.2 to 2.3 μm, and were perfused by oxygenated K-H solution, containing 1 mM $[Ca^{2+}]_o$, at 22-23 °C. (Further details of muscle dissection and solution preparation are given in Chapter 2).

3.1.3 RESULT

Typical force and $Ca^{2+}$ transient traces recorded from the right ventricular (RV) trabecula of rats are shown in Figure 3.1-1. The smooth trace represents the force of contraction and the noisy trace represents the intracellular $Ca^{2+}$ transient. The force and the $Ca^{2+}$ transient traces at 0.2 and 2 Hz are superimposed in figure 3.1-2. This figure shows the increased amplitude and the accelerated kinetics of the two parameters at 2 Hz (compared to 0.2 Hz). The frequency-dependent alteration in the kinetics of the force and the $[Ca^{2+}]_i$ is even more clearly shown in figure 3.1-3, where the traces are scaled.

Summaries of the rate-dependent changes in the force and the $Ca_i$ transient from five experiments, over a range of frequencies of stimulation, are shown in Figure 3.1-4. The parameters were allowed to equilibrate following a change in the frequency. The twitch duration (TD) and the $[Ca^{2+}]_i$ duration (CaD) shown in this figure were the time from 50% contraction to 50% relaxation. Figure 3.1-4A shows that the trabeculae showed a biphasic force-frequency relationship, negative from 0.2 to 0.5 Hz and positive from 0.5 to 2 Hz. A statistically significant ($P<0.05$) decrease in the peak force was observed when the frequency of stimulation was increased from 0.2 to 0.5 Hz ($21.79 \pm 2.61$ at 0.2 Hz to $18.04 \pm 1.36$ mN/mm²)
Figure 3.1-1: Twitch force and $[\text{Ca}^{2+}]_i$ transients recorded from rat cardiac trabecula.

The force and the $[\text{Ca}^{2+}]_i$ transients (as labeled) at 0.2 Hz (A) and 2 Hz (B).
(Figure 3.1-1)
Figure 3.1-2: Superimposed force and [Ca^{2+}] traces.

"A" represents the superimposed twitch force at 0.2 (solid line) and 2 Hz (dotted line). Superimposed [Ca^{2+}] traces at 0.2 and 2 Hz are shown in "B". Notice the difference in the amplitude of these two parameters at the two different frequencies of stimulation.
(Figure 3.1-2)
**Figure 3.1-3:** Scaled force and \([\text{Ca}^{2+}]_i\), traces.

The force traces at 0.2 and 2 Hz are scaled in "A" and the corresponding \([\text{Ca}^{2+}]_i\), transient traces are scaled in "B". Notice the fast rate of relaxation of both parameters at 2 Hz compared to 0.2 Hz. The rate of contraction, however, was only accelerated in the twitch force and not in the \([\text{Ca}^{2+}]_i\).
(Figure 3.1-3)
**Figure 3.1-4:** Frequency-dependent changes in the peak force, peak \([Ca^{2+}]_i\), twitch duration and \([Ca^{2+}]_i\) duration.

A) The peak force showed a biphasic response to an increase in the frequency of stimulation. B) Peak \([Ca^{2+}]_i\) continued to increase as the frequency of stimulation was increased. C and D) Twitch duration (TD) and \([Ca^{2+}]_i\) duration (CaD) decreased as the rate of stimulation was increased regardless of the positive or negative force-frequency relationship. TD and CaD were the time from 50% contraction to 50% relaxation.
A

Peak Force (mN/mm²)

0.0 0.5 1.0 1.5 2.0
Frequency of stimulation (Hz)

B

Peak [Ca²⁺] (μM)

0.0 0.5 1.0 1.5 2.0
Frequency of stimulation (Hz)

C

Twitch duration (msec)

0.0 0.5 1.0 1.5 2.0
Frequency of stimulation (Hz)

D

[Ca²⁺] duration (msec)

0.0 0.5 1.0 1.5 2.0
Frequency of stimulation (Hz)

(Figure 3.1-4)
at 0.5 Hz) and a significant increase was observed when the frequency was increased from 0.5 to 1 and then to 2 Hz (23.44 ± 5.49 at 2 Hz). Peak [Ca\textsuperscript{2+}] \textsubscript{i} transient, however, did not follow the changes in the force of contraction (Fig. 3.1-4B). The peak [Ca\textsuperscript{2+}] \textsubscript{i} (μM) increased significantly (P<0.05) as the frequency of stimulation was increased from 0.2 to 0.5 Hz (0.52 ± 0.03 to 0.53 ± 0.02) and to 2 Hz (0.73 ± 0.03). The TD and the CaD continued to decrease as the frequency of stimulation was increased (Fig. 3.1-4C and D). The TD (msec) declined significantly from 0.2 Hz to 2 Hz (from 296.75 ± 19.4 to 177.50 ± 21.76), so did the CaD (from 218.25 ± 31.14 to 94.50 ± 8.91 msec).

In order to further characterize the frequency-dependent alterations in the kinetics of the force and the [Ca\textsuperscript{2+}] \textsubscript{i}, the duration of the contraction phase (time to peak) and the relaxation phase (time from peak to 80% relaxation) were measured over a range of stimulation frequencies. Figure 3.1-5A shows the interval that was measured as the contraction phase. The summary of the frequency-dependent changes in the duration of the contraction phase are shown in figure 3.1-5A ii (n=5). The time to peak force significantly decreased from 187.33 ± 16.62 msec at 0.2 Hz to 124.33 ± 15.18 msec at 2 Hz (P<0.05). The decrease in the time to peak [Ca\textsuperscript{2+}] \textsubscript{i} (msec), however, was not statistically significant (from 46.33 ± 2.02 at 0.2 Hz to 38.67 ± 2.73 at 2 Hz, P=0.09). Figure 3.1-5B shows the interval that was measured as the duration of the relaxation phase in the force and the [Ca\textsuperscript{2+}] \textsubscript{i} traces. The rate of relaxation (msec) of force and Ca\textsuperscript{2+} transient significantly accelerated as the frequency of stimulation was increased from 0.2 to 2 Hz (Fig. 3.1-5B ii). The relaxation phase of force shortened from 272.0 ± 29.5 to 187.33 ± 13.44 msec (P<0.05) and that of Ca\textsuperscript{2+} transient shortened from 512.33 ± 142.67 to 142.67 ± 6.84 msec (P<0.05). Therefore, the frequency-dependent shortening of twitch force is due to acceleration of both the contraction and the relaxation phase. However, the frequency-dependent [Ca\textsuperscript{2+}] \textsubscript{i} transient duration shortening is mainly due to acceleration of the relaxation phase. These observations show that the kinetics of the twitch force do not precisely follow those of the [Ca\textsuperscript{2+}] \textsubscript{i} transient, which suggests a contribution of the contractile proteins in mediating the frequency-dependent shortening of the twitch duration.
**Figure 3.1-5:** Frequency-dependent changes in the contraction and relaxation phases of the twitch force and the Ca$^{2+}$ transient.

**Ai)** Contraction phase was considered as the time from the onset of contraction to the peak value (force or Ca$^{2+}$). **Aii)** The contraction phase of the twitch force significantly shortened as the frequency of stimulation was increased from 0.2 to 2 Hz, but that of the Ca$^{2+}$ transient did not.

**Bi)** The relaxation phase was considered as the time from peak to 80% relaxation. The rate of relaxation was significantly accelerated in both force and [Ca$^{2+}$]$_i$ as the frequency of stimulation was increased from 0.2 to 2 Hz (**Bii**).
(Figure 3.1-5)
3.1.4 DISCUSSION

Frequency-Dependent Changes in the Amplitude of Force and \([\text{Ca}^{2+}]_i\)

We found that the staircase phenomenon in rat cardiac trabeculae is biphasic, negative from 0.2 to 0.5 Hz and positive from 0.5 to 2 Hz (Figure 3.1-4). This observation is consistent with previous studies that also reported a biphasic force-frequency relationship in rat myocardium (Borzak et al., 1991; Tang et al., 1996). However, different ranges for the positive and the negative inotropic effects have been reported. Consistent with our observation, Tang et al. (1996) found that the force-frequency relationship in rat cardiac trabeculae was negative from 0.1 to 0.5 Hz and positive above 0.5 Hz. Whereas, Borzak et al. (1991) found that the staircase effect, in rat single myocytes, was negative up to 1 Hz and was positive from 1 to 6 Hz. This difference could be due to the different experimental conditions and also the preparations. Borzak et al. experimented on ventricular single myocytes, whereas we and Tang et al., studied cardiac trabeculae (multicellular preparations).

The data obtained from single myocytes cannot be directly compared to those obtained from the cardiac trabecula. In isolated myocytes, the force of contraction is estimated by extent of cell shortening, which does not necessarily mirror the contractile force measured in our trabeculae. The relaxation of cell shortening in isolated myocytes is limited by the decline of the \([\text{Ca}^{2+}]_i\) transient (Spurgeon et al., 1992). However, in cardiac trabeculae, force rise and relaxation are limited by the contractile filaments and not just by the \([\text{Ca}^{2+}]_i\) handling (Backx et al., 1995). Differences in intracellular \(\text{Ca}^{2+}\) handling between single myocytes and multicellular preparations (papillary muscles) have also been reported (Ravens et al., 1996).

It has been shown that the \(\text{Ca}^{2+}\) recirculation fractions were significantly larger in single myocytes than in papillary muscles. Therefore, a reported change in the force recorded from single myocytes might not be directly comparable to the force recorded from trabeculae.

It has been suggested that the negative phase of the F-frequency relationship, during low stimulation rates, in rat myocardium is due to the relatively avid SR \(\text{Ca}^{2+}\) loading (Bers and Bridge, 1989; Schouten, 1990; Borzak et al., 1991; Schouten and ter Keurs, 1991). This
suggestion is consistent with the observation that ryanodine treatment (which stimulate SR Ca\textsuperscript{2+}
release) abolished the negative phase without affecting the positive phase of the force-frequency relationship (Borzak et al., 1991; Tang et al., 1996). There are, however, controversial reports regarding the source of this extra Ca\textsuperscript{2+}. Based on the experiments where the extracellular Ca\textsuperscript{2+} was depleted during rest. Banijamali et al. (1991) suggested that extra Ca\textsuperscript{2+} enters the cell via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Others have hypothesized redistribution of Ca\textsuperscript{2+} from mitochondria (Cooper et al., 1992; Isenberg et al., 1993), which, however, is not supported by all investigators (Bassani et al., 1993). The SR Ca\textsuperscript{2+} overload could also be due to decreased activity or rapid adaptation of the SR Ca\textsuperscript{2+} release channels (the mechanism of action of this channel is discussed in chapter 1). Contribution of I\textsubscript{Ca} to SR Ca\textsuperscript{2+} overload is not very likely, since blockade of this channel did not alter the negative phase of the force-frequency relationship in rat myocyte (Borzak et al., 1991) or trabecula (Tang et al., 1996).

The positive staircase effect at the higher range of the frequency of stimulation could be due to elevation of the cytoplasmic Ca\textsuperscript{2+} concentration resulting from increased Ca\textsuperscript{2+} influx via the I\textsubscript{Ca,l} as previously suggested (Borzak et al., 1991: Schouten and ter Keurs, 1991: Tang et al., 1996). Alternatively, the increase in the force of contraction could also be due to frequency-dependent alterations in the contractile proteins. however. no data confirming this hypothesis has been reported yet.

Unlike the peak force, the peak [Ca\textsuperscript{2+}]\textsubscript{i} continually increased as the frequency of stimulation was increased. This observation is consistent with previous reports that the peak Ca\textsubscript{i} transients in rat heart increased despite the negative inotropic force-frequency response (Morii et al., 1996). The increased [Ca\textsuperscript{2+}]\textsubscript{i} could be due to the increased Ca\textsuperscript{2+} influx via the I\textsubscript{Ca,l}. Moreover, increased frequency of stimulation is accompanied by elevation of [Na\textsuperscript{+}]\textsubscript{i} (Cohen et al., 1982: Bountra et al., 1988) which can decrease the electrochemical gradient of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger to extrude Ca\textsuperscript{2+}. as it normally happens in rat myocardium. and therefore cause a frequency-dependent increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Morii et al., 1996).
**Frequency-Dependent Changes in the Kinetics of Force and 
\([Ca^{2+}]_i\)**

The TD and the CaD (time from 50% contraction to 50% relaxation) continually decreased as the frequency was increased from 0.2 to 0.5, 1 and 2 Hz, regardless of the positive or negative force-frequency response. The decrease in the duration of force and \([Ca^{2+}]_i\) in response to increased frequency of stimulation, even during a negative force-frequency phase, was also previously reported (Borzak et al., 1991; Tang et al., 1996). The CaD abbreviation has been suggested to be due to increased rate of \(Ca^{2+}\) uptake by the SR \(Ca^{2+}\)-ATPase pump due to CaM Kinase II-mediated phosphorylation of phospholamban which subsequently causes TD abbreviation (Shouten et al., 1990; Bassani et al., 1995). The involvement of CaM Kinase II-induced phosphorylation of the SR \(Ca^{2+}\) ATPase pump or phospholamban in mediating the frequency-dependent CaD abbreviation has not been confirmed by other investigators (Frampton and Orchard, 1992; Odermatt et al., 1996). Figure 3.1-5 shows that following elevation of the frequency of stimulation, the time to peak and the relaxation phase of the twitch force were significantly accelerated whereas only acceleration of the relaxation phase of the \(Ca^{2+}\) transient was statistically significant. Therefore, the kinetics of the twitch force do not simply follow those of the \([Ca^{2+}]_i\) transients. This observation further suggests involvement of the contractile proteins in the frequency-dependent twitch kinetics alterations.

The significantly accelerated rate of relaxation of the \(Ca_i\) transient (\([Ca^{2+}]_i\)), when the frequency of stimulation was increased from 0.2 to 2 Hz, could be due to the \(Ca^{2+}\)-dependent activity of the SR \(Ca^{2+}\)-ATPase pump. It has been demonstrated previously that the time constant of \([Ca^{2+}]_i\) decline (\(t_{1/2}\)) is highly dependent on the range of the free \([Ca^{2+}]_i\) measured (Bers and Berlin, 1995). This is due to the nonlinear relationship between free \([Ca^{2+}]_i\) and both SR \(Ca^{2+}\)-ATPase pump flux and \(Ca^{2+}\) binding. Thus, a slow \(t_{1/2}\) for \([Ca^{2+}]_i\) decline of a smaller \(Ca_i\) transient is not necessarily due to any inherent difference in the \(Ca^{2+}\) transport properties (Bers and Berlin, 1995). In other words, a higher peak \([Ca^{2+}]_i\) is associated with a faster \([Ca^{2+}]_i\) decline because the time constant of \([Ca^{2+}]_i\) decline (\(\tau\)) is smaller when the \(Ca_i\) transient has a higher peak value. Bers and Berlin further explained that SR \(Ca^{2+}\)-ATPase pump functions in a \([Ca^{2+}]_i\)-dependent manner, so that its rate of \(Ca^{2+}\) uptake is higher at a higher peak \([Ca^{2+}]_i\).
However, the observation that the relaxation phase of the Ca$\text{\textsubscript{i}}$ transients at 0.2 and 2 Hz cross over (Figure 3.1-2B) suggests that the [Ca$^{2+}$]$\text{\textsubscript{i}}$-dependent upregulation of the SR Ca$^{2+}$-ATPase is not the only mechanism activated by the increased frequency of stimulation. If the [Ca$^{2+}$]$\text{\textsubscript{i}}$-dependent upregulation of the SR Ca$^{2+}$-ATPase pump were the only factor responsible for the accelerated relaxation of the Ca$^{2+}$ transient (and the twitch force), one would expect the rate of Ca$\text{\textsubscript{i}}$ transient relaxation to be equal after the crossing point where the [Ca$^{2+}$]$\text{\textsubscript{i}}$ is the same in both cases. We, therefore, conclude that the frequency-dependent acceleration of [Ca$^{2+}$]$\text{\textsubscript{i}}$ decline and twitch duration shortening must be mediated by other factors besides the increased rate of Ca$^{2+}$ uptake by the SR due to the elevated [Ca$^{2+}$]$\text{\textsubscript{i}}$. 
SECTION 3.2

The Sarcomere Length-Dependence of the Frequency-Dependent Twitch Kinetics Alterations

3.2.1 INTRODUCTION

The activation process in cardiac muscle is highly dependent on the muscle or the sarcomere length (Allen and Kentish, 1985). Sarcomere length (SL) is a major determinant of the force of contraction and the velocity of muscle shortening in rat myocardium (Daniels et al., 1984; Schouten et al., 1990). A decrease in the SL results in decreased twitch force and twitch duration (Backx and ter Keurs, 1993). These changes could be due to SL-dependent changes in the sensitivity of the myofilaments to Ca²⁺ (Hibberd and Jewel, 1982) and/or the affinity of Troponin C (Tn-C) for binding to Ca²⁺ (Hoffmann and Fuchs, 1987a, 1987b, 1988; Allen and Kentish, 1988; Saeki et al., 1993). Hence, the changes in twitch and [Ca²⁺]; transient duration might be modified by sarcomere length. Therefore, I studied the frequency-dependent changes in twitch and [Ca²⁺]; transient duration at short and long SLs.

3.2.2 SARCOMERE LENGTH-DEPENDENT CHANGES IN MYOFILAMENT Ca²⁺ SENSITIVITY

The SL-dependence of the myofilament Ca²⁺ sensitivity was originally hypothesized to reside in the number of attached cross bridges (Hofmann and Fuchs, 1988). However, this hypothesis was challenged by the observation made by Allen and Kentish that Ca²⁺ sensitivity was increased not only along the ascending limb of the force-length curve but also along the descending limb, where the number of cross-bridge attachments would be expected to decrease with increasing length (1985). Myofilament lattice shrinkage was later suggested to be the mechanism that accounts for the SL-dependence of myofilament Ca²⁺ sensitivity in cardiac muscle (Harrison et al., 1988). The role of interfilament spacing as a significant component of the length-dependent Ca²⁺ sensitivity was confirmed by two independent studies (McDonald and Moss, 1995; Wang
These reports were further supported by Fuchs and Wang's observation that in skinned bovine cardiac muscle bundles, over the SL range of 1.7-2.3 μm, the Ca\(^{2+}\) sensitivity was closely correlated with interfilament spacing, so that with relatively constant interfilament spacing changes in sarcomere length had virtually no effect on Ca\(^{2+}\) sensitivity (Fuchs and Wang. 1996). Thus, the degree of separation between actin and myosin filaments is the dominant factor in the length-dependent modulation of Ca\(^{2+}\) sensitivity, and both Ca\(^{2+}\) sensitivity and Ca\(^{2+}\)-troponin C affinity are correlated with interfilament spacing rather than with myofilament overlap or sarcomere length per se (Fuchs and Wang. 1996).

Regardless of whether it is filament overlap or interfilament spacing (or both) that is the important factor in Ca\(^{2+}\) sensitivity of the myofilaments, it is cross-bridge attachment that controls Ca\(^{2+}\) regulatory protein function to muscle fiber length (Wang and Fuchs, 1995). The specific role of the cross bridges in myofilament Ca\(^{2+}\) sensitivity upon decreased interfilament spacing is still not clear, although it has been suggested that moderate lattice compression mainly acts to stabilize the strong binding actin.myosin.ADP (A.M.ADP) complex which is considered to be a force-generating cross-bridge state (Zhao and Kawai, 1993). This hypothesis accounts for the increased force generation seen with compression of both fully activated and partially activated fibers (Wang and Fuchs, 1995).

This dominant role of the myofilament spacing in the Ca\(^{2+}\) sensitivity of the myofilaments does not deny the role of the SL-dependent changes in the myofilament Ca\(^{2+}\) sensitivity and Ca\(^{2+}\) binding affinity of Tn-C, because in vivo and under normal conditions decreased (or increased) sarcomere length (preload) is associated with an increase (or decrease) in the interfilament spacing (Elliot, 1973).

Due to the influence of the SL on the peak force and the twitch kinetics, we became interested in studying whether the frequency-dependent TD shortening was
sustained at different sarcomere lengths. We compared the TD at 0.2 to 2 Hz at a slack SL of 1.9 µm to that at the SL of 2.2-2.3 (the control data).

3.2.3 RESULT

Twitch forces at two different sarcomere lengths (1.9 and 2.2 µm) are compared in figure 3.2-1 are compared at 0.2 Hz (A) and 2 Hz (B). At 0.2 Hz, decreasing the SL from 2.2 to 1.9 µm, significantly decreased the peak force from 21.79 ± 2.61 to 8.5 ± 1.22 mN/mm² (P<0.05, n=4). When stimulated at 2 Hz, the peak force at SL of 1.9 µm remained significantly smaller than that at 2.2 µm (23.44 ± 5.49 mN/mm² at 2.2 µm and 11.41 ± 1.55 mN/mm² at 1.9 µm; P<0.05, n=4). These SL-dependent changes in the peak force were accompanied by alterations in the twitch kinetics. Twitch duration was significantly shorter at the shorter SL at both low and high frequencies of stimulation. At 0.2 Hz, TD (50% contraction to 50% relaxation) was 296.75 ± 19.4 msec at the SL of 2.2 µm and 191.63 ± 21.32 msec at 1.9 µm (P<0.05, n=4). At 2 Hz it was 177.50 ± 21.76 and 141.25 ± 11.21 msec at sarcomere lengths of 2.2 and 1.9 µm, respectively (P<0.05, n=4).

Twitch forces recorded at the slack SL (1.9 µm), at 0.2 and 2 Hz are superimposed in figure 3.2-2A. This figure shows an increase in the amplitude and a decrease in the duration of the twitch force following elevation of the frequency of stimulation. The TD shortening as a result of increased frequency of stimulation is more evident in figure 3.2-2B where the force traces at the two frequencies are scaled. At 1.9 µm, twitch duration decreased from 191.63 ± 21.32 to 141.25 ± 11.21 following elevation of the frequency of stimulation from 0.2 to 2 Hz (P<0.05, n=4). The rate-dependent changes in the amplitude and kinetics of the twitch force at the slack SL of 1.9 µm are therefore identical to those at the control SL of 2.2 µm (Compare figure 3.2-2A to 3.1-2A and figure 3.2-2B to 3.1-3A). Thus, the frequency-dependent alterations in twitch kinetics are present at short as well as long sarcomere lengths.
Figure 3.2-1: Twitch force at two different sarcomere lengths.

A) Twitch forces at sarcomere lengths (SL) of 1.9 (solid line) and 2.2 (broken line) μm. at 0.2 Hz. The peak force was significantly smaller at the shorter SL. B) Force traces at 1.9 and 2.2 μm (solid and broken line, respectively) at 2 Hz. At both sarcomere length the peak force increased in response to an increase in the frequency of stimulation. however, the difference between the peaks was still significant at 2 Hz.
Figure 3.2-1

(A) 0.2 Hz

(B) 2 Hz

Force (mN/mm²) vs. Time (sec)

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SL=1.9 μm

SL=2.2 μm

(Figure 3.2-1)
Figure 3.2-2: Twitch force at SL of 1.9 μm at 0.2 and 2 Hz.

A) Superimposed force of contraction at 0.2 (solid line) and 2 Hz (broken line) at 1.9 μM. This figure shows the increase in the peak force following elevation of the frequency of stimulation. B) Scaled force of contraction at 0.2 (solid line) and 2 Hz (broken line). Notice the acceleration of the rate of contraction as well as the rate of relaxation phase at 2 Hz compared to 0.2 Hz.
SL = 1.9 μm

0.2 Hz

2 Hz

(Figure 3.2-2)
3.2.4 DISCUSSION

The force of contraction is regulated by a number of factors such as [Ca$^{2+}$]$_o$, temperature and SL. We compared the force-frequency and the twitch duration-frequency relations at two different sarcomere lengths, 2.2 and 1.9 μm. We observed that decreasing the SL caused an initial decrease in force and TD, however it did not affect the pattern of their frequency-dependent changes. Therefore, the rate-dependent changes in the force kinetics are present in different sarcomere lengths and are not altered by a change in the SL.
Section 3.3

$\text{Ca}^{2+}$-Activated Intracellular Kinases

3.3.1 INTRODUCTION

Integrated Time-Averaged $[\text{Ca}^{2+}]_i$

Integrated time-averaged intracellular $\text{Ca}^{2+}$. $\langle [\text{Ca}^{2+}]_i \rangle$, is the mean level of $[\text{Ca}^{2+}]_i$, averaged over several tens of seconds. $\langle [\text{Ca}^{2+}]_i \rangle$ was measured at the different frequencies of stimulation by using the following formula:

$$\langle [\text{Ca}^{2+}]_i \rangle = \frac{1}{T} \int_0^T [\text{Ca}^{2+}]_i (t) \, dt$$

where, $T$ is the inter-pulse interval. In other words, $\langle [\text{Ca}^{2+}]_i \rangle$ is the product of the area under the $[\text{Ca}^{2+}]_i$ transient curve and the frequency of stimulation. The time-averaged $[\text{Ca}^{2+}]_i$ as a function of the frequency of stimulation in shown in figure 3.3-1. The data were fitted by a linear regression model ($P=0.004$). As seen in figure 3.3-1. $\langle [\text{Ca}^{2+}]_i \rangle$ increased linearly (from $0.094 \pm 0.023 \text{ to } 0.255 \pm 0.030 \text{ M/sec}$. $P<0.05$. $n=4$) by increasing the rate of stimulation from 0.2 to 2 Hz. Such a linear increase has also been reported by other investigators (Bountra et al., 1988: Kaye et al., 1996). These data show that increasing the frequency of stimulation not only increases the peak $[\text{Ca}^{2+}]_i$ transient but also increases the amount of $\text{Ca}^{2+}$ in the cytoplasm at any given time. The elevated $[\text{Ca}^{2+}]_i$ can activate $\text{Ca}^{2+}$-dependent enzymes such as calmodulin (Bassani et al., 1995), nitric oxide synthase (NOS) (Kaye et al., 1996), and adenylate cyclase (AC), which consequently activate $\text{Ca}^{2+}$-calmodulin-dependent protein kinase II (CaM/kinase II), cGMP-dependent protein kinase (PKG), and cAMP-dependent protein kinase (PKA), respectively. These kinases phosphorylate a number of proteins among which PLB, SR $\text{Ca}^{2+}$-ATPase, $I_{\text{Ca}}$, and Tn-l are involved in force development and $\text{Ca}^{2+}$ handling in cardiac myocytes.
Figure 3.3-1: Integrated time-averaged $[\text{Ca}^{2+}]_i$ as a function of the frequency of stimulation.

The integrated time-averaged $[\text{Ca}^{2+}]_i$, $\langle [\text{Ca}^{2+}]_i \rangle$, increased linearly as the frequency of stimulation (Freq.) was increased from 0.2 to 2 Hz. The data were fitted by a linear regression model. $\langle [\text{Ca}^{2+}]_i \rangle = 0.09 \text{ (Freq.)} + 0.08$. $P=0.005$, $r = 0.99$. 
Frequency of stimulation (Hz)

Integrated time-averaged $[\text{Ca}^{2+}]_i$ (μM/sec)

(Figure 3.3-1)
3.3.2 ACTIVATION OF CAMKINASE II

Calcium-calmodulin dependent protein kinase (CaM Kinase) is activated in the presence of elevated \( [\text{Ca}^{2+}]_i \). Calmodulin (CaM) alters its structure following binding of four \( \text{Ca}^{2+} \) ions to its \( \text{Ca}^{2+} \) binding sites. The \( \text{Ca}^{2+} \)-bound CaM then activates a number of CaM kinases including CaM Kinase II which is present in cardiac cells (Kennedy and Greengard, 1981; Edman and Schulman, 1994) where it phosphorylates a number of intracellular proteins. It phosphorylates and regulates three major SR proteins: SR \( \text{Ca}^{2+} \) release channel or ryanodine receptor (RyR) (Takasago et al., 1991; Xu et al., 1993; Hawkins et al., 1994; Netticadan and Naryanan, 1996). SR \( \text{Ca}^{2+} \) ATPase pump at Ser\(^{38} \) (Xu et al., 1993; Hawkins et al., 1994; Toyofuku et al., 1994; Odermatt et al., 1996; Netticadan and Naryanan, 1996). phospholamban (Bilezikian et al., 1981; Baltals et al., 1995; Naryanan and Xu, 1997; Xu et al., 1997), and Troponin T at Thr\(^{100} \) (Jaquet et al., 1995). Phosphorylation of these proteins can influence the excitation-contraction (E-C) coupling. Phosphorylation of phospholamban will increase the rate of \( \text{Ca}^{2+} \) uptake by the SR. Therefore, can accelerate the rate of relaxation of the \( \text{Ca}^{2+} \) transient. The functional consequences of phosphorylation of \( \text{Ca}^{2+} \) release channel proteins has not yet been established. Li et al. recently reported that phosphorylation of this channel results in a change in its sensitivity to activation by \( \text{Ca}^{2+} \) such that greater SR \( \text{Ca}^{2+} \) efflux occurs for a given \( \text{l}_{\text{Ca}} \) trigger (1997). This conclusion was drawn from the observation that activity of the SR \( \text{Ca}^{2+} \) release was decreased following application of a specific CaM Kinase II inhibitor, KN-93.

Based on the previously reported role of the CaM kinase II in intracellular \( \text{Ca}^{2+} \) handling, as well as its role in the activity-dependent acceleration of relaxation in rat ventricular myocytes (Bassani et al., 1995), it was logical to hypothesize that activation of this kinase by the increased \( [\text{Ca}^{2+}]_i \) following increased frequency of stimulation, could play a role in the frequency-dependent acceleration of the kinetics of the twitch force. In order to test this hypothesis, the \( F \) and the \( \text{Ca}^{2+} \) transient duration were recorded over a range of frequencies of stimulation after CaM Kinase II was inhibited by a specific inhibitor.
3.3.2i **METHOD**

CaM Kinase II was inhibited by a water soluble potent inhibitor, KN-93 (Sumi et al., 1991). KN-93 specifically inhibits CaM Kinase II (K_i=0.37 μM, K_i for other enzymes >30 μM), as well as autophosphorylation of its α- and β-subunits, in a competitive fashion against calmodulin. It selectively and directly binds to the CaM-binding site of CaM Kinase II or its vicinity, and prevents the association of CaM and CaMKII (Sumi et al., 1991). KN-93 was dissolved in distilled water to make a 2 mM stock solution. 37.5 μL of the stock solution was diluted in 75 mL K-H solution to obtain a final concentration of 1 μM. We also used higher concentrations of KN-93, up to 5 μM (187 μL of the stock added to 75 mL K-H).

We originally purchased KN-93 from Research Biochemicals International (RBI). Because of the absence of any significant change in the kinetics of the force or the Ca^{2+} transient in the presence of this inhibitor, we also purchased this compound from the company where the drug was originally developed, Seikagaku America Inc. Similar results were observed after using KN-93 from Seikagaku America. The data presented here is the pooled data of the KN-93 from the two different sources. The data were collected 8-10 minutes after application of the drug.

3.3.2ii **RESULTS**

Figure 3.3-2A shows the force and the [Ca^{2+}]_i traces under control conditions (solid line) and in the presence of 5 μM KN-93 (broken line) at 0.2 and 2Hz. None of the used concentrations of KN-93 (1 to 5 μM) caused a significant change in the kinetics of the force and the [Ca^{2+}]_i. At 0.2 Hz, 5 μM KN-93 changed the TD from 312.25 ± 28.46 to 319.75 ± 28.32 (P=0.82, n=4), and the CaD changed from 158.33 ± 22.45 to 196.33 ± 29.09 msec (P=0.11, n=4).

Figure 3.3-2B shows the TD- and CaD-frequency relationship (left and right panels, respectively) in the absence (solid line) and presence (broken line) of KN-93 (n=4). Inhibition of CaM kinase II did not alter the pattern of the frequency-dependent change in TD and CaD.
Figure 3.3-2: Effect of 5 μM KN-93 (CaM Kinase II inhibitor) on the frequency-TD relationship.

A) Force and [Ca$^{2+}$]_i traces in the absence (solid line) and presence (broken line) of KN-93 at 0.2 Hz (left panel) and 2 Hz (right panel). B) TD- and CaD- frequency relationship in control conditions (solid line) and in the presence of KN-93 (broken line). KN-93 did not make a significant difference in these relationships. The TD and the CaD were the time from 50% contraction to 50% relaxation.
Figure 3.3-2
In the presence of KN-93, TD decreased from $319.75 \pm 28.32$ (at 0.2 Hz) to $194.25 \pm 9.98$ msec at 2Hz ($P<0.05$, $n=4$), and CaD decreased from $196.33 \pm 29.09$ (at 0.2 Hz) to $101.0 \pm 4.58$ msec at 2 Hz ($P<0.05$, $n=4$). Therefore, the frequency-dependent activation of CaM Kinase II is not the underlying mechanism of the frequency-dependent TD shortening.

### 3.3.3 Activation of Nitric Oxide Synthase (NOS)

Nitric oxide (NO) is produced by the conversion of L-arginine and oxygen into NO and citrulline, a reaction that is catalyzed by a constitutive Ca$^{2+}$-sensitive NO synthase (cNOS) (Grocott-mason and Anning, 1994; Kaye, 1996). NO is released from vascular endothelial cells (VEC) (Vane, 1990) and relaxes vascular smooth muscle by lowering levels of intracellular ionized free Ca$^{2+}$ through activation of a soluble guanylate cyclase (Ignarro \textit{et al.}, 1987; Moncada \textit{et al.}, 1988; Palmer \textit{et al.}, 1987, 1988). The heart contains large numbers of endothelial cells, which line both the inner cavity surface (endothelial cells, EEC) and the coronary blood vessels (VEC). There is approximately a 1:1 ratio between the number of capillaries and cardiac myocytes, and any myocyte is only a few microns away from a capillary VEC (Berne and Rubio, 1980). It has been shown that NO is elevated in isolated rat ventricular myocytes in a frequency-dependent manner (Finkel \textit{et al.}, 1995; Kaye \textit{et al.}, 1996). NO has also been reported to be responsible for the negative force-frequency relationship in rat and hamster papillary muscles and its inhibition by L-NMMA ($N^G$-monomethyl-L-arginine) reversed this relationship to positive (Finkel \textit{et al.}, 1995).

The main subcellular action of NO is activation of soluble guanylate cyclase (GC) causing elevation of intracellular guanosin 3',5'-cyclic monophosphate (cGMP) (Moncada \textit{et al.}, 1991) which subsequently activates cGMP-dependent protein kinase (PKG). Substrates for PKG include $I_{Ca,L}$ (Mery \textit{et al.}, 1991). Tn-I (Pfitzer \textit{et al.}, 1982), phospholamban and SR Ca$^{2+}$ release channels. Phosphorylation of phospholamban by PKG increases the uptake of Ca$^{2+}$ into the SR (Raeymaekers \textit{et al.}, 1988), but phospholamban does not appear to be a good substrate for PKG in intact
cardiac tissue (Huggins et al., 1989). PKG may also regulate cardiac contractility through phosphorylation of Tn-I which will decrease myofilament Ca\(^{2+}\) sensitivity (Pfizer et al., 1982). The physiological significance of phosphorylation of the SR Ca\(^{2+}\) release channel by PKG is not yet known (Takasago et al., 1991), although it has been suggested to be involved in the NO-induced negative force-frequency relation in hamsters (Finkel et al., 1995). It has also been suggested that cGMP predominantly inhibits L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) via a mechanism involving PKG (Levi et al., 1989; Lohmann et al., 1991; Mery et al., 1991).

It has been shown that release of NO in isolated ferret papillary muscles induced earlier myocardial relaxation and a small decrease in peak force, but no change in the early phase of contraction was observed (Smith et al., 1991). These NO-mediated changes were accompanied by a rise in myocardial cGMP. Kaye et al. also demonstrated that repetitive depolarization accompanied by contraction in isolated adult rat ventricular myocytes was associated with enhanced generation of NO due to activation of a Ca\(^{2+}\)-activated constitutive isoform of NOS in these cells (1996). Moreover, Kaye et al. found that inhibition of NOS by L-NA (nitro-L-arginine) increased the amplitude of shortening at 3 Hz (1996).

Therefore, the frequency-dependent elevation of NO could play a role in the frequency-dependent acceleration of the force and the Ca\(^{2+}\) transient kinetics. If this hypothesis were correct, inhibition of NO formation by inhibiting NOS should have abolished the frequency-TD and frequency-CaD relationship.

3.3.3i **METHOD**

NOS was inhibited by L-NOARG (N\(^{G}\)-Nitro-L-Arginin) a potent reversible inhibitor of NOS (K\(_i\)=200 nM). L-NOARG was dissolved in distilled water to obtain a 50 mM stock solution. 200 μL of the stock solution was added to 100 mL of the
perfusion solution (K-H) to give a final concentration of 100 μM. The data was collected 8-10 minutes after application of the drug.

3.3.3ii RESULTS

Inhibition of NOS and therefore inhibition of NO accumulation did not cause significant changes in the amplitude or kinetics of the force of contraction and the Ca\(^{2+}\) transient. 50 μM L-NOARG changed the TD (msec) from 328.0 ± 2.0 to 315.1 ± 5.5 (P=0.15, n=4), and the CaD (msec) 179.3 ± 4.6 to 161.2 ± 27.7 msec (P=0.17, n=4), at 0.2 Hz (Figure 3.3-3A). The twitch duration as well as the CaD continued to decrease as the frequency of stimulation was increased. Figure 3.3-3B shows that despite inhibition of NOS, increasing the frequency of stimulation from 0.2 to 2 Hz significantly decreased the TD from 315.1 ± 5.5 to 242.5 ± 12.5 msec (P<0.05, n=4) and the CaD from 161.2 ± 27.7 to 104.5 ± 0.5 msec. Therefore, frequency-dependent accumulation of NO is not responsible for the frequency-TD and frequency-CaD relations.
Figure 3.3-3: Effect of 100 μM L-NOARG (NOS inhibitor) on the frequency-TD relationship.

A) Force and \([\text{Ca}^{2+}]_i\) transient traces in control (solid line) and in the presence of L-NOARG (broken line), at 0.2 (left panel) and 2 Hz (right panel). B) TD- and CaD-frequency relationships in the absence (solid line) and presence (broken line) of L-NOARG. These relationships were not altered in the presence of this drug. The TD and the CaD were the time from 50% contraction to 50% relaxation.
(Figure 3.3-3)
3.3.4 ADENYLATE CYCLASE (AC)

Adenylate cyclase (AC) is the enzyme that converts ATP to cAMP. To date, nine distinct isoforms of mammalian AC (designated I to IX) have been cloned and characterized (Scholich et al., 1997). The various AC isoforms are divided into five groups based on their regulations. Group one consists of type I, III and VIII AC which are stimulated by Ca\(^{2+}\) or calmodulin (Bakalyar and Reed, 1991; Tang et al., 1991; Choi et al., 1992; Cali et al., 1996). Group two contains type II and IV which are stimulated by βγ subunits of the heterotrimeric G-protein (G\(_{\alpha}\)) (Feinstein et al., 1991; Gao and Gilman, 1991; Tang and Gilman, 1991). Type II and type VII isoforms are phosphorylated and activated by PKC and constitute group three (Choi et al., 1992; Krupinski et al., 1992; Cali et al., 1996). Group four is comprised of type V and VI AC which are inhibited by Ca\(^{2+}\) (Ishikawa et al., 1992; Permont et al., 1992; Yoshimura and Cooper, 1992). Group five contains only type IX AC which is regulated by calcineurin (Paterson et al., 1995). All various AC isoforms share one common regulatory feature. They are all stimulated by the active, GTP-bound, G\(_{\alpha}\) (Iyengar, 1993; Taussig and Gilman, 1995).

Every tissue contains more than one isoform of AC. For instance, as many as seven different AC isoform are expressed in rat heart, liver, and kidney (Krupinski et al., 1992). Rat heart has been reported to predominantly contain type VII isoform (50%), and other isoforms in smaller proportions (18% type VI, 8% type V, 10% type III, 10% type IV, 2% type I, and 2% type II). DNA sequencing method did not show any presence of type VIII AC isoform in rat heart. Therefore, from the total AC found in rat heart, type I and III (about 12% of the total cardiac AC) are stimulated by Ca\(^{2+}\) and types V and VI (26% of the cardiac AC) are inhibited by Ca\(^{2+}\). Therefore, the effect of elevated \([\text{Ca}^{2+}]_i\) and \(\langle [\text{Ca}^{2+}] \rangle\) as a result of increased frequency of stimulation cannot be clearly predicted.

Activation of AC results in activation of cAMP-dependent protein kinase (PKA) via elevation of the intracellular [cAMP]. PKA phosphorylates a number of proteins such as phospholamban (Katz, 1990; Nadiani et al., 1996; Tada and Toyofuku, 1996), SR Ca\(^{2+}\) ATPase pump (Tada et al., 1974; LePeuch et al., 1979), SR Ca\(^{2+}\) release channel (Valdivia et al., 1995).
L-type Ca\(^{2+}\)-channels (\(I_{\text{CaL}}\)) (Gao et al., 1997; Puri et al., 1997), troponin C (Kiss et al., 1997) and troponin I (Solaro et al., 1976; Johns et al., 1997; Malhorta et al., 1997). Phospholamban functions as an inhibitory co-factor for cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump (SR Ca\(^{2+}\)-ATPase). PKA-mediated phosphorylation of phospholamban results in its dissociation from the SR Ca\(^{2+}\)-ATPase, thus augmenting the ATPase activity and increasing the Ca\(^{2+}\) uptake by the SR. Phosphorylation of \(I_{\text{CaL}}\) results in increased Ca\(^{2+}\) influx via this channel which could contribute to the elevated intracellular Ca\(^{2+}\) transient associated with \(\beta\)-AR stimulation. Phosphorylation of Tn-I causes a decrease in calcium sensitivity of the contractile proteins which has been suggested to be responsible for the \(\beta\)-AR-induced twitch abbreviation (Suard et al., 1994; Malhorta et al., 1997).

Different mechanisms have been proposed to be responsible for the inotropic effect of the \(\beta\)-AR stimulation. \(\beta\)-AR-mediated alterations in the intracellular Ca\(^{2+}\) handling was previously believed to be responsible for its inotropic effects (Endoh and Blinks, 1988; Suga, 1990). Recent studies have shown that the direct effect of the \(\beta\)-AR stimulation on the number (Katz, 1979; Winegrad, 1984; Sperelakis, 1988) or the rate of cross-bridge cycling (Berman et al., 1988; Hoh et al., 1988; Saeki et al., 1990, 1991, 1997) could be responsible for the inotropic effect of the \(\beta\)-AR stimulation. It has been suggested that \(\beta\)-AR stimulation increases the rate of cross-bridge cycling and therefore shortens the TD by increasing the rate of cross-bridge detachment from actin through a PKA-mediated mechanism (Hofman and Lang, 1994; Saeki et al., 1997). This suggestion was based on the observation that PKA, which mimics the action of \(\beta\)-agonists (Garvey et al., 1988), in demembranated preparations, increased \(V_{\text{max}}\), which serves as a measure of the rate of ATP turnover (i.e. the maximum rate of cross-bridge cycling). PKA also shifted the tension-pCa\(^{2+}\) relation rightward (i.e. decreased myofilament Ca\(^{2+}\) sensitivity) without changing the steepness of the tension-pCa\(^{2+}\) relation (the cooperativity) and the maximum Ca\(^{2+}\)-activated tension (\(F_{\text{max}}\)) (Saeki et al., 1997). The \(\beta\)-AR-mediated increase in \(V_{\text{max}}\) has not been confirmed by other investigators (Chiu et al., 1989; de Tombe and ter Keurs, 1991; de Tombe and Steinen, 1995). Chiu et al. as well as de Tombe and ter Keurs studied the effect of \(\beta\)-agonists on intact cardiac muscles and observed no significant change in \(V_{\text{max}}\). The
absence of a change in $V_{\text{max}}$ in these experiments could be because of the used preparation (intact trabecula) which could have caused the potentiating effect of the β-agonists on the $V_{\text{max}}$ to be overlooked because of the difficulties in determining $V_{\text{max}}$ during a twitch, in which the level of activation is continually changing with time (Saeki et al., 1997). De Tombe and Steinen (1995), however, used chemically skinned cardiac trabeculae (similar preparation to that used by Saeki et al., 1997) and observed a rightward shift in the $F_{\text{max}}$-[Ca$^{2+}$]$_i$ curve without any change in the steepness of the curve or the rate of ATP consumption, following PKA treatment. The absence of a change in the rate of ATP consumption following PKA application means that the rate of cross-bridge turnover remained unchanged. According to de Tombe and Steinen (1995), the mechanism underlying the profound reduction in the duration of twitch contraction following β-AR stimulation is unknown, and it could be related in part to alterations in the kinetics of Ca$^{2+}$ binding to Tn-C, a phenomenon that is unrelated to cross-bridge cycling rate. Dobrunz et al. (1995) also reported a rightward shift in the force-pCa$^{2+}$ relation and no change in the $F_{\text{max}}$ in the presence of isoproterenol suggesting an elevation in the off-rate of Ca$^{2+}$ unbinding from Tn-C or cross-bridge detachment. However, Dobrunz et al. further reported an isoproterenol-induced decrease in the cooperativity (decreased slope of the force-pCa$^{2+}$ relation) which was not observed by Saeki et al. or de Tombe and Steinen. This difference could be due to the different experimental preparations. Saeki et al. and de Tombe and Steinen used skinned rat trabeculae whereas Dobrunz et al. experimented on intact rat cardiac trabecula.

Therefore, acceleration of intracellular Ca$^{2+}$ handling (by phosphorylation of PLB and SR Ca$^{2+}$-ATPase: McIvor et al., 1988; Endoh et al., 1988) and decreased Ca$^{2+}$ binding affinity of Tn-C (due to phosphorylation of Tn-I (Swederek et al., 1988; Zhang et al., 1995 a,b) are the two major proposed mechanisms underlying the β-AR-induced acceleration of force kinetics. Some authors have suggested that phospholamban phosphorylation could be prominent in the explanation of the isoproterenol-mediated relaxation (McIvor et al., 1988; Endoh et al., 1988). These authors reasoned that force kinetics follow Ca$^{2+}$ transient kinetics, therefore, phospholamban phosphorylation that accelerates Ca$^{2+}$ transient decline also increases the rate of
twitch relaxation. Other authors, however, suggested that the isoproterenol-mediated TD shortening was due to changes in the contractile proteins. Two residues on cardiac troponin I (CTn-I), serine 22 and 23, are phosphorylated by PKA upon stimulation of the heart by β-AR agonists (Cole and Pettry, 1975; Swiderek et al., 1988; Zhang et al., 1995a; Zhang et al., 1995b). Phosphorylation of Tn I by PKA has been shown to decrease the affinity of the single regulatory Ca\(^{2+}\)-specific site of cardiac Tn-C for Ca\(^{2+}\) (Robertson et al., 1982; Zhang et al., 1995a,b). It was also demonstrated that phosphorylation of Tn-I had little effect on the time to peak or the time to half-peak Ca\(^{2+}\) saturation of Tn-C, but the time to half-relaxation decreased dramatically (Robertson et al., 1982). The exact mechanism as to how Tn-I phosphorylation affects the dissociation of Ca\(^{2+}\) is still not clear, but may involve a change in the interaction between Tn-I and Tn-C (Liao et al., 1990). Exposure of skinned cardiac muscle preparations to PKA, where SR Ca\(^{2+}\) pump and phospholamban where chemically removed, enabled Zhang et al. to determine contribution of Tn-I phosphorylation, by PKA, to acceleration of the rate of relaxation (1995). This experiment showed that relaxation time shortened significantly following Tn-I phosphorylation despite the absence of the Ca\(^{2+}\) uptake mechanism demonstrating a decrease in the Ca\(^{2+}\) sensitivity of the contractile filaments. These investigators also showed that the half relaxation time (t\(_{1/2}\)) changed by only 10% in response to phosphorylation of PLB alone, and changed by 40% in response to phosphorylation of both PLB and cardiac Tn-I. This experiment shows that even if the SR is able to reduce the Ca\(^{2+}\) transient faster, it will not speed up relaxation very much unless Ca\(^{2+}\) also dissociates faster from Tn-C, keeping pace with the faster pumping of the SR (Zhang et al., 1995a). The significant role of CTn-I phosphorylation in acceleration of the relaxation phase in response to β-AR stimulation was further confirmed by the transgenic mice whose PKA phosphorylation sites on Tn-I (Serine 22 and 23) where mutated to alanine (Zhang et al., 1995b). This study demonstrated that PKA phosphorylation of Tn-I, and not other proteins, is responsible for the decrease in Ca\(^{2+}\) sensitivity.

The inotropic effect of isoproterenol is well established. Application of 100 nM isoproterenol in isolated rat hearts increased the developed pressure (Ebihara and Karmazyn.
1996), the contractility (Kiss et al., 1997) and the Ca\(^{2+}\) transient. It also decreased the twitch and the Ca\(^{2+}\) transient duration in isolated myocytes (Ebihara and Karmazyn, 1996). Thus, the effects of β-AR stimulation on the force of contraction and the intracellular Ca\(^{2+}\) transient resemble those of elevated frequency of stimulation. They both increase the amplitude and accelerate the kinetics of the force and the Ca\(^{2+}\) transient.

Although the increased [Ca\(^{2+}\)]\(_i\) would not result in net activation of the cardiac AC. similarity of the effect of the increased rate of stimulation and AC activation (via β-AR stimulation) suggests that the elevated frequency of stimulation could activate this enzyme via a different pathway or could activate other factors in the β-AR agonist-activated pathway downstream from AC. Therefore, in order to investigate whether increased frequency of stimulation and β-AR stimulation share the same pathway to accelerate the TD, we first compared the effect of these two factors on the twitch force and the [Ca\(^{2+}\)]\(_i\) transient and their kinetics, and then measured the frequency-mediated twitch duration changes following interruption of the isoproterenol-activated pathway, by a PKA inhibitor.

3.3.4i METHOD

**ISOPROTERENOL.**

Isoproterenol, a non-selective β-AR agonist (β\(_1\)- and β\(_2\)-AR agonist), was dissolved in distilled water to make a 1mM stock solution. The stock solution was stored in the dark at -20° C for no longer than one month. 10 µL of the stock solution was diluted in 100 mL oxygenated K-H solution to give a final concentration of 100 nM.

**NOREPINEPHRINE**

Norepinephrine, an adrenergic agonist (α\(_1\)- and β\(_1\)-AR), was dissolved in distilled water to give a 1mM stock solution that was stored at -20° C. 10 µL of the stock solution was diluted in the oxygenated K-H solution to a final concentration of 100 nM.

**PHENTOLAMINE MESYLATE**
Phentolamine mesylate (PhM), an α-AR antagonist, was used before application of norepinephrine in order to block the α₁-AR activity so that pure β-AR activation could be studied. 1 mM stock solution was made by dissolving PhM in distilled water and was kept at room temperature. 100 μL of the stock solution was added to 100 mL oxygenated K-H solution to a final concentration of 1 μM.

K252-a

100 μg of K252-a, a non-selective inhibitor of a number of kinases including PKA, PKC, PKG and CaM Kinase, was dissolved in 15 μL DMSO (Dimethyl Sulfoxide) and was diluted in 75 mL K-H solution to a final concentration of 2.8 μM. K252-a was kept in dark and at -20°C.

3.3.4ii RESULT

β-ADRENOCEPTOR STIMULATION

A) ISOPROTERENOL

Application of 100 nM isoproterenol at 0.2 Hz. significantly (P<0.05, n=5) increased the force of contraction (from 21.79 ± 2.61 to 35.22 ±0.83 mN/mm²) and the [Ca²⁺]i transient (from 0.52 ± 0.03 to 0.79 ± 0.12 μM). It also significantly decreased the TD (from 296.75 ± 19.39 to 191.25 ± 8.09 msec) and the CaD (from 218.25 ± 31.14 to 120 ± 7.79 msec) (Figure 3.3-4A). Figure 3.3-4B shows the percent change in the force and the [Ca²⁺]i transient following β-AR stimulation by isoproterenol. 100 nM isoproterenol at 0.2 Hz. increased the peak twitch force by 64.3 ± 20.1 % and the peak [Ca²⁺]i transient by 48.15 ± 14.74 %. The kinetics of the force and the Ca²⁺ transient were abbreviated by 35.2 ± 2.7 and 42.7 = 7.6, respectively.
Figure 3.3-4: Effect of 100 nM isoproterenol on the force and the $[Ca^{2+}]_i$ transient.

A) The amplitude of the force and the $[Ca^{2+}]_i$ transient were increased and their kinetics were accelerated in the presence of isoproterenol. B) The percentage change in the amplitude and kinetics of the force and the $[Ca^{2+}]_i$ transient in the presence of isoproterenol. TD and CaD were the time from 50% contraction to 50% relaxation.
A

CONTROL

ISOPROTERENOL

Time (sec)

Force (mN/mm²)

Time (sec)

[Ca²⁺] (μM)

B

% change in peak force

% change in peak [Ca²⁺]

% change in twitch duration

% change in [Ca²⁺] duration

(Figure 3.3-4)
FREQUENCY-DEPENDENT ALTERATIONS IN THE FORCE AND THE $Ca^{2+}$-TRANSIENT IN THE PRESENCE OF ISOPROTERENOL

The changes in the force and the $[Ca^{2+}]_i$ and their kinetics as the frequency of stimulation was increased from 0.2 to 2 Hz under control conditions (solid line) and in the presence of 100 nM isoproterenol (broken line) are shown in Figure 3.3-5. Isoproterenol induced a negative force-frequency and a positive $[Ca^{2+}]_i$-frequency relationship (Fig. 3.3-5A and B). In the presence of 100 nM isoproterenol ($n=5$), the force of contraction (mN/mm$^2$) decreased significantly from $35.22 \pm 0.83$ at 0.2 Hz to $26.23 \pm 1.46$ at 2 Hz ($p<0.05$), and the $[Ca^{2+}]_i$ ($\mu$M) increased significantly from $0.79 \pm 0.11$ at 0.2 Hz to $1.20 \pm 0.20$ at 2 Hz ($p<0.05$).

The negative force-frequency relationship in the presence of isoproterenol thus was not due to a decrease in the $[Ca^{2+}]_i$, but the combination of isoproterenol and increased frequency of stimulation could have caused SR $Ca^{2+}$ overload which would cause spontaneous $Ca^{2+}$ release (Fabiato and Fabiato, 1975; Allen et al., 1985; Mulder et al., 1989). This in turn could have attenuated the expected inotropic effect (Kart and Lakatta, 1988). This negative force-frequency relationship could also be due to alterations in the contractile protein activity due to the presence of isoproterenol and increased frequency of stimulation, so that their responsiveness to the increased $[Ca^{2+}]_i$ was diminished. The TD and the CaD were significantly shorter in the presence of isoproterenol (compared to control) and continued to decrease as the frequency of stimulation was increased (Fig. 3.3-5 C and D). In the presence of isoproterenol, TD (msec) decreased from $191.25 \pm 8.09$ at 0.2 Hz to $136.50 \pm 4.41$ at 2 Hz ($P<0.05$), and CaD (msec) decreased from $120.00 \pm 7.79$ at 0.2 Hz to $76.50 \pm 13.62$ at 2 Hz ($P<0.05$). Interestingly, figures 3.3-5 C and D show that the TD-frequency and the CaD-frequency curves in the presence and absence of isoproterenol tend to converge at higher frequencies of stimulation. This observation suggests that the same mechanism may be involved in isoproterenol- and frequency-induced TD shortening.
Figure 3.3-5: Frequency-dependent alterations in the force and $[\text{Ca}^{2+}]_i$ in the presence and absence of 100 nM isoproterenol.

At all the frequencies of stimulation, the peak force and the peak $[\text{Ca}^{2+}]_i$ were significantly higher and their duration were significantly shorter in the presence of isoproterenol (broken line) compared to the control (solid line). In the presence of isoproterenol, a negative peak force-frequency relationship was observed despite a positive peak $[\text{Ca}^{2+}]_i$-frequency relationship. The TD-frequency and CaD-frequency curves in control and in the presence of isoproterenol tend to converge at higher frequencies of stimulation, which suggests involvement of the same mechanism in these two cases.
(Figure 3.3-5)
B) NOREPINEPHRINE

We also examined the effect of β-AR stimulation on the twitch force and the intracellular Ca^{2+} transient by application of norepinephrine (NE), an adrenergic receptor agonist. NE stimulates both β₁- as well as α₁-AR. Presence of α-AR in the myocardium and the inotropic effect of its stimulation has been reported in humans (Landzberg et al., 1991; Parker et al., 1995) and in different animal species (Benfey, 1990 [rat, guinea pig, and dogs]; Nakanishi et al., 1989 [rat and rabbit]; Shavit et al., 1986 [rat]). Although NE has been used by other investigators as a β₁-AR agonist with no concern regarding its α-AR activation function (Gilbert et al., 1993; Port et al., 1992; Xiao and Lakatta, 1993; Xiao et al., 1994), we used phentolamine mesylate (PhM), a selective α-AR blocker in order to study pure β₁-AR stimulation. PhM was added to the perfusion solution (K-H) 3-5 minutes before NE was added. Stimulation of β₁-AR (by NE) changed the force and the [Ca^{2+}]_i transient similar to isoproterenol. Figure 3.3-6A shows that 100 nM NE (following 1 μM PhM) at 0.2 Hz. significantly increased the force of contraction (mN/mm²) from 21.85 ± 1.86 to 35.06 ± 2.04, and the peak [Ca^{2+}]_i transient (μM) from 0.63 ± 0.07 to 0.95 ± 0.08. It also decreased the duration of the twitch force and the Ca^{2+} transient (50% contraction to 50% relaxation) from 310.13 ± 4.03 to 182.77 ± 27.39 and from 264.25 ± 37.33 to 147.25 ± 21.67 msec. respectively. Figure 3.3-6B shows that stimulation of β₁-AR increased the force and the [Ca^{2+}]_i by 61.2 ± 4.7 and 50.75 ± 8.84 %, respectively, and decreased the TD and the CaD by 41.3 ± 8.1 and 42.7 ± 7.2 %, respectively. These values are comparable to the changes induced by isoproterenol (Fig. 3.3-4B). The similarity between the inotropic effect of isoproterenol (β₁- and β₂-AR stimulation) and NE (only β₁-AR) is consistent with the general belief that β₁-ARs, which comprise about 80% of the total cardiac β-AR, are the major contributor to the myocardial inotropic effect following sympathetic stimulation and β₂-AR plays a minor role.
Figure 3.3-6: Effect of β₁-AR stimulation on the force and the $[\text{Ca}^{2+}]_i$ transient.

Norepinephrine (NE) (α- and β₁-AR agonist) was used in the presence of phentolamine mesylate (PhM) (α-AR blocker) in order to solely stimulate the β₁-AR. (A) represents that β-AR stimulation increased the amplitude and accelerated the kinetics of the force and the $[\text{Ca}^{2+}]_i$ transient. The percentage changes in the peak force, peak $[\text{Ca}^{2+}]_i$, twitch duration and $[\text{Ca}^{2+}]_i$ duration due to β₁-AR stimulation are shown in (B).
(Figure 3.3-6)
FREQUENCY-DEPENDENT CHANGES IN THE FORCE AND THE [Ca$^{2+}$]$_i$ IN THE PRESENCE OF PhM AND NE

Stimulation of $\beta_1$-AR by NE (in the presence of PhM) resulted in a negative force-frequency relationship while the positive peak [Ca$^{2+}$]$_i$-frequency relationship was maintained (Fig. 3.3-7 A and B). In the presence of NE, peak force (mN/mm$^2$) decreased from $35.06 \pm 2.04$ at 0.2 Hz to $27.68 \pm 0.54$ at 2 Hz ($P<0.05$, $n=5$), whereas the peak [Ca$^{2+}$]$_i$ (μM) increased from $0.95 \pm 0.08$ to $1.21 \pm 0.16$ at 2 Hz ($P<0.05$, $n=5$). Therefore, the negative force-frequency relationship in the presence of NE was not due to a decrease in the [Ca$^{2+}$]$_i$, but it could be due to a decrease in the contractile function of the contractile proteins, mediated by a combination of the used drugs (PhM and NE) and the increased frequency of stimulation. It could also be due to SR Ca$^{2+}$ overload and the consequent spontaneous Ca$^{2+}$ release (as suggested for isoproterenol).

Kinetics of the force and the Ca$^{2+}$ transient were significantly abbreviated as the frequency of stimulation was increased from 0.2 to 2 Hz in the presence of NE. Twitch duration (msec) (50% contraction to 50% relaxation) decreased from $182.77 \pm 27.39$ to $125.37 \pm 24.31$ ($P<0.05$, $n=5$), and Ca$^{2+}$ transient duration (msec) (50% contraction to 50% relaxation) decreased from $147.25 \pm 21.67$ to $93.5 \pm 15.4$ following elevation of the frequency of stimulation from 0.2 to 2 Hz ($P<0.05$, $n=5$) (Fig. 3.3-7C and D). The TD- and the CaD-frequency curves in control experiments and in the presence of NE tend to converge at higher frequencies of stimulation (identical to the effect of isoproterenol, figure 3.3-5C and D). This observation further confirms the possibility of involvement of the same mechanism in the $\beta$-AR-mediated and the frequency-mediated TD shortening.

The rate of [Ca$^{2+}$]$_i$ decline at elevated frequency of stimulation and in the presence of isoproterenol are shown in Figure 3.3-8. The Ca$^{2+}$ transients at 0.2 (solid line) and 2 (broken line) Hz are superimposed in figure 3.3-8A, and the transients in the absence (solid line) and presence (broken line) of isoproterenol, at 0.2 Hz, are superimposed in figure 3.3-8B. Comparing these two figures shows that the rate of [Ca$^{2+}$]$_i$ decline is altered similarly when the
**Figure 3.3-7**: The frequency-dependent changes in the peak force, peak $Ca^{2+}$ transient, twitch and $[Ca^{2+}]_i$, duration in control and in the presence of NE and PhM.

A negative force-frequency relation was observed in the presence of NE and PhM (A) despite a positive $[Ca^{2+}]_i$-frequency relationship (B). The TD-frequency and CaD-frequency relationships in control and in the presence of NE and PhM tend to converge at higher frequencies of stimulations (C and D), suggesting activation of the same mechanism in response to increased frequency of stimulation and β-AR stimulation. These observations are very similar to the effect of isoproterenol.
A

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.3-7a}
\caption{Peak Force (mN/mm²) vs Frequency of stimulation (HZ) for Control and PhM + NE}
\end{figure}

B

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.3-7b}
\caption{Peak \([Ca^{2+}]\) (µM) vs Frequency of stimulation (HZ) for Control and PhM + NE}
\end{figure}

C

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.3-7c}
\caption{Twitch duration (msec) vs Frequency of stimulation (HZ)}
\end{figure}

D

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.3-7d}
\caption{\([Ca^{2+}]\) duration (msec) vs Frequency of stimulation (HZ)}
\end{figure}

(Figure 3.3-7)
frequency of stimulation was increased to 2 Hz and when isoproterenol was applied. Increased frequency of stimulation and isoproterenol increased the amplitude and accelerated the rate of $[Ca^{2+}]_i$ decline so that the relaxation phases at low and high frequencies of stimulation and before and after isoproterenol application cross over.

In order to demonstrate whether $\beta$-AR stimulation and increased frequency of stimulation activate the same pathway for inducing abbreviation of the force and the $[Ca^{2+}]_i$ kinetics, the $\beta$-AR-activated pathway was interrupted by inhibition of PKA and then the TD- and CaD-frequency relations were measured.

**Inhibition of PKA.**

PKA was inhibited by 2.8 $\mu$M K252-a ($K_i = 25$ nM), an inhibitor of cyclic nucleotide-dependent protein kinases (PKA, PKG), as well as PKC, CaM Kinase and MLC kinase. K252-a inhibits these kinases by competing for ATP (Kase et al., 1987). It was only at this concentration of K252-a (2.8 $\mu$M) that the $\beta$-AR-induced inotropic effect could be completely blocked. The complete blockade of this kinase was verified by the absence of an inotropic effect following $\beta$-AR stimulation (Figure 3.3-9). Figure 3.3-9A shows that the force increased in the presence of 100 nM norepinephrine (NE) (and PhM) and equilibrated at the new elevated level. The bottom two panels of figure 3.3-9A show the force and the $Ca^{2+}$ transient traces before and after $\beta$-AR stimulation. As expected, force and $Ca^{2+}$ amplitudes increased and their duration decreased following NE application. Figure 3.3-9B shows the same data, in the presence of 2.8 $\mu$M K252-a. The inotropic effect of 100 nM NE was blocked in the presence of this inhibitor. The two bottom panels in figure 3.3-9B show that the force and the $Ca^{2+}$ transient traces remained unchanged following NE application in the presence of 2.8 mM K252-a.

Phentolamine mesylate and norepinephrine (and not isoproterenol) were used to verify complete inhibition of the PKA molecules because NE inserts its inotropic effect via $\beta_1$-AR stimulation and it has been established that $\beta_1$-ARs act via activation of PKA. However, isoproterenol is a non-selective $\beta$-AR ($\beta_1$ and $\beta_2$) agonist and $\beta_2$-AR has been suggested to act
Figure 3.3-8: The effect of increased frequency of stimulation and β-AR stimulation on $[Ca^{2+}]_i$ transients.

A) At 2 Hz, the peak $[Ca^{2+}]_i$ was increased and its rate of relaxation of the was accelerated compared to 0.2 Hz, so that the two traces cross. $[Ca^{2+}]_i$ changed similarly in the presence of isoproterenol as in response to an increase in the frequency of stimulation (B).
(Figure 3.3-8)
Figure 3.3-9: The effect of β-AR stimulation in control and in the presence of K252-a (a PKA inhibitor).

A) In control conditions, application of NE in the presence of PhM resulted in a significant inotropic effect and elevation of peak $\left[Ca^{2+}\right]_i$ (the bottom panels). In the presence of K252-a (B), NE and PhM did not induce an inotropic effect, which is more evident in the bottom panels which show the force and the $[Ca^{2+}]_i$ traces before and after β-AR stimulation.
via pathways other than PKA (Lemoine and Kaumann. 1991; Xiao et al., 1995). Therefore, isoproterenol would not be an appropriate drug to verify inhibition of PKA.

The force and the Ca\(^{2+}\) transients in the absence (solid line) and presence (broken line) of K252-a are superimposed in figure 3.3-10 at 0.2 and 2 Hz. K252-a did not cause any change in the duration of the twitch and the Ca\(^{2+}\) transient. 2.8 μM K252-a changed the TD from 301 ± 66 to 275.5 ± 25.5 msec (P=0.22, n=4), and changed the CaD from 215 ± 15 to 191 ± 29 msec (P=0.18, n=4), at 0.2 Hz. Absence of any change in the frequency-dependent changes of the force and the Ca\(^{2+}\) transient despite complete inhibition of PKA is clearly demonstrated in figures 3.3-9B. In the presence of K252-a TD decreased significantly from 275.5 ± 25.5 at 0.2 Hz to 197.5 ± 10.5 msec at 2 Hz (P<0.05), and the CaD decreased significantly 215 ± 15 at 0.2 Hz to 78 ± 12 msec at 2 Hz. (P<0.05, n=5). Inhibition of PKA and thereby interruption of the isoproterenol-activated pathway, did not disrupt TD- and CaD-frequency relationship. Therefore, PKA does not play a major role in the frequency-dependent changes of the kinetics of the force and the Ca\(^{2+}\) transient.

Moreover, K252-a also inhibits other protein kinases such as PKC (K\(_{i}\)=25 nM), PKG (K\(_{i}\)=20 nM), CaM Kinase (K\(_{i}\)=1.8 nM) and myosin light chain (MLC) kinases (K\(_{i}\)=17 nM) in addition to PKA (K\(_{i}\)=25 nM). The fact that the TD-frequency relationship remained unaltered in the presence of this inhibitor supports that intracellular protein kinases are not involved in the frequency-dependent twitch kinetic acceleration.
Figure 3.3-10: The effect of K252-a (PKA inhibitor) on the TD-frequency relationship.

A) Force and \([Ca^{2+}]_i\) traces in control (solid line) and in the presence of 2.8 \(\mu\)M K252-a (broken line), at 0.2 (right panel) and 1 (left panel) Hz. The TD- and CaD-frequency relationships (B) were not significantly altered in the presence of K252-a.
Figure 3.3-10
3.3.5 **DISCUSSION**

The purpose of this series of experiments was to investigate the role of the Ca\(^{2+}\)-activated kinases in the HR-mediated TD shortening, since increasing the frequency of stimulation resulted in significant elevation of the integrated time-averaged [Ca\(^{2+}\)]. Frequency-dependent elevation of \(\langle [\text{Ca}^{2+}] \rangle\) could activate Ca\(^{2+}\)-activated kinases, CaM Kinase II, PKG. These kinases are involved in phosphorylation of a number of intracellular proteins among which phospholamban, SR Ca\(^{2+}\) ATPase, and Tn-I are directly involved in regulation of the force and the [Ca\(^{2+}\)] kinetics. The Ca\(^{2+}\)-dependent activation of CaM Kinase II following increased frequency of stimulation can phosphorylate SR Ca\(^{2+}\)-ATPase pump and phospholamban whereby it can accelerate the rate of Ca\(^{2+}\) uptake by the SR and the relaxation phase of the [Ca\(^{2+}\)], transient and the force of contraction.

Inhibition of CaM/Kinase, using KN-93, did not abolish the TD-frequency relationship (Fig. 3.3-2). Our data are consistent with Frampton and Orchard who found no change in the half-time of the decline of the [Ca\(^{2+}\)], transients or the twitch when they inhibited calmodulin activity, by W7, in rat ventricular myocytes (1992). However, it has been shown that withdrawal of calmodulin from the bathing solution results in a decrease in the amplitude and slowing of the time course of the Ca\(^{2+}\) transient (Fabiato, 1985). Our data do not agree with the findings of Bassani et al. and Li et al. who found that inhibition of CaM/Kinase II, by KN-62 (Bassani et al., 1995), and KN-93 (Li et al., 1997) completely eliminated the differences in the time courses of [Ca\(^{2+}\)], decline during steady state and post-rest twitches, in rat ventricular myocytes, by prolonging the rate of [Ca\(^{2+}\)], decline in steady state (SS) twitches. These authors concluded that CaM Kinase II is responsible for activity-dependent acceleration of relaxation in rat ventricular myocytes. The argument used in these two studies is that the prolonged rate of relaxation of the post rest (PR) twitches is due to the dephosphorylation of the phospholamban during the rest period. The difference between the findings in the above studies and our study can be explained by the
difference between the experimental preparations and the protocols. Bassani et al. and Li et al. used cardiac single myocytes whereas we used cardiac trabeculae. The differences between the isolated single myocytes and the cardiac trabeculae were discussed in Section 3.1 (Discussion). In summary, the force of contraction is estimated by the extent of cell shortening in single myocytes. Moreover, the rate of relaxation in myocytes is limited by the rate of $[\text{Ca}^{2+}]$, whereas the contractile filaments are the limiting factors in the contractile proteins (Backx et al., 1995).

The difference between the protocols used by Bassani et al. and Li et al. and those used in our study is that, in those studies, the rate of relaxation of the steady state twitches were compared to those of the PR (post rest) twitches prior to which there was a 3 minute (Bassani et al., 1995) and 60 second (Li et al., 1997) rest period. This time period is sufficient for dephosphorylation of the phosphorylated proteins (such as phospholamban) to take place. Whereas, the slowest frequency of stimulation used in our study was 0.2 Hz, i.e. 5 second interpulse interval, which is not long enough for dephosphorylation to take place. Moreover, the rest-dependent dephosphorylation of phospholamban can not explain the shorter TD at 2 Hz compared to 1Hz. Therefore, a mechanism other than the CaM Kinase II-mediated phosphorylation of the phospholamban and/or the SR Ca$^{2+}$ ATPase is responsible for the TD shortening following increased frequency of stimulation.

cNOS is another Ca$^{2+}$-dependent enzyme that is activated as the frequency of stimulation is increased and catalyzes production of NO. Action of NO is to activate soluble guanylate cyclase and to elevate cGMP. cGMP activatesPKG which phosphorylates a number of proteins, such as sarcolemmal and/or SR Ca$^{2+}$-channels (Moncada et al. 1991). cGMP has been shown to rise within five minutes of increasing the frequency of stimulation (Kaye, 1996). It could either act by activating PKG or by regulating intracellular cAMP level by cGMP-regulated phosphodiesterase. The
elevated level of cGMP at increased frequency of stimulation is expected to play a role in the rate of [Ca^{2+}], decline and twitch relaxation.

Inhibition of NOS, and thereby inhibition of NO accumulation, did not affect the TD-frequency relationship, and did not significantly alter the amplitude of the [Ca^{2+}], transient or the force. In the presence of the NOS inhibitor (L-NOARG), TD continued to decrease as the frequency of stimulation was increased (Fig. 3.3-3). Kaye et al. suggested that the elevated intracellular cGMP at higher pacing frequencies (>3Hz) resulted in a decrease in the amplitude of shortening (Kaye et al., 1996). They further explained that at pacing frequencies at or below 2 Hz only minimal effect of an NO antagonist or NOS inhibitor was observed. Balligand et al. (1993) also reported that L-NA (a NOS inhibitor) had no effect on the basal amplitude of shortening of isolated electrically paced adult rat ventricular myocytes at 2 Hz. Weyrich et al. (1994) used a stimulation frequency of 0.25 Hz and reported that neither NOS inhibitors nor a variety of pharmacological NO donors delivered at "physiological" concentrations had any effect on the contractile force developed by isolated rat papillary muscles or isolated adult rat cardiac myocytes. Therefore, NO seems not to affect the twitch shortening unless at frequencies at or above 3 Hz. The purpose of our study has been to discover the underlying mechanism of rate-dependent TD abbreviation regardless of the range of the frequency of stimulation. Therefore, our data is consistent with the previous findings that NO does not play a role in the frequency-dependent abbreviation of the TD.

We induced β-AR stimulation by application of isoproterenol, a non-selective β-AR agonist, and NE (in the presence of PhM) for pure stimulation of β_1-AR. β-AR activation affected the force and the Ca^{2+} transient in a similar fashion to increased frequency of stimulation (Figures 3.1-1 and 3.3-4 and 3.3-6). The force of contraction is regulated by two factors: 1) the intracellular Ca^{2+} handling and 2) the contractile proteins. We found that the change in the intracellular Ca^{2+} handling was comparable
when the frequency of stimulation was increased from 0.2 to 2 Hz and following application of isoproterenol (Fig 3.3-8). Furthermore, TD- and CaD-frequency relations in the absence and presence of β-AR stimulation tend to converge at higher frequencies of stimulation (Fig. 3.3-5 and 3.3-7. C and D). These observations suggest involvement of the same mechanism in these two cases. However, inhibition of PKA by 2.8 μM K252-a did not abolish the TD-frequency relationship and the TD continued to decrease as the frequency of stimulation was increased (Fig. 3.3-10). These data are consistent with the previous finding that PKA is not responsible for the activity-dependent acceleration of relaxation in rat ventricular myocyte (Bassani et al., 1995). Therefore, increased frequency of stimulation and β-AR activation although affect the force and the Ca^{2+} transient similarly, they do not share the same mechanism.
SECTION 3.4
Time Course of the Frequency-Dependent Changes

3.4.1 Sarcomere Length of 2.2 μm

In order to further investigate the frequency-dependent TD shortening, we studied the time course of this phenomenon because it could be an important factor in identifying its underlying mechanism. We measured the peak force and the TD of every individual beat as the frequency of stimulation was changed from 0.2 to 2 Hz and back to 0.2 Hz (Figure 3.4-1). This experiment was performed on five trabeculae and figure 3.4-1 represents typical force and TD alterations following changes in the frequency of stimulation. The vertical dotted lines show where the frequency was changed. Figure 3.4-1A shows that by increasing the rate of stimulation from 0.2 to 2 Hz, the force of contraction underwent an undershoot and then increased to a new steady state level that was higher than that at 0.2 Hz (from 21.8 ± 2.6 to 23.4 ± 5.5, n=5). When the frequency of stimulation was changed back to 0.2 Hz, the force first underwent an overshoot, followed by a decrease to below its starting level at 0.2 Hz. The peak force then gradually increased and reached its control level (at 0.2 Hz) after 20 to 30 minutes. The other trabeculae behaved similarly following an increase and a decrease in the frequency of stimulation. They showed an undershoot and an overshoot in the peak force when the frequency was increased and decreased, respectively. In two trabeculae, the equilibrated peak force at 2 Hz was significantly higher than that at 0.2 Hz. This increase, however, was not significant in the other three trabeculae.

The frequency-dependent changes in the TD, on the other hand, were consistent in all the trabecula (Fig. 3.4-1B). The TD always rapidly and significantly shortened within 1-2 beats, after an increase in the frequency of stimulation to 2 Hz, and rapidly returned to the baseline level when the frequency was returned to 0.2 Hz. The twitch duration did not undergo any undershoot or overshoot as a result of a change in the rate of stimulation. Figure 3.4-1B clearly shows that the frequency-dependent changes in the twitch duration occur at a faster
Figure 3.4-1: The time course of the frequency-dependent changes in the peak force and the twitch duration at a sarcomere length of 2.2 μm.

The dotted lines show when the frequency of stimulation was changed. A) The peak force underwent an undershoot and an overshoot when the frequency of stimulation was increased and decreased, respectively. B) The changes in the twitch duration in response to a change in the frequency of stimulation was very rapid and no undershoot or overshoot was observed upon a change in the rate of stimulation.
time scale than the peak force. Therefore, different mechanisms must be responsible for the rate-dependent changes in these two parameters (peak force and twitch duration).

3.4.2 Slack Sarcomere Length of 1.9 µm

Since sarcomere length (SL) is one of the regulating factors of the force of contraction and its kinetics (Section 3.2), we decided to investigate if the time course of the rate-dependent changes in the peak force or the twitch duration were altered at a SL other than the optimal SL of 2.2 µm. Individual beats following a change in the rate of stimulation (from 0.2 to 2 and back to 0.2 Hz) were recorded at a slack SL of 1.9 µm.

Figure 3.4-2 shows the frequency-dependent changes in the peak twitch force (A) and the twitch duration (B) at the SL of 1.9 µm. Decreasing the SL from 2.2 (Fig. 3.4-1) to 1.9 µm. caused a significant decrease in the peak force of contraction at 0.2 Hz (from 21.79 ± 2.61 to 8.5 ± 1.22 mN/mm²) as well as in the twitch duration (296.75 ± 19.4 to 191.63 ± 21.32 msec). However, following elevation of the frequency of stimulation from 0.2 to 2 Hz (and its decrease back to 0.2 Hz) the force of contraction and the twitch duration followed the same pattern of changes as they did at the sarcomere length of 2.2 µm. At 1.9 µm. the twitch duration significantly decreased from 191.63 ± 21.32 msec at 0.2 Hz to 141.25 ± 11.21 msec at 2 Hz (P<0.05. n=4). and the peak force increased from 8.51 ± 1.22 mN/mm² at 0.2 Hz to 11.41 ± 1.55 mN/mm² at 2 Hz (compared to 296.75 ± 19.4 to 177.50 ± 21.76 msec. and 21.79 ± 2.61 to 23.44 ± 5.49 mN/mm² at SL of 2.3 µm).

Similar to the observations made at the SL of 2.2 µm. the peak force. and not the TD. underwent an undershoot and an overshoot when the frequency of stimulation was increased to 2Hz and decreased to 0.2 Hz. respectively. The rate-dependent change in the TD was still very rapid at the short SL (1.9 µm) and reached its new steady state within 1-2 contractions. These data demonstrated that the sarcomere length does not affect the time course of the frequency dependent changes in the kinetics of the force of contraction.
Figure 3.4-2: The time course of the frequency-dependent changes in the peak force and the twitch duration at a sarcomere length of 1.9 μm.

The dotted lines show where the frequency of stimulation was changed. Upon an increase and a decrease in the frequency of stimulation the peak force underwent an undershoot and an overshoot, respectively (A). The frequency-dependent changes in the TD were very rapid with no undershoot or overshoot (B). The time course of the rate-dependent changes of these two parameters at the short SL (1.9 μm) is identical to that at the longer SL (2.2 μm) (Figure 3.4-1)
(Figure 3.4-2)
3.4.3 DISCUSSION

In order to study the time course of the frequency-dependent TD shortening, individual beats, immediately following a change in the frequency of stimulation, were recorded. Upon increasing the frequency of stimulation from 0.2 to 2 Hz, the peak of the first 2-3 contractions declined and it increased in the following contractions to reach a new higher steady state (Figure 3.4-1A). This undershoot in the peak force can be explained by the two-compartment model of the sarcoplasmic reticulum (Burkhoff et al., 1984; Schouten et al., 1987). When the frequency of stimulation was increased, the Ca\(^{2+}\) in the release compartment was released into the cytoplasm before the Ca\(^{2+}\) sequestered into the uptake compartment could be transferred to the release compartment. Therefore, the first beat after increasing the frequency of stimulation was very small. As the muscle continued to be stimulated at the high frequency (2 Hz), more Ca\(^{2+}\) was sequestered by the uptake compartment, which led to increased rate of transfer from the uptake to the release compartment. After a few contractions, the [Ca\(^{2+}\)]\(_{i}\) was elevated (due to increased \(I_{Ca,i}\) influx and increased SR Ca\(^{2+}\) release) and resulted in elevation of the force of contraction. Similarly, when the frequency of stimulation was decreased back to 0.2 Hz (from 2 Hz), the inter-stimulus interval was prolonged, allowing a longer time for the Ca\(^{2+}\) transfer to take place. Moreover, the rate of intra-compartment transfer could still be high due to the high Ca\(^{2+}\) content of the uptake compartment (from the previous fast rate). Therefore, the first stimulus at the low frequency of stimulation resulted in the release of a large quantity of Ca\(^{2+}\) which caused the first beat at 0.2 Hz to be very large. As the muscle continued to contract at the low rate, the amount of the sequestered Ca\(^{2+}\), the rate of Ca\(^{2+}\) transfer from the transfer compartment to the release compartment, and therefore the amount of Ca\(^{2+}\) released from the SR decreased. This interval-dependent Ca\(^{2+}\) release from the SR, which causes the interval-dependent changes in the amplitude of [Ca\(^{2+}\)]\(_{i}\) transient, and consequently the developed force (or pressure) has also been shown by other investigators (Zaugg et al., 1995).

We observed that the rate-dependent changes of the TD, however were much more rapid than those of the peak force at both SL (Fig. 3.4-1B and 3.4-2B). This observation shows that the rate-dependent TD shortening is regulated by factors other than just the rate of SR activity.
which is responsible for the rate-dependent changes of the peak force of contraction. A change in the twitch duration could therefore, be due to frequency-dependent alterations in the contractile proteins. Because of the very rapid changes in the TD in response to changes in the frequency of stimulation, we hypothesize that the mechanism responsible for this rate-dependent TD abbreviation should have a very short time-constant and be independent of the SL. The very rapid time course of the frequency-dependent change in TD (within 1 to 2 sec) is consistent with the observed absence of contribution of the intracellular Ca\(^{2+}\)-activated kinases to this phenomenon (Section 3.3). Activation of these kinases and the subsequent phosphorylation of their substrate proteins, involved in the development of the force of contraction, generally take many seconds to minutes (longer than the time course of the frequency-dependent TD shortening).
SECTION 3.5
The Role Of The Frequency-Dependent pH\textsubscript{i} Decline In The Frequency
Dependent Twitch Duration Shortening

3.5.1 INTRODUCTION

It has been shown that increased rate of stimulation causes a decrease in the intracellular pH (pH\textsubscript{i}) (Allen et al., 1986; Bountra et al., 1988 a,b). Camilion de Hurtado et al. (1996) however, reported that increments in the frequency of contraction in cat papillary muscle induced a reversible increase in pH\textsubscript{i} in the presence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-buffered medium and a decrease in the absence of bicarbonate (in HEPES-buffered Tyrode solution). Whereas, Bountra et al. (1988) reported that a fall in pH\textsubscript{i} following a rate increase occurred also when the sheep purkinje fibers were bathed in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-mediated Tyrode solution. Camilion de Hurtado et al. (1996) suggested existence of an electrogenic Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} transport mechanism that mediates bicarbonate influx upon membrane depolarization during an action potential. An increase in heart rate therefore, would result in increased bicarbonate influx, which would increase the myocardial cell’s ability to recover from an enhanced proton production. Presence of an electrogenic Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} exchanger was not confirmed in sheep purkinje fibers and this exchanger was also found to be voltage insensitive (Dart and Vaughan-Jones, 1992). Bountra et al., showed that inhibition of the Na\textsuperscript{+}/H\textsuperscript{+} antiport or the Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} did not abolish the rate-dependent acidosis in sheep Purkinje fibers, indicating no participation by these transporters (1988). These authors further demonstrated that the rate-dependent acidosis was inhibited by the organic calcium antagonist (D600) which also inhibited twitch tension. Therefore, Bountra et al. (1988) concluded that the rate-dependent acidosis is related to the activation of developed tension by Ca\textsuperscript{2+}, and is generated metabolically via stimulation of glycolysis, following an increase in contraction. Allen et al. (1986) also attributed the intracellular acidosis associated with an increase in the frequency of stimulation, in ferret heart, to increased lactic acid production.
Decreased pH, has been shown to increase the amplitude of the Ca, transients (Orchard. 1987: Allen et al., 1989: Gamassi and Capogrossi, 1992: Than et al., 1994) and decreases the sensitivity of the myofilaments to Ca²⁺ (Blanchard and Solaro, 1984; Orchard and Kentish, 1990; Gambassi and Capogrossi, 1992; Morii et al. 1996). Blanchard and Solaro (1984) suggested that much of the shift in myofilament Ca²⁺ sensitivity could be attributed to a decrease in the affinity of Ca²⁺ binding to cardiac troponin C. The effect of pH on Ca²⁺ binding to troponin C is also amplified by a pH-sensitive change in the affinity of troponin I for troponin C (El-Saleh and Solaro, 1988; Solaro et al., 1989). A decrease in the myofilament Ca²⁺ sensitivity or the affinity of Tn-C for Ca²⁺ could abbreviate the interaction between the contractile proteins (Tn-C) and Ca²⁺ and therefore accelerate the twitch kinetics (as it happens in the presence of isoproterenol, Saeki et al., 1997). Therefore, the decreased affinity of Ca²⁺ binding to Tn-C at decreased pH, associated with elevated HR, could be responsible for the rate-dependent TD shortening and the elevation in [Ca²⁺], transient amplitude. The influence of the rate-dependence of pH, on the magnitude and the time course of an inotropic response to a change in the HR has also been previously reported (Bountra et al., 1988a).

The rate-dependent pH, decline and its reported contribution to the elevated Ca²⁺ transient amplitude and myofilament Ca²⁺ sensitivity suggests that this reduction in pH, could underlie the change in the twitch duration as a function of stimulation rate. Therefore, to examine how closely the frequency-dependent changes in the twitch duration and the pH, accord, the time course of the changes in these two parameters following a change in the frequency of stimulation were compared.

3.5.2 METHOD
We used SNARF-AM, a membrane permeable dual emission dye, and the ratiometric technique to measure the pH,. Details of making the SNARF solution, the loading and the recording technique is given in chapter 2. The trabeculae were excited by 540 nm ultraviolet light and the emitted lights, at 590 and 640 nm, were collected by an objective and transmitted
to two photomultiplier tubes. The data was displayed on a computer and sampled via an analog to digital (A/D) converter installed in the computer. The recorded data was later imported to an analysis program, "origin", where the fluorescence ratio (640/590) was calculated. Figure 3.5-1A shows the raw traces of the autofluorescents (background fluorescence of the muscle in the absence of the dye) (solid line), and the fluorescence of the loaded muscle (dotted line), at 640 nm (left) and 590 nm (right). These two figures show that the fluorescent signal was markedly enhanced upon loading the trabecula with SNARF-AM (compared to the autofluorescence). A movement artifact was present in the fluorescent signals (dotted lines) but not in the autofluorescent signals (solid lines). The autofluorescence was manually subtracted from the corresponding signals, i.e. the autofluorescence recorded at 590 nm was subtracted from the signals recorded at 590 nm, and similarly for 640 nm. After this subtraction, the ratio of 640 nm signal over 590 nm signal was calculated, which caused the movement artifact of the signals to be cancelled out as shown in figure 3.5-1B.

The advantage of using the ratiometric technique to measure the fluorescent signal is that the measurements are independent of the dye concentration in the muscle (more details in chapter 2). The pH, values are reported as the fluorescent ratio (640/590), because in this experiment we are interested in the time course of the frequency-dependent changes in pH, and not its absolute value. The simultaneous measurement of the fluorescent signals at two wavelength enabled us to study the time course of the frequency-dependent changes in the pH, (i.e. the fluorescent ratio) by measuring the fluorescent ratio in each individual beat following a change in the frequency of stimulation. The fluorescent ratio (640/590) is a function of the pH, (and not [H\(^+\)]), that is, a decrease in the fluorescent ratio represents a decrease in pH, (and not [H\(^+\)]). To establish that SNARF would respond to changes of the pH, we first tried addition and removal of NH\(_4\)Cl which have been established to result in transient intracellular alkalosis and acidosis, respectively (Roos and Boron, 1981; Bountra and Voughan-Jones, 1989). Changes in the fluorescent ratio following changes in the frequency of stimulation were recorded.
Figure 3.5-1: The raw traced of SNARF-AM fluorescent recordings.

A) The raw traces of autofluorescence (solid line) and fluorescent signal in loaded trabeculae (dotted line) emitted at 640 nm (left) and 590 nm (right). B) The ratio of the fluorescent signal at 640 to 590 nm after the autofluorescence was subtracted (the noisy line), and the corresponding force trace.
Figure 3.5-1
3.5.3 RESULT

It has been established that addition and removal of NH₄Cl to the perfusion solution transiently alters the pHᵢ of cardiac muscles at a constant pHᵢ, and these pHᵢ changes are accompanied by temporally correlated changes in the force of contraction (Roos and Boron, 1981: Bountra and Voughan-Jones, 1989). In order to demonstrate that SNARF-AM can yield the expected results in cardiac trabeculae, we examined changes in the SNARF fluorescent ratio following addition and removal of 10 mM NH₄Cl (Fig. 3.5-2). The vertical dotted lines in this figure show the interventions. Addition of 10 mM NH₄Cl resulted in a transient increase in the pHᵢ (alkalosis) and in the force of contraction followed by a subsequent decrease (Fig 3.5-2). Removal of the NH₄Cl resulted in a transient acidosis and a decrease in the force of contraction which returned to the control level after a few minutes. Previous reports also showed that addition of 10 mM NH₄Cl transiently decreased the pHᵢ of guinea pig papillary muscles at a constant pHᵢ, and a transient fall of pHᵢ was induced by NH₄Cl removal (Bountra and Voughan-Jones, 1989). These studies also reported the concurrent change in the pHᵢ and the force (as seen in figure 3.3-1) following addition and removal of NH₄Cl. Therefore, we were able to reproduce the expected pHᵢ changes in the presence and absence of NH₄Cl by using SNARF-AM. We further confirmed that the fluorescent ratio (640/590) is a function of pHᵢ (and not [H⁻]).

Figure 3.5-3 shows the beat-to-beat changes in the peak force, twitch duration, and SNARF fluorescent ratio (a function of pHᵢ) as the frequency of stimulation was changed from 0.2 to 2 Hz and back to 0.2 Hz. This figure shows that the twitch duration shortened rather immediately (within 2 to 3 beats), by 28%, after increasing the rate of stimulation from 0.2 Hz to 2.0 Hz, whereas the pHᵢ decline (by 2.6%) occurred far more slowly. The time course of the frequency-dependent change in the pHᵢ was much slower than that of the TD, so that 86% of the TD decrease occurred within the first two beats whereas only 7.7% of the pHᵢ decrease had occurred by this time. Recovery of pHᵢ to the control level after returning the frequency to 0.2 Hz also occurred very slowly compared
Figure 3.5-2: The effects of NH₄Cl wash-in and wash-out on the pHᵢ.

The broken line shows where NH₄Cl was washed in and out. Peak force is presented in panel “A” and the fluorescent ratio (640/590), which represents the change in pHᵢ (and not [H⁻]) is shown in panel B. NH₄Cl wash-in caused a transient increase in pHᵢ (alkalosis) and the peak force which returned back to the control level after about 10 seconds. NH₄Cl wash-out resulted in a transient decrease in pHᵢ (acidosis) and peak force which again returned to the control level.
CI
I
O
NH₄Cl wash-in
NH₄Cl wash-out

A

Peak Force (mN/mm²)

Time (min)

B

Fluorescent Ratio (640/590)

Time (min)

(Figure 3.5-2)
**Figure 3.5-3:** Frequency-dependent changes in the peak force and TD in comparison to pH$_i$.

Peak force (A), twitch duration (B), and pH$_i$ (C) were measured as the frequency of stimulation was increased from 0.2 to 2 Hz and back to 0.2 Hz (indicated by the broken lines). Panels B and C show that the frequency-dependent pH$_i$ decline and TD shortening are temporally unrelated.
(Figure 3.5-3)
to the TD. Therefore, the frequency-dependent change in the pH<sub>i</sub> does not temporally correlate with those of the twitch duration.

### 3.5.4 DISCUSSION

Intracellular pH (pH<sub>i</sub>) in cardiac muscle is not constant. The value of pH<sub>i</sub> depends upon the frequency of stimulation: pH<sub>i</sub> is lower at higher frequencies of repetitive activity (Bountra <em>et al.</em>. 1988). In this study we observed that pH<sub>i</sub> decreased following elevation of the frequency of stimulation from 0.2 to 2 Hz and recovered when the frequency of stimulation was decreased back to 0.2 Hz. This observation is consistent with the previous work done by Vanheel and deHemptinne (1985) as well as Bountra and Vaughan-Jones (1988 a,b) who found that pH<sub>i</sub> in the sheep heart Purkinje fiber decreases linearly with increase in the frequency of stimulation.

We further found that the time scale of the rate-dependent changes in the pH<sub>i</sub> was much slower than that of the twitch duration, so that when pH<sub>i</sub> started to decline, twitch duration had already decreased to its new steady state. This observation is consistent with previous reports that the fall in pH<sub>i</sub> is achieved within a few minutes of a rate increase (Bountra and Vaughan-Jones. 1988; Camilon de Hurtado <em>et al.</em>. 1996). Thus, although an increase in the frequency of stimulation is accompanied by a decline in pH<sub>i</sub>, the latter is not likely responsible for the rate-dependent abbreviation of the twitch duration since it occurs on a much slower time scale compared to twitch duration.
SECTION 3.6

CONTRIBUTION OF THE CONTRACTILE PROTEINS TO THE RELAXATION PHASE OF A TWITCH FORCE

3.6.1 INTRODUCTION

Our data so far showed that the frequency-dependent acceleration of the intracellular Ca\(^{2+}\) handling kinetics was not responsible for the frequency-dependent TD shortening. Contractile proteins are the other factor that regulate development of the force of contraction in a muscle. In order to evaluate contribution of the contractile proteins to the frequency-dependent changes in the rate of force development and relaxation we used a technique that was previously demonstrated by Backx et al. (1995). Backx et al. (1995) measured the steady state (SS) F-[Ca\(^{2+}\)] relationship in tetanized rat cardiac trabeculae. The trabeculae were tetanized by stimulating them at a high frequency of stimulation (8Hz), in the presence of cyclopiazonic acid (CPA), a selective inhibitor of SR Ca\(^{2+}\) ATPase pump. The force and the [Ca\(^{2+}\)] were measured three seconds after tetanus at different [Ca\(^{2+}\)]\(_o\). The data points were then fitted to the Hill equation:

\[
F = F_{\text{max}} \frac{[\text{Ca}^{2+}]}{K_D^{N} + [\text{Ca}^{2+}]^{N}}
\]

where, \(F_{\text{max}}\) is the maximal force, \(K_D\) is the Ca\(^{2+}\) concentration for half-maximal activation, and N is the Hill coefficient which characterizes the steepness of the relation and is a measure of the amount of molecular cooperativity of Ca\(^{2+}\) and Tn-C (Moss. 1992: Backx et al., 1995). The steady state force-[Ca\(^{2+}\)] relationship shows the dependence of the force on the [Ca\(^{2+}\)]\(_i\). Along the SS curve, the force of contraction is regulated only by [Ca\(^{2+}\)]\(_i\). Any shift from this curve suggests involvement of other factors (e.g. the contractile protein) in the rate of force development and relaxation.

Backx et al. (1995) demonstrated that in the twitches measured at different [Ca\(^{2+}\)]\(_o\) and in the presence of 100 \(\mu\)M CPA at 1 Hz, there was a total overlap of the force-[Ca\(^{2+}\)]\(_i\) curve during the relaxation period despite the marked differences in the level of activation.
(peak force) (Fig. 3.6-1). Thus, regardless of activation level, the force-[Ca\textsuperscript{2+}]\textsubscript{i} relationship was unique during most of the relaxation period. Moreover, this unique relaxation period (in the presence of CPA) was found to coincide identically with the steady state relationship measured during tetani (open circles, Fig. 3.6-1).

We, therefore, used the relaxation period of a twitch, measured in the presence of 100 μM CPA, as the SS F-[Ca\textsuperscript{2+}]\textsubscript{i} relationship. By superimposing the F-[Ca\textsuperscript{2+}]\textsubscript{i} phase plots of the single twitches, recorded at 0.2 and 2 Hz, on this SS curve, we showed whether [Ca\textsuperscript{2+}]\textsubscript{i} is the only limiting factor in the rate of force development or relaxation.

3.6.2 METHOD

60 mM cyclopiazonic acid (CPA) stock solution was made by dissolving 10 mg of (CPA) in 500 μL DMSO (Dimethyl Sulfoxide). 125 μL of the stock was then diluted in 75 mL oxygenated K-H solution to a final concentration of 100 μM. [Ca\textsuperscript{2+}]\textsubscript{i} had to be increased to 8 mM to compensate for the decrease in the peak force due to the presence of CPA.

3.6.3 RESULTS

100 μM CPA significantly prolonged the force of contraction and the Ca\textsuperscript{2+} transient, so that a rate of stimulation higher than 0.1 Hz would have caused elevation of the diastolic tone. In figure 3.6-2A the raw force and [Ca\textsuperscript{2+}]\textsubscript{i} traces in the presence of CPA at 0.1 Hz are shown. TD and the CaD were significantly prolonged to 1100.79 ± 85.53 msec and 496.62 ± 45.22 msec, respectively, in the presence of CPA. The peak [Ca\textsuperscript{2+}]\textsubscript{i} and the peak force were higher in the presence of CPA than in the control condition because of the high external [Ca\textsuperscript{2+}]. The forces in figure 3.6-2 are normalized to the F\textsubscript{max}. The F\textsubscript{max} is the force generated by the muscle when [Ca\textsuperscript{2+}]\textsubscript{i} is saturated, so that the [Ca\textsuperscript{2+}]\textsubscript{i} is not a limiting factor in the level activity of the
(Figure 3.6-1)
**Figure 3.6-2:** A) Force and Ca$^{2+}$ transient of a single twitch in the presence of 100 μM CPA and 8 mM external [Ca$^{2+}$] at 0.1 Hz. The force was normalized by the F$_{\text{max}}$. B) The relaxation phase of the force-[Ca$^{2+}$]$_i$ relationship was fitted to Hill equation. K$_D$ = 0.595 ± 0.001 μM. N=6.01 ± 0.08. This fit represents the steady state force-[Ca$^{2+}$]$_i$ relationship, where the force of contraction is regulated by intracellular Ca$^{2+}$ transient (see text).
(Figure 3.6-2)
muscle. \( F_{\text{max}} \) was measured here by tetanizing the muscle at 10 Hz. in the presence of CPA.

Figure 3.6-2B shows the relaxation phase of the \( F-[\text{Ca}^{2+}] \) relationship in the presence of CPA fitted to the Hill equation. The force was normalized to the \( F_{\text{max}} \). The fit of this relationship yielded values for \( K_D \) and \( N \) of 0.595 ± 0.001 \( \mu \text{M} \) and 6.01 ± 0.09, which are statistically comparable to the values reported by Bach et al. (1995) in CPA-tetanized trabeculae (\( K_D=0.63 \pm 0.11 \ \mu \text{M}, \ N=4.59 \pm 1.26 \)).

The phase plots of single twitches recorded under control conditions (\( [\text{Ca}^{2+}]_0=1 \ \text{mM}, \ SL=2.2-2.3, \ \text{Temperature}=22-23^\circ \text{C} \)), at 0.2 and 2 Hz. are shown in figure 3.6-3A. The small arrows show the direction of the twitch development. Following a stimulation (i.e. when an action potential reaches a myocyte), the \( [\text{Ca}^{2+}]_i \) starts to rise and reaches its peak value when the force has just started to increase. This delay in the force development is due to the more rapid kinetics of the \( \text{Ca}^{2+} \)-transients compared to the force of contraction (Backx et al., 1995). By the time the force reaches its peak value (contraction phase), \( [\text{Ca}^{2+}]_i \) has decreased to almost half of its peak towards its resting level. Then the force declines and \( [\text{Ca}^{2+}]_i \) will continue to decrease until they reach their diastolic level (relaxation phase), that is where the loop is completed.

In figure 3.6-3B. the phase loops of single twitches at 0.2 (solid line) and 2 Hz (dotted line), recorded under control conditions, are superimposed on the relaxation phase of the CPA-twitch which represents the SS \( F-[\text{Ca}^{2+}] \) relationship. At 2 Hz, the right half of the phase loop (the contraction phase) is shifted rightward, and the relaxation phase (the left half of the phase loop) of the twitch is shifted leftward relative to the SS relationship. This observation shows that force development and relaxation lags behind the rise and the relaxation of the \( [\text{Ca}^{2+}]_i \) transient. At 2 Hz, the contraction and the relaxation phase of the twitch force are shifted further away from
Figure 3.6-3: Contraction and relaxation phase of twitch force with respect to the steady state relationship.

A) Phase loops of twitches at 0.2 (solid line) and 2 Hz (broken line). The force was normalized by $F_{\text{max}}$. The small arrows show the direction of the change in the force and the $[\text{Ca}^{2+}]_i$ during development of a twitch. The peak force and the peak $[\text{Ca}^{2+}]_i$ are labeled. B) Phase loops of the twitches at 0.2 and 2 Hz superimposed with the SS relationship shows that neither the contraction nor the relaxation phase of a twitch overlap with the SS curve. Therefore, the contractile proteins must be the limiting factors during these phases and not the $[\text{Ca}^{2+}]_i$. Moreover, at 2 Hz, these phases are shifted even further away from the SS curve which suggests further contribution of the contractile proteins at the elevated frequency of stimulation.
(Figure 3.6-3)
the SS curve than at 0.2 Hz, suggesting that at elevated frequency of stimulation the kinetics of the force contraction and relaxation are still limited by the contractile filaments. Any shift towards the SS curve at 2 Hz would have suggested increased regulatory effect of the intracellular Ca\(^{2+}\) transient in limiting the rate of force development and relaxation. However, such a change was not observed.

3.6.4 DISCUSSION

In a normal twitch recorded at 0.2 Hz, the contraction and the relaxation phase of the \(F-Ca^{2+}\) plot was shifted to the right and left relative to the SS curve, confirming that force relaxation in cardiac muscle is not limited by the rate of decay of the \([Ca^{2+}]_i\) transient. Thus, force relaxation lags behind the relaxation of the \([Ca^{2+}]_i\) transient, which is consistent with the notion that mechanical relaxation is limited by the contractile filaments themselves and not the \([Ca^{2+}]_i\) (Backx et al., 1995). Various cross-bridge models have shown that, in heart, kinetic responses are slower than \([Ca^{2+}]_i\), changes during a twitch (Backx et al., 1995). At 2 Hz, the contraction and the relaxation phase of the \(F-Ca^{2+}\) phase loop were shifted further away from the SS relationship, confirming further contribution of the contractile proteins in limiting the rate of force development and relaxation at this high frequency of stimulation.

Therefore, the mechanism underlying the frequency-dependent acceleration of the force kinetics must be at the level of the contractile proteins. Further understanding of this mechanism requires detailed investigation of the effect of the frequency of stimulation on the property of the contractile elements (actin, myosin, troponin complex, tropomyosin, etc.) and/or their interaction.
3.6.5 CONCLUSION

The force of contraction is regulated by two main factors: intracellular Ca\(^{2+}\) handling ([Ca\(^{2+}\)]\(_i\) transient) and contractile proteins. Frequency-dependent acceleration of the twitch force was due to acceleration of its contraction as well as relaxation phase, whereas only the relaxation phase of the Ca\(^{2+}\) transient was accelerated. Moreover, the rate-dependent shortening of the TD occurred too fast (within 1-2 beats) to be due to the activation of the intracellular kinases and the phosphorylation of their substrate proteins (i.e. PLB. SR Ca\(^{2+}\) pump, etc.). We observed that neither the contraction nor the relaxation phase of the force-[Ca\(^{2+}\)]\(_i\) phase plot, at 0.2 Hz, overlapped with the SS F-[Ca\(^{2+}\)]\(_i\) curve (where the [Ca\(^{2+}\)]\(_i\) is the only regulator of the force of contraction). Therefore, the rate of the force development and relaxation is limited by the contractile proteins. At 2 Hz, the relaxation and the contraction phase are shifted further away from the SS curve, suggesting an increasing contribution of the contractile proteins to the kinetics of the twitch force at the higher frequency of stimulation. The frequency-dependent alterations in the contractile filaments that could cause acceleration of the force and kinetics could include decreased affinity of Tn-C to bind to Ca\(^{2+}\). decreased sensitivity of the contractile proteins to Ca\(^{2+}\). increased rate of Cross-bridge cycling (V\(_{\text{max}}\)). Therefore, although the rate of [Ca\(^{2+}\)]\(_i\) decline is accelerated at high frequencies of stimulation, it cannot be the limiting factor in determining the rate of force relaxation.
CHAPTER 4
Force of Contraction and Intracellular Calcium transient in
Hypertrophic Rat Heart

4.1 INTRODUCTION
Sudden cardiac death claims a considerable number of lives in the world each year. In 75% of cases, the underlying pathology causing heart failure in patients with sudden cardiac death is coronary heart disease and myocardial infarction. Idiopathic dilated cardiomyopathy is the cause in the 15%, and a mixture of hypertensive left ventricular hypertrophy, hypertrophic obstructive cardiomyopathy, and chronic valvular heart disease in the remaining 10% (Pye et al., 1992).

Acute myocardial infarction (MI) induces functional, morphological and biochemical damages to the myocardium which include ventricular mechanical dysfunction, disturbance of heart rhythm, formation of scar tissue and failure of myocardial energy production in humans as well as experimental animals (Sanbe et al., 1993). These changes are known to be heterogeneous and thus pathological alterations are complex depending upon the severity and extent of myocardial ischemia provoked (Sanbe et al., 1993).

4.2 NEUROHUMORAL AND MECHANICAL COMPENSATIONS
Following MI, alterations occur at many different levels. Such alterations include mechanical and neurohumoral compensations. recapitulation of fetal gene expression, and most importantly functional alterations, that is, attenuation in the pumping ability of the heart which is determined by the force of contraction generated by the cardiac muscles, which in turn depends on the intracellular \( \text{Ca}^{2+} \) handling and contractile protein properties (Piano, 1994).

Neurohumoral and mechanical compensations are evoked to maintain systemic circulation. The neurohumoral compensation includes activation of vasopressin and
sympathetic nervous systems (Goldsmith et al., 1983; Raya et al., 1990), activation of the renin-angiotensin-aldosterone systems (Fabris et al., 1990; Hirsch et al., 1991). The synthesis and release of atrial natriuretic peptide (ANP) is also elevated in congestive heart failure (Hodsman et al., 1988; Drexler et al., 1990; Ackermann et al., 1992). The rise in plasma level of ANP correlates with severity of cardiac failure and with elevated atrial pressures, and is considered an important adaptive mechanism that helps unload the failing myocardium through systemic vasodilation, decreased venous return and reduced vascular volume as well as promoting salt and water excretion by the kidney in the face of myocardial failure (Winaver et al., 1995).

The mechanical compensatory response of the myocardium includes myocyte hypertrophy and nonmyocyte (fibroblast) hyperplasia. Following an infarction, the surviving (un-infarcted) myocytes assume the additional work load for the lost (infarcted) myocytes and undergo hypertrophy which normalizes wall stress by adding new sarcomeres and mitochondria for contraction and energy production, respectively (Weber et al., 1991). Therefore, myocardial mass will increase which will result in ventricular wall thickening (Piano, 1994).

The non-myocyte component of the myocardium includes endothelial cells, vascular cells, fibroblasts, macrophages and mast cells (Opie, 1991; Weber et al., 1991). Fibroblasts are one of the more abundant nonmyocyte cells and undergo hyperplasia during cardiac hypertrophy (Weber et al., 1991). Fibroblast cells proliferate and produce excess fibrillar collagen that, in long term, leads to structural (ventricular) remodeling of the cardiac interstitium and decreased myocardial compliance (Chien et al., 1991; Piano, 1994). The resultant neurohumoral alterations and ventricular hypertrophy compensate for the original changes in the cardiac workload, but eventually lead to the progression of disease and heart failure. Ventricular remodeling, which is the principal long term change, is referred to the pathomorphological changes occurring in the aftermath of myocardial infarction (Lee.
1993). It can be described as progressive ventricular dilation eventually leading to pump failure.

It has been well documented that left ventricular myocardial infarction induces a compensatory hypertrophy of the surviving left ventricular myocardium (Anversa et al., 1986; Greenen et al., 1987). Right ventricular hypertrophy has also been observed in studies concerning the left ventricular overload and has been linked to the hemodynamic state of the left ventricle (Anversa et al., 1984; Pfeffer et al., 1985). Because morphological adaptation of the right ventricle in animals with large left ventricle infarcts is characterized by concentric hypertrophy (Anversa et al., 1984), whereas that of the viable nonischemic left ventricle is considered as both concentric and eccentric hypertrophy (Anversa et al., 1986; Greenen et al., 1989), it can be argued that changes in the left ventricle may differ from those in the right ventricle.

4.3 ALTERATIONS IN THE GENE EXPRESSION

It is well known that the adaptive changes in myocardial function that occur after the initiation of hemodynamic stress are associated with alterations in the steady state levels of mRNA encoding cardiac proteins (Feldman et al., 1993). In rodents, myocardial hypertrophy is characterized by recapitulation of the fetal pattern of gene expression (Shwartz et al., 1986). Development of hypertrophy has been associated with a decrease in the mRNA level of the α-myosin heavy chain (α-MHC) and a reciprocal increase in β-MHC (Buttrick et al., 1991; Imamura et al., 1991; Orenstein et al., 1996), an increase in the ventricular levels of α-skeletal actin (Shwartz et al., 1986), atrial natriuretic peptide (ANP) (Mercadier et al., 1989), angiotensin-converting enzyme (ACE) (Schunkert et al., 1990) and a diminution in the level of phospholamban (Nagai et al., 1989) and SR Ca^2+-ATPase (de la Bastie et al., 1990; Feldman et al., 1993). According to Katz (1991), the functional significance of the reappearance of these fetal genes (or isoforms) is not well understood, but it might further contribute to the cardiomyopathy of overload and deterioration of the failing heart.
4.4 **RESTING MEMBRANE POTENTIAL AND ACTION POTENTIAL DURATION**

The most consistent electrical abnormality which has been described in association with cardiac hypertrophy and heart failure (in human as well as animal models) is prolongation of the duration of the action potential (APD) (Hart, 1994). Prolonged AP will lead to prolonged depolarization of the sarcolemma and prolonged \( \text{Ca}^{2+} \) entry via the voltage-dependent L-type \( \text{Ca}^{2+} \) channels and \( \text{Na}^-/\text{Ca}^{2+} \) exchange. \( \text{Ca}^{2+} \) acts as an oncogene and activates growth hormones which could result in myocyte hypertrophy. Therefore, AP prolongation could play an important role in the development of cardiac hypertrophy.

In a ventricular action potential (AP), rapid upstroke (phase 0) is caused by rapid opening of \( \text{Na}^- \) channels (associated with propagation of membrane depolarization); early repolarization (phase 1) is caused by transient outward \( \text{K}^- \) current (\( I_{\text{to}} \)) and closure of the \( \text{Na}^- \) channels by a process of inactivation; the plateau phase (phase 2) is caused by \( \text{Ca}^{2+} \) influx (via L-type \( \text{Ca}^{2+} \) channels and \( \text{Na}^-/-\text{Ca}^{2+} \) exchanges, functioning at a forward mode); repolarization (phase 3) is caused by opening of delayed rectifier \( \text{K}^- \) channels (\( I_k \)) due to depolarization; the resting membrane potential (RMP) (phase 4) is maintained by inward rectifier channels (\( I_{\text{K,R}} \)).

Prolongation of the AP could be due to decreased hyperpolarizing currents (\( I_{\text{to}}, I_{\text{K}}, \) and/or \( I_{\text{K,R}} \)), increased depolarizing currents (\( I_{\text{Ca,L}}, \) and/or \( I_{\text{Na,Ca}} \)) or both. The ionic basis of AP prolongation in cardiac hypertrophy has been studied and controversial results have been reported depending on the species as well as the type and the stage of hypertrophy. Brooksby *et al.* (1993) reported a reduction in \( I_{\text{K,R}} \) in hypertrophic spontaneously hypertensive rats (16 weeks old) as the cause of the AP prolongation, whereas Cerbai *et al.* (1994) reported that to be due to a substantial reduction in \( I_{\text{to}} \). We have found in our laboratory Kaprielian *et al.* (1997) that the significant AP prolongation in coronary
ligated rats (LAD-ligated, 8-weeks post-operation) was due to a significant reduction in $I_\text{to}$. The significant reduction in $I_\text{to}$ in LAD ligated rats was also reported by Qin et al. (1996) 3-4 weeks following induction of coronary ligation. No significant reduction in $I_\text{k1}$ was reported in this model (Kaprielian et al., 1997; Qin et al., 1996).

Studies in rat hypertrophy models, such as abdominal aortic constriction moderate hypertrophy (Sen et al., 1990), Gh3 severe hypertrophy (Xu and Best, 1991), hypertrophic spontaneously hypertensive rats (SHR) (Brooksby et al., 1993), hypertrophic and HF SHR (Cerbai et al., 1994), LAD-igation induced hypertrophy (Qin et al., 1996), and hypertrophied and failing SHR (Gomez et al., 1997) showed no change in $I_{\text{Ca,L}}$ activity. Moreover, despite the significant prolongation of the AP, the resting membrane potential was not significantly changed in hypertrophic rats (Brooksby et al., 1993; Kaprielian et al., 1997) or syrian hamsters (Li et al., 1995).

4.5 MYOCARDIAL FUNCTIONAL ALTERATIONS

The velocity and extent of ventricular contraction and the rate of pressure development are decreased in hypertrophic (Litwin and Morgan, 1992, Brooks et al., 1994) and failing hearts (Gwathmey et al., 1987; Leger et al., 1990; Beuckelman, 1992, De Tombe et al., 1996). It is generally accepted that the ventricular dysfunction, that is characteristic of almost all heart failure models, is a consequence of diminished contractile function of the myocytes (Capasso et al., 1992; Capasso et al., 1993; Cheung et al., 1994). As a result of the observation that the primary defect in heart failure is decreased pump function, investigators have concentrated their efforts on determining systolic $[Ca^{2+}]_\text{i}$, as the causative mechanism for contractile dysfunction.

4.5.1 $Ca^{2+}$ transient and Intracellular $Ca^{2+}$ handling

Some investigators found no difference in peak systolic $[Ca^{2+}]_\text{i}$, between failing and nonfailing human myocardium (Gwathmey et al., 1989), whereas others found the peak $[Ca^{2+}]_\text{i}$ to be significantly reduced in human failing hearts (Beuckelmann, 1992).
The intracellular Ca\(^{2+}\) transient has been reported to be elevated during the early stages of hypertrophy. 6 weeks after myocardial infarction (Litwin and Morgan, 1994) and 6 weeks after aortic banding (Kagaya et al., 1996). A significant decrease in the systolic [Ca\(^{2+}\)] transient in guinea pigs at the end-stage heart failure (Siri et al., 1991), and following coronary artery narrowing in rats has been reported (Capasso et al., 1993). Therefore, the intracellular Ca\(^{2+}\) transient appears to be elevated during the early stage of heart failure and it gradually attenuates as the disease progresses, so that it is significantly diminished at the end-stage heart failure. Alterations in the intracellular Ca\(^{2+}\) transient could be due to changes in the activity of the SR Ca\(^{2+}\) uptake and release. Na\(^{+}\)/Ca\(^{2+}\) exchanger system and/or the L-type Ca\(^{2+}\) channels.

**SR Ca\(^{2+}\) ATPase pump**

The intracellular Ca\(^{2+}\) concentration in rat heart is primarily determined by the activity of the SR, which is responsible for release and uptake of 92% of the cytosolic Ca\(^{2+}\) during a contractile cycle (Bers, 1997). Accordingly, defects in Ca\(^{2+}\) release and uptake of the SR could result in the systolic and diastolic dysfunction seen in heart failure. A decrease in the mRNA level and activity of the SR Ca\(^{2+}\) ATPase, which could result in prolonged relaxation of the Ca\(^{2+}\) transient, has been reported in the hypertrophic hearts, induced by abdominal aortic banding (de la Bastie et al., 1990) and pressure overloaded rats (Komuro et al., 1989). Feldman et al. however, found no change in the mRNA level or the activity of the SR Ca\(^{2+}\) ATPase in the ascending aortic banded rats until development of decompensation and clinical symptoms of HF in rats (1993). SR Ca\(^{2+}\) uptake activity was decreased in rat left ventricle (LV) four weeks after left coronary artery ligation, while it was elevated in the right ventricle (RV) up to the 8th week. returned to the control level at 16 weeks and became depressed 20 weeks after the coronary ligation, at the HF stage (Afzal et al., 1992). According to this study, in rats with left coronary ligation, changes in the RV SR Ca\(^{2+}\) uptake are biphasic during development of heart failure, and when the LV has entered the failing stage, the RV is in a compensatory stage.
**Na⁺/Ca²⁺ Exchange System**

Na⁺/Ca²⁺ exchange system is the second important mechanism for Ca²⁺ efflux from the cardiac myocytes and may contribute to Ca²⁺ influx at positive potentials. The time course of decay of Ina/Ca as relaxation proceeds is determined principally by the rate of Ca²⁺ uptake by the SR (Cresp et al., 1990; Bers et al., 1990), and this rate is diminished in hypertrophied heart muscles (Sordahl et al., 1973; Whitmer et al., 1988). There is some evidence that Na⁺/Ca²⁺ exchange activity is decreased and that the Ca²⁺ sensitivity of the exchanger is reduced in sarcolemmal vesicles from hypertrophied rat hearts (Hang et al., 1990). Na⁺/Ca²⁺ exchange activity decreased in the rat LV four weeks after left coronary ligation and remained depressed (Dixon et al., 1992). Decreased activity of the Na⁺/Ca²⁺ exchanger and therefore decreased sarcolemmal Ca²⁺ efflux could contribute to intracellular Ca²⁺ overload.

Activity of sarcolemmal Ca²⁺ pump, another sarcolemmal Ca²⁺ transporter, was not altered in the hypertrophic rat hearts with MI (Dixon et al., 1992). The level of activity of the L-type Ca²⁺ channels has also been reported not to be affected in various hypertrophic models (Sen et al., 1990; Brooksby et al., 1993; Qin et al., 1996; Gomez et al., 1997).

4.5.2 **Force of Contraction**

Decreased force of contraction has been reported in hypertrophic (Litwin and Morgan, 1992; Kagaya et al. 1996) and failing hearts (de Tombe et al., 1996) subsequent to MI. The force of contraction is not regulated entirely by the intracellular Ca²⁺, but it also depends on the properties of the contractile proteins (Backx et al., 1995). Therefore, a defect in the pumping function of a diseased heart could also be due to alterations in contractile protein behavior. Aside from a decrease in [Ca²⁺], decreased force of contraction in a diseased heart could be due to one or more of the following factors:
1) Decreased myofilament sensitivity to Ca$^{2+}$.

Myofilament Ca$^{2+}$ sensitivity can be estimated from the force-[Ca$^{2+}$]$_i$ curve measured in chemically skinned cardiac fibers. A rightward shift of this curve (with respect to the control data) represents a decrease, and a leftward shift represents an increase in the sensitivity of the myofilaments to Ca$^{2+}$. Previous studies on the MI-induced hypertrophic (Litwin and Morgan, 1992) and failing hearts (De Tombe et al., 1996), as well as failing SHR (Perreault et al., 1992) did not show any change in the sensitivity of the myofilaments to Ca$^{2+}$. These authors observed a decrease in F$_{\text{max}}$ (maximal, Ca$^{2+}$-saturated force) but no significant change in cooperativity (Slope of the curve, Hill's coefficient, $n^H$) or myofilament Ca$^{2+}$ sensitivity ($K_D$, the [Ca$^{2+}$] at which the force is half of F$_{\text{max}}$). These observations show a reduction in maximal force-generating capacity of the muscle, and no change in the cooperativity or sensitivity of the contractile proteins to Ca$^{2+}$.

2) Conversion of the $\alpha$-MHC to $\beta$-MHC.

Mammalian heart's contractile apparatus is composed of two major components, thin and thick filaments. The thick filaments consist primarily of myosin which is composed of two heavy chains ($\alpha$-MHC and $\beta$-MHC) and four light chains. MHC exists as a homologous or heterologous dimer and can contain three isoforms (V1, V2, V3) which differ only in their MHC composition. The V1 isoform is composed of two $\alpha$-MHCs ($\alpha$.$\alpha$), V3 has two $\beta$-MHCs ($\beta$.$\beta$), and V2 has one MHC of each type ($\alpha$.$\beta$) (Hoh et al., 1978). The relative amounts of the three isoforms vary depending upon the developmental stage, the cardiac compartment, the animal, and the physiological status of the heart (Jones et al., 1996). V3 isoform has the slowest ATPase activity. In small rodents (e.g. rat and mouse) the predominant atrial MHC isoform is V1 before and after birth. The ventricles, however, contain V3 isoform during gestation. At or shortly after birth $\alpha$-MHC expression increases rapidly and V1 almost completely replaces V3 seven days after birth (Morkin, 1993; Palermo et al., 1996), so that V1 is the primary isoform expressed in both atria and ventricles in adult murine heart. In these animals, the
predominant ventricular MHC isoform changes from α-MHC to β-MHC in hypertrophic cardiomyopathy. This change has been observed in infarcted rat heart (Orenstein et al., 1996; Geenen et al., 1989), as well as in hypertrophied and failing spontaneously hypertensive rats (SHR) (Boluyt et al., 1994), and aortic-banded mouse (Dorn II et al., 1994) and rat (Swoap et al., 1995). Switching over to the isozyme with slower ATPase activity helps the affected fiber(s) save energy and is considered adaptive. β-MHC has a lower ATPase activity (than α-MHC), therefore will decrease the rate of cross-bridge formation. The switch of MHC isoform from α- to β- has been observed as early as one day after coronary ligation (Orenstein et al., 1996).

Despite the general belief that the predominant MHC isoform in human ventricles is β-MHC (Clark Jr. et al., 1982), data from 14 non-failing human hearts have recently shown that non-failing human heart expresses substantial amounts of alpha-MHC (23-34%) (Lowes et al., 1997; Nakao et al., 1997). In heart failure (in human) alpha-MHC is downregulated (by 67-84%) and β-MHC gene expression is upregulated (Lowes et al., 1997). Therefore, the data regarding the replacement of the alpha-MHC by β-MHC in the disease states, and its functional consequences, which has so far been assumed to be restricted to small rodents are indeed applicable to human studies as well.

The amplitude of the force of contraction depends on the number and the rate of cross-bridge formations. so that a decrease in either of these two factors can diminish the generated force of contraction. Due to β-MHC’s low ATPase activity, its predominance in the ventricles during the cardiac hypertrophy will decrease the rate of cross-bridge formation which could contribute to the decreased force of contraction in the hypertrophic muscles.

3) Decrease in the number of force generating units

Hypertrophy induces alterations in the extracellular (EC) matrix of the heart, such as increased interstitial fibrosis (Weber, 1991). An increase in EC collagen might lower
the number of force generating units (sarcomeres) per cross-sectional area (Litwin and Morgan, 1992; De Tombe et al., 1996). and could also adversely affect the availability of oxygen or metabolic substrates to the myocytes.

4.5.3 **Kinetics of the Force of Contraction**

Diastolic dysfunction, characterized by a delay in the rate of relaxation and a decreased filling of the left ventricle (Grossman, 1992), occurs early in the development of hypertrophy and usually precedes changes in systolic function (Piano, 1994). Some of the specific changes in diastolic function during hypertrophy include decreased ventricular compliance, increased end-diastolic pressure relative to diastolic volume, decreased rate of early diastolic filling and prolonged filling phase and delayed relaxation (Douglas et al., 1989; Lorell et al., 1990; Piano, 1994). Prolonged twitch duration (TD) has been reported to be associated with most of the animal models of hypertrophy and human heart failure (Jouannot and Hyatt, 1975; Gwathmey et al., 1992; Hajjar et al., 1992; Arai et al., 1993; De Tombe et al., 1996). In clinical terms, a delay in the rate of relaxation translates into a shorter diastole and a longer systole. Thus, there is less time for ventricular filling and coronary artery perfusion and greater chance for the development of subendocardial ischemia (Piano, 1994).

The potential candidates responsible for the prolongation of the twitch duration are as follows:

1) **Prolongation of the Ca\(^{2+}\) transient duration**

In most of the previous studies, the prolonged rate of relaxation of the force of contraction in hypertrophic and failing hearts has been attributed to defects in the rate of Ca\(^{2+}\) removal from the cytosol, more specifically, decreased activity of the SR Ca\(^{2+}\)-ATPase pump (Mercadier et al., 1990) and/or decreased density of the SR Ca\(^{2+}\)-ATPase (de la Bastie et al., 1990). Most of these studies, however, were done on isolated cardiac myocytes. In single cardiac myocytes, the relaxation of cell shortening is limited by the decline of the intracellular Ca\(^{2+}\) transient (Spurgeon et al., 1992). However, in cardiac
trabeculae (multicellular preparations). It has been demonstrated that, during rising phase and relaxation force lags behind the increases and relaxation of the $[Ca^{2+}]_{i}$ transient, respectively (Backx et al., 1995). This observation is consistent with the notion that mechanical relaxation is limited by the contractile filaments themselves and not by the rate of $Ca^{2+}$ decline (Backx et al., 1995). Therefore, a defect in the rate of force development or relaxation could be due to alterations in contractile protein behavior as well as the $[Ca^{2+}]_{i}$ transients.

2) **Downregulation of the β-adrenergic receptors**

Down-regulation of the cardiac β-adrenergic receptors (β-ARs) has been repeatedly reported in hypertrophy and heart failure (Baumann et al., 1981 and 1982; Bristow, 1988; Altschuld et al., 1995; Kagaya et al., 1996). Cardiac β-ARs are composed of about 80% β₁- and about 20% β₂-AR (Bristow, 1988). Stimulation of cardiac β-ARs has inotropic and chronotropic response on the heart. At a cellular level, β-AR activation results in elevation of the F and the $Ca^{2+}$ transient amplitude and acceleration of their kinetics. In vivo, in the absence of sympathetic stimulation, always a fraction of the β-ARs are in the activated state which contribute to the amplitude and the kinetics of the force and the $Ca^{2+}$ transient. Thus, the amplitude and kinetics of the force and the $Ca^{2+}$ transient, under basal conditions, is in fact under the effect of the active β-AR. Downregulation of the β-ARs would therefore, lead to the number of the β-ARs that can be in an activated state to be decreased. That will diminish the contribution of the β-AR to the amplitude and kinetics of the force and the $Ca^{2+}$ transient in the hypertrophic hearts. Therefore, decreased total number of the β-ARs could be partly responsible for the prolonged rate of mechanical relaxation that has been observed in diseased hearts.

3) **Replacement of the ventricular α-MHC with β-MHC**

Cardiac hypertrophy is associated with recapitulation of the fetal gene program, among which is elevated expression of the β-MHC and a reciprocal decrease in α-MHC expression (Schwartz et al., 1986; Fareh et al., 1996). Increases in the percentage of the
"slow activity" V₃ (β,β) myosin isoforms have been shown to occur in the noninfarcted myocardium in the MI-induced hypertrophic hearts (Gay et al., 1988; Michel et al., 1988; Orenstein et al., 1996). The importance of the MHC isoform switch is that it occurs in the early stage of cardiac hypertrophy (Wiesner et al., 1997). An increase in the mRNA level of the β-MHC has been shown as early as one day after induction of myocardial infarction (Orenstein et al., 1996) and an increase in its protein level has been observed as early as seven days (Wiesner et al., 1997) and 3-4 weeks (Reddy et al., 1996) after aortic banding. Therefore, it could be responsible for the alterations in the cardiac function observed during the early stage of hypertrophy. The low ATPase activity of the β-MHC causes a decrease in the rate of cross bridge cycling and could therefore contribute to prolongation of twitch duration. Predominance of the β-MHC in the hypertrophic ventricles has been the classical cause of TD prolongation in hypertrophic and failing hearts (Gay et al., 1988; Kagaya et al., 1996; Orenstein et al., 1996).

4.6 **FORCE-FREQUENCY RELATIONSHIP in HYPERTROPHY and HEART FAILURE**

The mechanism of F-frequency relationship and its biphasic nature in rat myocardium was fully discussed in chapter 3. The F-frequency relationship is critical for cardiac function as HR is increased. A defect in this relationship would lead to deficient cardiac function or even death at high heart rates. Numerous studies have shown that in end-stage HF, the F-frequency relationship of cardiac muscles is blunted or reversed in human (Feldman et al., 1988; Gwathmey et al., 1990; Mulieri et al., 1992; Schwinger et al., 1993), as well as in animal experimental models (Jauannat and Hyatt, 1975; Narayan et al., 1995). The F-frequency relationship depends on the developmental stage of heart failure, so that the positive relationship becomes gradually attenuated with the degree of HF (Schmidt et al., 1994). The negative F-frequency relationship in terminally failing hearts has been suggested to be due to differences in Ca²⁺ handling between the failing and the nonfailing myocardium, so that Ca²⁺ uptake is diminished in failing myocardium and that is aggravated at higher stimulation rate (Gwathmey et al., 1990). This
Earlier in this study, we observed that in normal rats, increased frequency of stimulation resulted in a biphasic force-frequency relationship, accompanied by a linear rise in peak [Ca\(^{2+}\)]\(_i\) and a significant decrease in the twitch duration and Ca\(^{2+}\) transient duration. We further demonstrated that the frequency-dependent acceleration of twitch kinetics has a very rapid time scale and is not affected by the sarcomere length, and is not caused by activation of the intracellular kinases or the rate-dependent pH\(_i\) decline. Having studied the mechanism of the frequency-TD relation, we decided to investigate whether this relationship is maintained in hypertrophic hearts. This would have very important clinical implications and has not previously been studied. Before studying the frequency-dependent alterations in the kinetics of the twitch force, we needed to characterize the used hypertrophy model by comparing the developed force of contraction and the Ca\(^{2+}\) transient and their kinetics to those of control (sham-operated, see method) rats.

4.8 METHOD

Left ventricular myocardial infarction (MI) was induced by a ligation around the left anterior descending artery (LAD) of male LBN-F1 rats (250-280 g at the time of surgery). The surgery was performed in Dr. P. Liu's laboratory by Drs. F. Dawood and W.H. Wan. Animals were anesthetized with ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/Kg) intraperitoneally. After adequate anesthesia, they were intubated with a 14-gauge polyethylene catheter and ventilated with room air using a small animal ventilator. A left thoracotomy was performed in the fifth intercostal space and the pericardium was opened. The proximal left coronary artery under the tip of the left atrial appendage was encircled and ligated using a 6-0 silk suture. Proximal left anterior descending artery ligation in a rat model creates a reproducibly large lateral wall infarction. The muscle and skin were closed in layers. In sham operated animals, the left coronary artery was encircled but not ligated and the muscle layers and skin were closed similarly (Orenstein et al., 1996).
6-7 weeks after the surgery. 350-400 g sham and ligated rats were anaesthetized and the RV trabeculae were dissected, mounted and perfused with K-H solution as explained in Chapter 2. Right ventricular hypertrophy in these hearts was developed by the compensatory neurohumoral and mechanical factors that were activated following induction of left ventricular myocardial infarction. The whole animals, whole heart and RV wall were weighed. The lungs were also dissected out, rinsed in K-H solution (to wash out the blood), and weighed.

To measure the infarction size of the left ventricle, the whole left ventricle (LV) and the infarcted area were traced on a piece of paper. The papers were weighed and the ratio of the infarct paper over the whole LV paper was expressed as a percentage. 40-50% of the left ventricle of the ligated hearts that were used in this study was infarcted. Force of contraction and intracellular Ca^{2+} transients were measured in the right ventricular trabeculae by using the techniques described in chapter 2. These rats were studied during the early stage of cardiac hypertrophy, when none of the clinical symptoms of heart failure were detectable. Therefore, this study provides information regarding development of cardiac hypertrophy. In this chapter, the LAD-ligated rats will be referred to as “ligated rats” and the sham-operated rats will be referred to as “sham rats.”

4.9 SOLUTIONS

The dissected trabeculae were perfused by oxygenated K-H solution as explained in Chapter 2. The experiments were performed at room temperature (22-23°C), at a sarcomere length of 2.2 to 2.3 μm and in the presence of 1 mM CaCl_{2} ([Ca^{2+}]_o=1 mM). 10 mM norepinephrine (a β_{1}- and α_{1}-AR agonist) stock solution was made by dissolving 2.1 mg NE in 10 mL distilled water. 10 μL of the stock solution was diluted in 100 mL K-H solution to obtain a final concentration of 100 nM. 1mM stock solution of phentolamine mesylate (PhM) (a α-AR antagonist) was made by dissolving 5 mg PhM in 13 mL distilled water. 100 μL of the stock solution was diluted in 100 mL K-H solution to the final concentration of 1 μM.
4.10 **STATISTICAL ANALYSIS**

All results are presented as means ± SE. Statistical comparisons of the sham and the LAD-ligated rats were made with unpaired t-test. Comparison of the parameters within the same group was made by paired t-test. The differences were statistically significant when P<0.05.

4.11 **RESULTS**

When the animals were sacrificed, the heart, RV and lung were weighed and normalized to the body weight (Table-4.1). Only the rats with an infarct size of 40-50% of the left ventricle were used. At 6-7 weeks post-operation, the whole heart as well as the right ventricle of the LAD-ligated rats (n=5) were significantly hypertrophic (p<0.05) compared to the sham rats (n=5). The whole heart weight normalized to the body weight increased from 0.27±0.01% in sham to 0.37±0.03% in ligated rats, and the RV weight normalized to the body weight increased from 0.035±0.003% to 0.100±0.013. However, the increase of the normalized lung weights in the ligated rats (0.34±.02 vs. 0.31±0.01%, P=0.41) was not statistically significant. Therefore these rats had not yet developed pulmonary hypertrophy which is a symptom of heart failure.

4.11.1 **Amplitude of Force and Intracellular Ca²⁺ transient**

Typical force and [Ca²⁺]ᵢ transient traces, recorded at 1 Hz, in sham and ligated rats are shown in figures 4-1 A and B, respectively. It is clear from these figures that in the ligated rats, the force of contraction was smaller but the Ca²⁺ transient was larger than the sham rats. Systolic and diastolic force and [Ca²⁺]ᵢ in sham and ligated rats are compared in figures 4-1 C and D. At 1 Hz, the peak force of contraction (mN/mm²) was significantly smaller in ligated rats (16.57 ± 2.46) than in the sham rats (26.35 ± 2.12):
Table 4.1- Hypertrophy-induced alterations in the heart and lung weight. All values are presented as mean ± SE. * represents significant difference between the sham and the ligated rats (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Sham-operated rats</th>
<th>LAD-ligated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>372.5 ± 28.5</td>
<td>395.5 ± 19.3</td>
</tr>
<tr>
<td>Whole heart weight</td>
<td>0.27 ± 0.01</td>
<td>0.37 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td>normalized to body</td>
<td></td>
</tr>
<tr>
<td></td>
<td>weight (%)</td>
<td></td>
</tr>
<tr>
<td>Right ventricular</td>
<td>0.035 ± 0.003</td>
<td>0.100 ± 0.013*</td>
</tr>
<tr>
<td></td>
<td>weight (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>normalized to body</td>
<td></td>
</tr>
<tr>
<td></td>
<td>weight (%)</td>
<td></td>
</tr>
<tr>
<td>Lung weight normalized to body weight (%)</td>
<td>0.31 ± 0.01</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 4-1: Force and $[Ca^{2+}]_i$ transients in sham and hypertrophic rats.

Force and $[Ca^{2+}]_i$ transients traces in sham rats (A) and in LAD-ligated rats (B). C) The peak force and the peak $[Ca^{2+}]_i$ are compared between the sham (white column) and the ligated (gray column) rats in the left and the right panel, respectively. D) The diastolic force and the diastolic $[Ca^{2+}]_i$ are compared between the sham (white column) and the ligated (gray column) rats in the left and the right panel, respectively. * P<0.05.
(Figure 4-1)
P<0.05, n=6), however, the peak \([Ca^{2+}]_t\) transient (\(\mu M\)) was significantly higher in the ligated rats (1.25 ± 0.22) compared to the sham rats (0.85 ± 0.05). The diastolic force (mN/mm²) and \(Ca^{2+}\) transient (\(\mu M\)) were slightly higher in the ligated rats (4.28 ± 1.04 vs. 3.96 ± 0.65 mN/mm² and 0.19 ± 0.04 vs. 0.13 ± 0.03 \(\mu M\) in sham rats), however, these differences did not reach statistical significance (P=0.49 and 0.18, respectively).

4.11.2 Kinetics of the Force and the \(Ca^{2+}\) transient

Twitch duration and \([Ca^{2+}]_t\) duration were measured in three different ways: 1) Contraction phase (\(TD_{p, CD_p}\)), the time from the onset of contraction to the peak. 2) Twitch (or \([Ca^{2+}]_t\) duration (\(TD_{50, CD_{50}}\)), the time from 50% contraction to 50% relaxation. 3) 80% Relaxation phase (\(TD_{R, CD_R}\)), the time from the peak to 80% relaxation. The durations were measured in milliseconds. Time to 80% relaxation was chosen (instead of 50%) because the prolongation of the force and the \(Ca^{2+}\) decline occurred mostly during the later phase of the relaxation.

Twitch Kinetics

Typical traces of the force of contraction from a sham and a ligated heart are superimposed in figure 4-2A (the solid line and the broken line, respectively). The decrease in peak force in the ligated heart can be seen in the left panel and prolongation of its kinetics is more clearly shown in the right panel where the two traces are scaled. In figure 4-2B, the three different ways that the kinetics of the twitch force were measured are shown: time to peak (\(TD_p\)), time from 50% contraction to 50% relaxation (\(TD_{50}\)), and relaxation time (\(TD_R\)). The kinetics of the force of contraction in sham and ligated rats at 1 Hz. are compared in figure 4-2C (white and gray columns, respectively). These graphs show that alterations in the kinetics in the hypertrophic hearts greatly depends on the way that the measurement was made. At 1 Hz, time to peak and time from half contraction to half relaxation were significantly prolonged (P<0.05) in the ligated hearts (\(TD_p=214.25 ± 5.76, TD_{50}=326.7 ± 19.1, n=5\)) compared to the sham hearts (\(TD_p=182.2 ± 17.8, TD_{50}=256.5 ± 28.8, n=5\)). However, prolongation of the relaxation phase in the
Figure 4-2: Twitch kinetics in sham and hypertrophic hearts at 1 Hz.

A) The left panel shows superimposed force traces from sham (solid line) and ligated (broken lines). These two traces are scaled in the right panel. B) The intervals that were measured in order to study the twitch kinetics in the ligated hearts. C) From left to right, time to peak, twitch duration and relaxation phase are compared between sham (white columns) and ligated (gray columns) rats. * P<0.05.
Figure 4-2
ligated hearts was not statistically significant (235.0 ± 23.4 in sham and 280.25 ± 29.81 in ligated rats. n=5, P=0.35). The time interval from 50% contraction to 80% relaxation is considered as the twitch duration which is significantly prolonged in the hypertrophic hearts.

**Intracellular Ca\(^{2+}\) transient Kinetics**

Typical traces of the intracellular Ca\(^{2+}\) transients (Ca\(^{2+}\)) from sham and ligated hearts, at 1 Hz, are superimposed in figure 4-3A. The difference of the Ca\(^{2+}\) transient duration between the two groups is more clear in the right panel of this figure where the two traces are scaled. This figure shows that the early relaxation phase is not very different between the sham and the ligated rats, but the late phase is prolonged in the ligated hearts. The three different ways in which the Ca\(^{2+}\) transient kinetics were measured are shown in figure 4-3B: contraction phase, from 50% contraction to 50% relaxation and peak to 80% relaxation. Ca\(^{2+}\) transient kinetics of sham and ligated rats, at 1 Hz, are compared in figure 4-3C (white and gray columns, respectively). Prolongation of the contraction phase (from 47.4 ± 3.7 in sham to 53.75 ± 2.46 in ligated. n=5), the Ca\(^{2+}\) duration (CD\(_{50}\)) (from 126.2 ± 10.6 to 142.6 ± 24.2), and the relaxation phase (from 285.2 ± 10.47 to 324.75 ± 38.33) were not statistically significant (P=0.41, 0.52 and 0.35, respectively).

4.11.3 **FREQUENCY-DEPENDENT ALTERATIONS**

In order to study the influence of cardiac hypertrophy on the frequency-dependent changes on the force and the Ca\(^{2+}\) transient, these parameters were measured at different frequencies of stimulation. It is important to appreciate that the sham rats used in this study were different from the normal rats used in the study described in chapter 3. The sham rats were age-matched with the ligated rats and at least twice as old as the normal rats. They had also undergone a surgical procedure although no ligation was made. Thus, the amplitude of force and [Ca\(^{2+}\)], and their duration as well as the frequency-
**Figure 4-3:** $[\text{Ca}^{2+}]_i$ transient kinetics in sham and hypertrophic hearts at 1 Hz.

A) The $[\text{Ca}^{2+}]_i$ transient traces from sham and ligated rats are superimposed in the left panel and are scaled in the right panel. B) The intervals that were measured to study the $[\text{Ca}^{2+}]_i$ transient kinetics in the ligated rats. C) From left to right, contraction phase duration, $[\text{Ca}^{2+}]_i$ transient duration and relaxation phase duration in sham (white columns) and ligated (gray columns) rats. No statistical significance was observed.
Figure 4-3
dependent changes could be different between the young normal rats and the sham-operated rats.

Force and intracellular Ca\(^{2+}\) transient at 0.2 and 2 Hz, in sham and ligated rats are shown in figure 4-4 A and B, respectively. This figure shows that the trabeculae from the sham and the ligated rats responded similarly to an increase in the frequency of stimulation, most importantly, the kinetics of the force and the Ca\(^{2+}\) transients seem to be accelerated at 2 Hz in both sham and ligated rats. The changes in the peak force and the peak [Ca\(^{2+}\)]\(_i\) transient over a range of the frequency of stimulation are plotted as functions of the frequency of stimulation in figure 4-4C. The force of contraction was significantly smaller in the ligated than in the sham rats at all frequencies of stimulation. Both groups showed a biphasic force-frequency relationship, negative from 0.2 to 0.5 Hz and positive from 0.5 to 1 Hz (Fig. 4-4C, the left panel). In the sham rats, the positive phase, but not the negative phase was statistically significant. The peak decreased from 26.87 ± 3.28 to 24.22 ± 2.07 (P=0.13) and increased to 28.55 ± 4.44 mN/mm\(^2\) (P<0.05) as the frequency of stimulation was increased from 0.2 to 0.5 and to 2 Hz. In the ligated rats, on the other hand, the negative phase, and not the positive phase was significant. The force decreased significantly from 19.10 ± 2.70 to 16.29 ± 6.01 (P<0.05) and increased to 18.52 ± 5.56 (P=0.23) when the rate of stimulation was increased from 0.2 to 0.5 and to 2 Hz. Therefore, the force-frequency relationship in the ligated hearts appears to be flat and not significantly biphasic although not negative either.

The peak Ca\(^{2+}\) transients in the ligated rats were significantly higher than the sham rats at all the frequencies of stimulation (Fig. 4-4C, right panel). Peak [Ca\(^{2+}\)]\(_i\) rose linearly as the frequency of stimulation was increased from 0.2 to 0.5, 1 and 2 Hz in the sham and the ligated rats. The data were fit to a linear regression model (P= 0.010 for sham and P=0.002 for ligated rats). Following increasing the frequency of stimulation from 0.2 to 2 Hz, the peak [Ca\(^{2+}\)]\(_i\) increased from 0.72 ± 0.07 to 1.06 ± 0.21 μM in sham, and from 1.01 ± 0.20 to 1.71 ± 0.44 μM.
Figure 4-4: Frequency-dependent changes in the peak force and the \([\text{Ca}^{2+}]_i\) transient.

A) Force and \([\text{Ca}^{2+}]_i\) transient traces at 0.2 (left panel) and 2 Hz (right panel) in sham rats. B) Force and \([\text{Ca}^{2+}]_i\) transient traces at 0.2 (left panel) and 2 Hz (right panel) in LAD-ligated rats. C) Left panel: Biphasic force-frequency relationship in the sham (solid line) and ligated (broken line) rats. Right panel: Linear \([\text{Ca}^{2+}]_i\)-frequency relationship in sham (solid line) and ligated rats (broken line). The \([\text{Ca}^{2+}]_i\) data were fitted by a linear regression model. Sham rats: \([\text{Ca}^{2+}]_i = 0.20 \text{ (Freq.)} + 0.65\). \(P = 0.01, r = 0.99\). Ligated rats: \([\text{Ca}^{2+}]_i = 0.44 \text{ (Freq.)} + 0.82\). \(P = 0.002, r = 0.99\).
(Figure 4-4)
Figure 4-5 shows the frequency-dependent changes in the force and the Ca$^{2+}$ transient diastolic tone in sham and ligated rats. Force and Ca$^{2+}$ transient at 0.2 and 2 Hz from sham rats are superimposed in figure 4-5A and those from ligated rats are superimposed in figure 4-5B. These figures do not show a noticeable elevation of the diastolic tone at 2 Hz compared to 0.2 Hz in sham or ligated rats. The frequency-dependent changes in the diastolic tone of the force and the Ca$^{2+}$ in sham (solid line) and ligated (broken line) rats are shown in figure 4-5C. Diastolic tones of the force and the Ca$^{2+}$ transient were not significantly different between the sham and the ligated rats at any of the frequencies of stimulation. Furthermore, increasing the frequency of stimulation from 0.2 to 2 Hz did not cause a significant change in the diastolic force in the sham rats ($4.33 \pm 0.61$ to $4.37 \pm 0.56$ mN/mm$^2$, $P=0.89$, $n=5$) or in the ligated rats ($4.62 \pm 1.09$ to $4.78 \pm 1.12$ mN/mm$^2$, $P=0.68$, $n=5$). Moreover, the frequency-dependent changes in the diastolic Ca$^{2+}$ transient were not statistically significant in either group. It increased from $0.12 \pm 0.04$ at 0.2 Hz to $0.18 \pm 0.03$ μM at 2 Hz ($P=0.13$) in sham rats, and from $0.21 \pm 0.04$ at 0.2 Hz to $0.26 \pm 0.05$ μM at 2 Hz ($P=0.25$) in ligated rats.

The kinetics of the force and the Ca$^{2+}$ transient accelerated as the frequency of stimulation was increased in both ligated and sham rats (Fig. 4-6 A and B). Upon increasing the frequency of stimulation from 0.2 to 2 Hz, the time from 50% contraction to 50% relaxation of the twitch force (TD$_{50}$) decreased from $329.0 \pm 28.3$ to $210.0 \pm 22.9$ msec ($P<0.05$) in sham rats and from $415.2 \pm 21.2$ to $111.1 \pm 4.9$ msec ($P<0.05$) and in the ligated rats (Fig 4-6 A). TD$_{50}$ was significantly prolonged in the ligated rats at 0.2, 0.5 and 1 Hz (compared to the sham rats) but not at 2 Hz ($P=0.21$). Therefore, despite the significant prolongation of the twitch kinetics in the ligated rats, it accelerated following elevation of the frequency of stimulation. To compare the frequency sensitivity of the twitch kinetics between the sham and the ligated rats, the slopes of the TD-frequency curves were compared between these two groups. The data in figure 4-7A were fitted by a linear regression model. There was a significant linear relationship between the TD and the frequency of stimulation in sham ($P=0.03$) and in the ligated rats.
Figure 4-5: Frequency-dependent changes in the diastolic force and \([\text{Ca}^{2+}]_i\) in sham and hypertrophic hearts.

A) Superimposed force (left) and \([\text{Ca}^{2+}]_i\) (right) traces at 0.2 (solid line) and 2 Hz (broken line), in sham rats. B) Superimposed force (left) and \([\text{Ca}^{2+}]_i\) (right) traces at 0.2 (solid line) and 2 Hz (broken line), in ligated rats. C) Frequency-dependent changes in diastolic force (left) and diastolic \([\text{Ca}^{2+}]_i\) (right), in sham (solid line) and ligated (broken line) rats. No significant frequency-dependent changes in the diastolic tone of either group was observed.
Figure 4-5

(A) Sham

(B) LAD-ligated

(C) Graphs showing diastolic force (mN/mm²) and diastolic [Ca²⁺] (μM) against frequency of stimulation (Hz).
Figure 4-6: Twitch and $[Ca^{2+}]_i$ duration in sham and ligated rats.

The duration presented in this figure are the time from 50% contraction to 50% relaxation. **A)** $TD_{50}$ in sham (solid line) and ligated (broken line) rats as functions of the frequency of stimulation.  **B)** $CaD_{50}$ in sham (solid line) and ligated (broken line) rats as functions of the frequency of stimulation.
Frequency of stimulation (Hz)

A

Twitch duration (TD₃₀) (msec)

B

[Ca²⁺] duration (CD₅₀) (msec)

(Figure 4-6)
**Figure 4-7**: Linear relationship between twitch and \([\text{Ca}^{2+}]_i\) duration and frequency of stimulation.

The data were fitted to a linear regression model.

**A)** Sham rats (solid line): $TD = -61.92 \text{ (Freq.)} + 327.8$. $P = 0.03$. $r = -0.97$.
Ligated rats (broken line): $TD = -103.2 \text{ (Freq.)} + 429.9$. $P = 0.002$. $r = -0.99$.

**B)** Sham rats (solid line): $\text{CaD} = -65.9 \text{ (Freq.)} + 207.8$. $P = 0.03$. $r = -0.97$.
Ligated rats (broken line): $\text{CaD} = -72.8 \text{ (Freq.)} + 237.7$. $P = 0.06$. $r = -0.94$. 
A

Twitch duration (TD<sub>50</sub>) (msec)

Frequency of stimulation (Hz)

Ligated

sham

B

[Ca<sup>2+</sup>]<sub>i</sub> duration (CaD<sub>50</sub>) (msec)

Frequency of stimulation (Hz)

Ligated

sham

(Figure 4-7)
The slope of the TD-frequency curve was \(-61.9 \pm 10.9\) in the sham and \(-103.2 \pm 5.1\) in the ligated rats. The slope was significantly larger in the ligated rats compared to the sham rats (P=0.03), which suggests that the frequency sensitivity of the twitch kinetics was stronger in the hypertrophic hearts than that in the control (sham) hearts.

Prolongation of the Ca\(^{2+}\) transient duration (CD\(_{50}\)) was not statistically significant at any of the frequencies of stimulation (Fig. 4-6 B). The CaD-frequency relationship was maintained in the hypertrophic hearts, so that increasing the frequency of stimulation from 0.2 to 2 Hz caused a significant decrease in the CD\(_{50}\) in sham (from 206.0 \(\pm\) 28.7 to 83.5 \(\pm\) 11.4 msec. P<0.05) as well as in ligated rats (from 244.3 \(\pm\) 24.1 to 103.7 \(\pm\) 12.0 msec. P<0.05). Moreover, when the data in figure 4-7B were fitted by a linear regression model, a linear relationship was found between CaD and frequency of stimulation sham (P=0.03) but not in ligated rats (P=0.06). The difference between the slope of these two curves (-65.9 \(\pm\) 10.6 in sham, and -72.8 \(\pm\) 17.8 in ligated rats), however, was not statistically significant (P=0.18). Therefore, frequency sensitivity of the Ca\(^{2+}\) transient shortening was not altered in the hypertrophic (ligated) hearts.

4.11.4 \(\beta\)-ADRENORECEPTOR ACTIVATION

Possible down-regulation of the \(\beta\)-ARs and its contribution to the prolonged twitch duration in the hypertrophic heart was examined by comparing the level of activity of these receptors in sham and ligated rats. Norepinephrine (NE), a \(\beta_1\)- and \(\alpha_1\)-AR agonist, was used to stimulate the \(\beta_1\)-ARs. In order to exclusively study the activity of the \(\beta_1\)-AR, 1 \(\mu\)M phenotolamine mesylate (PhM), a specific \(\alpha\)-AR antagonist, was applied 3-5 minutes before NE. PhM was used to block the \(\alpha\)-AR stimulation, instead of the commonly used \(\alpha\)-AR blocker, prazosin, because prazosin has fluorescent activities which would interfere with those of Fura-2 salt which was used to measure the \([Ca^{2+}]_i\). Application of PhM did not have any noticeable effect on the force of contraction. Application of 100 nM NE, following PhM, caused a significant inotropic effect in both sham (Fig. 4-8A) and ligated (Fig. 4-8B) hearts in less than one minute. Figure 4-8
shows that the inotropic effect of $\beta_1$-AR stimulation was intact in the hypertrophic hearts. 100 nM NE (in the presence of 1 $\mu$M PhM) at 1 Hz. significantly (P<0.05) increased the force of contraction (mN/mm$^2$) in sham (from $25.12 \pm 2.27$ to $39.85 \pm 4.51$, n=5) and in the ligated rats (from $16.29 \pm 2.46$ to $30.85 \pm 5.5$, n=5). $\beta$-AR stimulation also increased [$Ca^{2+}]_i$ ($\mu$M) significantly in sham (0.81 $\pm$ 0.07 to 1.19 $\pm$ 0.15, n=5) and in the ligated rats (1.19 $\pm$ 0.17 to 2.10 $\pm$ 0.11, n=5). The kinetics were also significantly accelerated in both groups following application of NE. TD$_{80}$ decreased from 292.00 $\pm$ 26.03 to 220.23 $\pm$ 14.1 milliseconds in sham rats, and from 367.5 $\pm$ 29.4 to 293.1 $\pm$ 16.5 milliseconds in the ligated rats. Intracellular $Ca^{2+}$ transient duration (CD$_{80}$) also decreased from 309.0 $\pm$ 11.6 to 232.5 $\pm$ 8.6 msec in sham, and from 342.75 $\pm$ 45.55 to 248.16 $\pm$ 25.42 msec in ligated rats.

The percentage change in force, [$Ca^{2+}]_i$, twitch duration (TD$_{80}$) and $Ca^{2+}$ duration (CD$_{80}$) in the sham and the ligated rats are shown in figure 4-9. Stimulation of $\beta_1$-ARs increased the force in the sham and the ligated rats by 63.0 $\pm$ 16.1% and 39.75 $\pm$ 15.02%, and the peak [$Ca^{2+}]_i$ by 49.3 $\pm$ 5.2% and 67.0 $\pm$ 13.4%, respectively. The TD$_{80}$ and the CD$_{80}$, respectively decreased to 71.0 $\pm$ 7.6% and 80.75 $\pm$ 1.89% of their control values in sham and to 77.5 $\pm$ 5.6% and 78.12 $\pm$ 7.87% of their control values in ligated rats. The NE-induced changes in the force and the $Ca^{2+}$ transient were statistically significant in both groups. These data show that $\beta_1$-AR activity was not decreased in the hypertrophic hearts.
Figure 4-8: β₁-Adrenergic receptor activity in sham and in ligated rats.

A) The inotropic effect of β₁-AR stimulation by norepinephrine (NE, α- and β₁-AR stimulator) and phentolamine mesylate (PhM, an α-AR inhibitor) in (sham operated) rats (chart recorder paper). PhM was added 3-5 minutes before NE. The two bottom panels show the increase in the amplitude of the force and Ca²⁺ transient in the control rats. B) The inotropic effect of β₁-AR stimulation (NE + PhM) in ligated hearts (chart recorder paper). The alterations in the force and the [Ca²⁺]ᵢ transient following activation of the β₁-AR are shown in the two bottom panels.
A

Normal

![Graph showing force vs. time for normal conditions.]

Control

![Graph showing force vs. time for control conditions.]

100 nM NE

![Graph showing force vs. time for 100 nM NE conditions.]

B

LAD Ligation

![Graph showing force vs. time for LAD ligated conditions.]

Control

![Graph showing force vs. time for control conditions in LAD ligated tissue.]

100 nM NE

![Graph showing force vs. time for 100 nM NE conditions in LAD ligated tissue.]

(Figure 4-8)
**Figure 4-9:** Percentage change in the peak and kinetics of force and $[\text{Ca}^{2+}]_i$ transient following $\beta_1$-AR stimulation.

The panels on the left show the changes in the sham rats and the panels on the right show the changes in the ligated rats. White columns represent the control sham, gray columns represent the control ligated, and the crossed-hatched columns represent the effect of $\beta_1$-AR stimulation. **A)** Percentage change in the peak force. **B)** percentage change in the peak $[\text{Ca}^{2+}]_i$ transient. **C)** percentage change in the twitch duration (50% contraction to 50% relaxation), and **D)** percentage change in the $[\text{Ca}^{2+}]_i$ transient duration (50% contraction to 50% relaxation) following stimulation of the $\beta_1$-ARs by NE (in the presence of PhM).
A Sham

B Ligated

C

D

(Figure 4-9)
4.12 DISCUSSION

In chapter 3, we investigated the underlying mechanism of the rate-dependent twitch duration abbreviation. We concluded that a frequency-dependent change in the contractile proteins was responsible for this phenomenon, although the details of such alteration(s) requires further investigation. Having studied this phenomenon in normal hearts, it was intriguing to find out whether the frequency-TD relationship was sustained in diseased states. We used hypertrophic rat hearts (hypertrophy induced by LAD-ligation), as a disease model, in order to study this relationship. These rats were hypertrophic but showed no overt sign of heart failure. Before we could study the frequency-dependent changes in the twitch force in these rats, we needed to characterize this hypertrophy model by studying the hypertrophy-induced changes in the force, $[\text{Ca}^{2+}]_i$ and their kinetics compared to the control rats.

Force of Contraction

The force of contraction generated by cardiac muscles is determined by a multitude of factors, such as sarcomere length, temperature, and $[\text{Ca}^{2+}]_o$. In this study, these factors were kept constant during experiments on the sham and ligated rats (SL = 2.2-2.3 μm. Temp. = 22-23°C. $[\text{Ca}^{2+}]_o$=1 mM), therefore, differences in the amplitude and kinetics of the force of contraction could be primarily due to alterations in the intracellular Ca$^{2+}$ transient and/or the contractile proteins. However, the significant decrease in the twitch force in the hypertrophic trabeculae cannot be explained by the simultaneous significant elevation in the peak intracellular Ca$^{2+}$ transient. This decrease in the force of contraction could therefore, be due to decreased myofilament sensitivity to Ca$^{2+}$, a decrease in the number or the rate of crossbridge formation (due to conversion of the α-MHC to β-MHC), and/or decreased number of force generating units (sarcomeres). The mechanism by which each of these factors could contribute to the decreased force of contraction, are described in Introduction (4.5b). The cause of this significant reduction in the force of contraction requires further investigation (See future directions).
**Force Kinetics**

In this study we observed that, at 1 Hz. duration of the twitch (from 50% contraction to 80% relaxation) was significantly prolonged in the ligated hearts compared to the sham hearts. We also observed that the rate of contraction (time to peak) was significantly prolonged in the hypertrophic hearts whereas prolongation of the relaxation phase (peak to 80% relaxation) was not statistically significant. Therefore, prolongation of the twitch duration was mostly due to the slowed rate of force development. The potential mechanism underlying prolongation of the force kinetics in these hypertrophic hearts will be discussed later.

**Intracellular Ca\(^{2+}\) Transient**

The peak Ca\(^{2+}\) transient was significantly elevated in the RV trabeculae of the ligated rats. Increased systolic [Ca\(^{2+}\)]\(_i\) in the early stage of hypertrophy has been previously reported in other models of hypertrophy, such as in MI induced by ligation of a proximal coronary artery (Litwin and Morgan, 1992) and aortic banding (Kagaya et al., 1996). The increase in the peak Ca\(^{2+}\) transient could be due to increased Ca\(^{2+}\) influx via \(I_{Ca,L}\) and \(I_{Na-Ca}\) or increased SR Ca\(^{2+}\) release. Increased \(I_{Ca,L}\) could lead to increased Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release from the SR as well as its direct contribution to the elevation of the cytosolic Ca\(^{2+}\). Majority of the studies have shown that \(I_{Ca,L}\) did not change in hypertrophic (Sen et al., 1990; Brooksby et al., 1993; Qin et al., 1996; Kaprielian et al., 1997) or in failing hearts (Cerbai et al., 1994; Gomez et al., 1997). Therefore, it is unlikely that the significant elevation of the [Ca\(^{2+}\)]\(_i\) was due to increased \(I_{Ca,L}\). Na\(^+\)/Ca\(^{2+}\) exchanger system plays a small role in the Ca\(^{2+}\) influx and efflux during a contractile cycle in rat myocardium (responsible for <8% of cytosolic Ca\(^{2+}\): Bers 1997). Therefore, alterations in the activity of this system could not cause significant changes in the Ca\(^{2+}\) handling. Moreover, this exchange system has been shown to be downregulated in rats with coronary artery ligation (Dixon et al., 1992), which could only cause a reduction in Ca\(^{2+}\) influx or efflux via this pathway. Therefore, involvement of this exchange system in the elevated [Ca\(^{2+}\)]\(_i\) is highly unlikely. Increased sensitivity of the SR ryanodine
receptors could cause increased Ca\(^{2+}\) release from the SR in response to the same level of Ca\(^{2+}\) influx via the I\(_{\text{Ca,L}}\), although such an alteration in hypertrophic hearts has not been yet reported.

It has been shown in our laboratory (Kaprielian et al., 1997) that action potential duration (APD) was significantly prolonged in the ligated rats. Prolongation of the APD would result in prolonged depolarization of the myocyte membrane and therefore prolonged Ca\(^{2+}\) influx via the voltage-dependent sarcolemmal pathways (I\(_{\text{Ca,L}}\) and Na\(^+\)/Ca\(^{2+}\) exchange) which could contribute to the elevated [Ca\(^{2+}\)]\(_i\). Therefore, APD prolongation appears to be the most logical explanation for the elevated systolic [Ca\(^{2+}\)]\(_i\) in the hypertrophic hearts.

[Ca\(^{2+}\)]\(_i\) transient kinetics

The prolongation of the [Ca\(^{2+}\)]\(_i\) kinetics (time to peak, time from peak to 80% relaxation, and time from 50% contraction to 50% relaxation) did not reach statistical significance. The rate of Ca\(^{2+}\) transient relaxation in rat myocardium has been suggested to be directly correlated with the level of activity of the SR Ca\(^{2+}\) ATPase pump (Hasenfus et al., 1996). We observed significant elevation of the peak [Ca\(^{2+}\)]\(_i\) in the hypertrophic hearts without a significant prolongation in the rate of relaxation which requires increased activity of the SR Ca\(^{2+}\) pump, the main factor in Ca\(^{2+}\) uptake from the cytosol. This assumption is very well supported by finding of Afzal et al. (1992) who showed that SR Ca\(^{2+}\) ATPase uptake activity in the RV of rats with left coronary artery ligation (similar to the model used in our study) was elevated up to 8 weeks after surgery (i.e. induction of MI). SR Ca\(^{2+}\) ATPase uptake activity declined to the control level 16 weeks after, and was depressed 20 weeks after the surgery. Therefore, according to Afzal et al. (1992), during the compensatory hypertrophy (up to 8 weeks post-operation) SR Ca\(^{2+}\) uptake activity was elevated, and its depression occurred when the rats of this hypertrophy model have been shown to enter heart failure (>20 weeks post-MI, De Tombe et al., 1996). This observation is consistent with that of Feldmann et al. (1993) who found that
downregulation of the SR Ca\(^{2+}\) pump activity took place after the animals had entered decompensated hypertrophy and heart failure. We used our ligated rats 6-7 weeks after the surgery when, according to Afzal et al. (1992), SR Ca\(^{2+}\) uptake activity was elevated, which explains the absence of a significant CaD prolongation. Moreover, due to the [Ca\(^{2+}\)]-dependent activity of the SR Ca\(^{2+}\)-ATPase pump (Bers and Berlin, 1995), the elevated [Ca\(^{2+}\)]\(_i\) in the hypertrophic hearts could also upregulate the SR Ca\(^{2+}\) pump activity which would accelerate Ca\(^{2+}\) uptake by the SR. Therefore, according to our findings, activity of the pumps and transporters (of Ca\(^{2+}\)) were not decreased 6-7 weeks after myocardial infarction (i.e. during compensatory hypertrophy).

Our data about Ca\(^{2+}\) transient contrasts a number of other animal and human studies that reported decrease of the amplitude and prolongation of the Ca\(^{2+}\) transient in the hypertrophic hearts (Gwathmey et al., 1984; Beuckelmann et al., 1992; Brooks et al., 1994). This difference could be related most to the degree of heart disease in the animals. we used ligated rats during the early stage of the disease when they were still in the compensatory hypertrophy. Our data are consistent with other studies, on infarcted and aortic-banded rats during the early stage of hypertrophy, which also found an elevation in the peak [Ca\(^{2+}\)]\(_i\) and no significant change in the duration of the Ca\(^{2+}\) transient (Litwin and Morgan, 1992; Kagaya et al., 1996). Therefore, we conclude that kinetics of the Ca\(^{2+}\) transient are not affected during the early stage of hypertrophy, when, according to the previous studies, down-regulation of the Ca\(^{2+}\) transport systems has not yet taken place.

In summary, left ventricular MI resulted in the death of a fraction of the left ventricular myocytes and hypertrophy of the surviving myocytes and hyperplasia of non-myocytes in the left and the right ventricles. Prolongation of the action potential (Kaprielian et al., 1997) led to prolonged Ca\(^{2+}\) entry via the voltage-dependent sarcolemmal I\(_{\text{Ca,L}}\) and Na\(^{+}\)/Ca\(^{2+}\) exchange which resulted in elevation of the peak [Ca\(^{2+}\)]\(_i\) transient. Elevated [Ca\(^{2+}\)]\(_i\) could activate proto-oncogenes and growth factors which would further contribute to progression of hypertrophy and fibrosis. Despite the elevated
[Ca\(^{2+}\)]_\text{i} \text{ transient, peak force was significantly diminished in the ligated rats. Thus, the decrease in the twitch force was due to alterations at the level of the contractile proteins and not due to a reduction in the available [Ca\(^{2+}\)]_\text{i}. We further observed a significant prolongation of the force kinetics, whereas prolongation of the [Ca\(^{2+}\)]_\text{i} transient kinetics did not reach statistical significance, possibly due to upregulation of the SR Ca\(^{2+}\) ATPase pumps which has been reported to occur during the early stage of hypertrophy (up to 8 weeks following MI) (Afzal \textit{et al.}, 1992). It could also be due to increased rate of [Ca\(^{2+}\)]-dependent activity of the SR Ca\(^{2+}\) ATPase pump (Bers and Berlin, 1995) since the [Ca\(^{2+}\)]_\text{i} was elevated in the ligated rats.

**FREQUENCY-DEPENDENT ALTERATIONS**

The frequency-dependent changes in the amplitude and kinetics of the force and the Ca\(^{2+}\) transient were similar, but not identical, in the ligated and the sham rats. The F-frequency relationship in the ligated rats. looked biphasic (negative from 0.2 to 0.5 Hz and positive from 0.5 to 2 Hz); however, the increase in the force during the positive phase was not statistically significant. In other words, the positive phase of the F-frequency relationship was blunted in the ligated rats. Although this relationship was not significantly biphasic in the ligated rats, it was not reversed either, as reported in end-stage heart failure (Narayan \textit{et al.}, 1995). Our observation agrees with a previous study that showed the F-frequency relationship was maintained in isoproterenol-induced hypertrophic rat hearts (Tang \textit{et al.}, 1996). Moreover, diastolic force remained unchanged following increased frequency of stimulation. Thus, the F-frequency relation in the ligated rats was attenuated in the ligated rats at this stage of hypertrophy. Since the extent of attenuation of the F-frequency relationship depends on the degree of progression of heart failure (Schmidt \textit{et al.}, 1994), it will be interesting to study these ligated rats during the later stages of hypertrophy (>8 weeks post-MI), when hypertrophy is more progressed or when heart failure is developed (~23-24 weeks post-MI).
The linear increase of the peak $[\text{Ca}^{2+}]_i$ as a result of increased frequency of stimulation was sustained in the hypertrophic hearts. Peak $[\text{Ca}^{2+}]_i$ increased linearly in response to increased frequency of stimulation in both sham and ligated rats. This observation suggests that the frequency-dependent activity of the factors that are involved in intracellular $\text{Ca}^{2+}$ handling (SR $\text{Ca}^{2+}$ ATPase pump, SR $\text{Ca}^{2+}$ release, $I_{\text{Ca,1}}$ and $I_{\text{Na,Ca}}$) is sustained in the ligated hearts. However, to confirm this hypothesis, the level of activity of these factors needs to be studied. Lack of a significant change in the relaxation rate or the diastolic tone of the $[\text{Ca}^{2+}]_i$ transient strongly suggests that the $\text{Ca}^{2+}$ uptake mechanism is not affected in the ligated rats.

Kinetics of the force and the intracellular $\text{Ca}^{2+}$ transient (TD and CaD) in both sham and ligated rats, decreased significantly in response to increased frequency of stimulation. The frequency-dependent linear decrease of the twitch and the $[\text{Ca}^{2+}]_i$ transient duration was maintained in the ligated rats at this stage of hypertrophy. The frequency sensitivity of the TD in the ligated rats was stronger than that in the sham rats (i.e. a larger slope). However, no significant difference in the frequency sensitivity of the CaD was observed between the two groups.

In chapter three, we proposed that the underlying mechanism of the frequency-dependent twitch duration shortening was due to a change in the property or behavior of the contractile proteins (and not merely $[\text{Ca}^{2+}]_i$ handling). Moreover, our study from the hypertrophic hearts suggested that the observed reduction in the amplitude and the rate of relaxation of the twitch force were most likely due to hypertrophy-induced alterations in the contractile proteins (decreased myofilament $\text{Ca}^{2+}$ sensitivity, fibrosis, or alpha-MHC to $\beta$-MHC switch). We also observed an increased frequency sensitivity of the twitch duration in the ligated rats. Therefore, the hypertrophy-induced alteration(s) in the contractile proteins must have increased their responsiveness (or sensitivity) to frequency of stimulation. The increased frequency of stimulation of the trabeculae from the hypertrophic hearts explains why despite the significant prolongation of the twitch
duration in the ligated rats, the peak force-frequency and the diastolic force-frequency relations were not attenuated in these rats and were very similar to those in the sham rats. Although the frequency sensitivity of the twitch duration was significantly increased in the hypertrophic hearts, that of the \([Ca^{2+}]_i\) duration was not significantly altered in the ligated rats compared to the sham rats. This observation further supports the minor role of the intracellular \(Ca^{2+}\) handling and the predominant role of the contractile proteins in the frequency-TD relationship. Therefore, the mechanism responsible for the frequency-dependent TD in normal rats needs to be discovered in order to fully understand the underlying mechanism of the hypertrophy induced hypersensitivity of the twitch kinetics to the frequency of stimulation.

THE UNDERLYING MECHANISM OF TWITCH DURATION PROLONGATION IN THE LIGATED RATS
The potential candidates responsible for the significant prolongation of the twitch duration in the ligated rats are:

1) Prolongation of the \(Ca^{2+}\) transient duration.
2) Down-regulation of the \(\beta\)-ARs
3) Replacement of the ventricular \(\alpha\)-MHC with \(\beta\)-MHC.

Prolongation of the action potential duration (APD) is not a potential candidate to underlie the prolonged twitch duration in the ligated rat myocardium. Rat AP is extremely short compared to the other mammals, so that even when it is prolonged by about three times in the ligated hearts (APD to 90% relaxation increased from \(29.0 \pm 5.2\) in sham to \(82.0 \pm 10.3\) msec in the ligated hearts; Kaprielian et al., 1997). membrane potential returned to the resting level before the force had even reached its peak (TD\(_p\)=182.2 \pm 17.8 in sham). Therefore, prolongation of the APD could not have caused the TD\(_{50}\) prolongation.
Duration of the Ca\(^{2+}\) transient was not significantly altered in the hypertrophic hearts, therefore, it could not have been responsible for the prolongation of the twitch duration either. We also observed that stimulation of the β\(_1\)-ARs significantly increased the amplitude, and accelerated the kinetics of the force and the [Ca\(^{2+}\)]\(_i\) transient in both sham and the ligated rats. Therefore, at this early stage of hypertrophy, the cardiac β\(_1\)-ARs were not down-regulated in the ligated rats. β\(_2\)-ARs constitute a small fraction of the cardiac β-ARs and therefore have a minor contribution to the β-AR-induced changes in the force and the [Ca\(^{2+}\)]\(_i\). However, the level of activity of the β\(_2\)-ARs need to be studied in the ligated hearts. That can be done by application of zinterol, a selective β\(_2\)-AR agonist.

Therefore, the strongest candidate to underlie the twitch duration prolongation in the ligated hearts is replacement of the ventricular α-MHC with β-MHC (the slower isoform), which is also the classical definition for the prolonged twitch kinetics in the hypertrophic and failing hearts (Gay et al., 1988: Kagaya et al., 1996: Orenstein et al., 1996). An increase in the β-MHC and/or a decrease in the α-MHC content in these ligated hearts still needs to be demonstrated in our study, although elevation of the β-MHC mRNA has been shown to occur one day after induction of MI (Orenstein et al., 1996). and elevated β-MHC protein level has been reported 7 days after aortic banding (Wiesner et al., 1997) and 4 weeks after aortic constriction (Reddy et al., 1996).
**FUTURE DIRECTIONS**

In order to discover the underlying mechanism of the frequency-dependent TD shortening, frequency-dependent alterations in the contractile proteins must be studied by evaluating the effect of the rate of stimulation on:

a) Sensitivity of the contractile proteins to $\text{Ca}^{2+}$ (by using chemically skinned cardiac trabecula where $[\text{Ca}^{2+}]$ is not a limiting factor in force generation).

b) The rate of binding and unbinding of $\text{Ca}^{2+}$ to Tn-C.

c) The rate of cross bridge turn over (by measuring the rate of ATP consumption).

If any of these factors were found to be altered in response to an increase (or decrease) in the frequency of stimulation, they should also be studied in the ligated rats in order to investigate the mechanism responsible for the stronger frequency-TD relationship in the ligated rats compared to the sham rats.

The changes in the force development and its kinetics, 6-7 weeks following myocardial infarction, appear to be due to alterations in the contractile proteins. To investigate the underlying mechanism of the significant reduction in the twitch force and the significant prolongation of its kinetics, sensitivity of the contractile proteins to $\text{Ca}^{2+}$ (in skinned cardiac trabecula), the extent of fibrosis (histological studies), the change in the mRNA and protein level of $\alpha$- and $\beta$-MHC (by southern and western blot, respectively) must be determined.

Further characterization of this hypertrophic model can be done by performing hemodynamic studies (using a Langendorff preparation) to measure systolic volume and pressure, end-diastolic volume and pressure, and the rate of pressure development and relaxation of the ventricles ($\pm \text{dP/dt}$). Plasma level of the hormones that have been reported to be elevated in heart failure or to contribute to its development (e.g. angiotensin II, norepinephrine, atrial natriuretic peptide (ANP), endothelin, etc.) should be measured in these rats. The level of activity of the $\beta_2$-ARs should also be evaluated in
these rats (by using, Zinterol, a selective β2-AR agonist) to investigate any upregulation of the activity of this receptor, which has previously been reported in hypertrophic hearts.

Performing these studies at different stages of cardiac hypertrophy and heart failure (eg. 6-7 wks, 12-13 wks, and 23-24 wks after induction of MI), will help to differentiate between the cause and the effect of heart failure development.
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