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**MECHANICAL ALTERNANS AND THE FORCE-FREQUENCY
RELATIONSHIP IN FAILING RAT HEARTS**

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * *

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ABSTRACT

Frequency potentiation of contractile force represents a potent inotropic mechanism in the myocardium of most species. This positive force-frequency relationship is altered in heart failure; where increased heart rates have a lesser effect and at times may even decrease the force of contraction. Failing hearts are also predisposed to alternans, an intriguing phenomenon characterized by alternating strong and weak beats. Mechanical alternans is also seen in patients with myocardial ischemia and tachycardia. In the laboratory alternans can reliably be induced by a number of ways such as making the myocardial preparation acidic, lowering bathing calcium, hypothermia and a high rate of pacing.

The present study examines the force-frequency relationship and mechanical alternans in Langendorff perfused ventricles from SHHF rats with end-stage dilated cardiomyopathy. The performance of these hearts was compared with those from young 3 month old Sprague Dawley rats as well as age-matched (20 month old) Wistar Furth rats. While a majority of the failing hearts showed alternans at physiological pacing rates viz. 5 Hz, the normotensive controls did so only at much higher pacing frequencies viz. 9 Hz. Agents such as caffeine and ryanodine which promote sarcoplasmic reticulum calcium release abolished alternans. Treatment with *beta* adrenergic agonists as well as increasing

trigger calcium also abolished alternans. Pre-treatment with 500 nM thapsigargin, an agent that inhibits sarcoplasmic reticulum Ca^{++} uptake, did not lower the threshold frequency for alternans in the control hearts. Furthermore examination of the mechanical restitution curves for the three types of hearts revealed a right shift in the half-time for restitution in the failing animals.

A model is proposed for the phenomenon of pacing induced alternans. Refractoriness of the sarcoplasmic reticulum calcium release channels following calcium induced calcium release seems to account for alternans. Longer refractory periods in the failing hearts may predispose them to alternans at lower pacing frequencies.

Dedicated to my parents

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TABLE OF CONTENTS

	<u>Page</u>
Dedication	iv
Acknowledgments.....	v
Vita.....	vi
List of Tables.....	xi
List of Figures.....	xii
Abbreviations.....	xv

Chapters:

1.	Introduction.....	1
1.1	The force-frequency relationship.....	1
1.2	Mechanical alternans.....	4
1.3	Mechanical restitution.....	7
1.4	The SHHF/Mcc- <i>fa^{cP}</i> rat model.....	10
1.5	Statement of Problems and Research	
	Goal.....	12

2.	Materials and Methods.....	14
2.1	Isolated Langendorff perfused rat hearts.....	14
2.2	Pacing protocols.....	16
2.3	^{31}P NMR.....	20
2.4	Isolation of cardiac myocytes.....	21
2.5	Pressure-Volume relationship.....	23
2.6	$[\text{Ca}^{++}]_i$ dependent Fura - 2 fluorescence ratio transients.....	23
2.7	Analysis of nucleotides, nucleosides and creatine.....	24
2.8	Protein analysis.....	25
3.	Results.....	26
3.1	The SHHF rat model.....	26
3.2	The force-frequency relationship and mechanical alternans.....	28
3.3	Modulation of MA by temperature.....	39
3.4	Effects of agents that disrupt SR function - caffeine, ryanodine and thapsigargin.....	39

3.5	^{31}P NMR and alternans.....	44
3.6	Modulation of MA by β adrenergic activity.....	51
3.7	Effect of trigger Ca^{++} on alternans.....	57
3.8	The force-interval relationship.....	60
3.9	Effect of thapsigargin on the F-I relationship.....	74
4.	Discussion.....	83
4.1	The SHHF rat model.....	83
4.2	The force-frequency relationship in rat hearts.....	84
4.3	Mechanical alternans.....	86
4.4	β - Adrenergic stimulation.....	89
4.5	A model for pacing induced alternans: Refractoriness of the SR Ca release channels to CICR.....	93
4.6	Mechanical restitution: A model based upon refractoriness of calcium release and SR Ca load.....	99
	Bibliography.....	102

LIST OF TABLES

<u>Table</u>		<u>Page</u>
3.1	Left ventricular pressure development in SD, WF and SHHF hearts.....	32
3.2	Intracellular nucleotide pools and total creatine content.....	55
3.3	Agents that abolish mechanical alternans.....	59

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
2.1	Experimental set-up and pacing protocol.....17
3.1	Pressure-volume relationship in SD, WF and SHHF rat hearts.....27
3.2	LVP recording from a SD heart in response to an increase in pacing frequency.....29
3.3	LVP recording from an SHHF heart in response to an increase in pacing frequency.....33
3.4	Fully relaxed mechanical alternans.....37
3.5	A plot of the occurrence of sustained mechanical alternans as a function of pacing frequency in SD, WF and SHHF rat hearts.....38
3.6	The “degree” of alternans is exacerbated as pacing frequency is increased.....40
3.7	Effect of caffeine on alternans.....41
3.8	Effect of thapsigargin on LVP.....45
3.9	Effect of thapsigargin on mechanical alternans.....47

3.10	2DG6P as a probe to monitor intracellular pH: Comparison with P_i	50
3.11	^{31}P NMR spectra of WF and SHHF hearts during mechanical alternans.....	52
3.12	Intracellular pH before and during mechanical alternans.....	54
3.13	Effect of isoproterenol on mechanical alternans.....	56
3.14	Effect of increasing $[\text{Ca}]_0$ on mechanical alternans.....	58
3.15	Response of a SD heart to test stimuli after varying intervals.....	61
3.16	Response of an SHHF heart to a premature test stimulus.....	65
3.17	$[\text{Ca}^{++}]_i$ and a premature test stimulus.....	66
3.18	Response of a WF heart to test stimuli after varying intervals.....	68
3.19	Mechanical restitution curves for SD, WF and SHHF rat hearts.....	72
3.20	Post-extrasystolic potentiation curves for SD, WF and SHHF rat hearts.....	75
3.21	Thapsigargin does not prevent force production in response to a premature stimulus.....	76
3.22	Thapsigargin treatment results in post-rest depression.....	77

3.23	Thapsigargin treatment corrects the anomalous trend seen in WF hearts.....	80
4.1	Signal cascade for β adrenergic agonists and key target proteins for phosphorylation by PKA.....	91
4.2	A model for pacing induced mechanical alternans.....	95

ABBREVIATIONS

F-F	Force-frequency
SR	Sarcoplasmic reticulum
F-I	Force-interval
MA	Mechanical alternans
PKA	Protein kinase A
2DG	2-deoxy-glucose
2DG6P	2deoxy-glucose-6-phosphate
ESI	Extrasystolic interval
MR	Mechanical restitution
PESP	Post-extrasystolic potentiation
EDP	End diastolic pressure
LVP	Left ventricular pressure
HEPES	hydroxyethyl-piperazine-N'-2-ethanesulphonic acid
ATP	Adenosine 5' - triphosphate
ADP	Adenosine 5' - diphosphate
AMP	Adenosine 5' - monophosphate
AN	Total adenine nucleotides

HPLC	High performance liquid chromatography
SHHF	Spontaneous hypertension and heart failure
IBMX	Isobutyl methylxanthine
PCr	Phosphocreatine
P _i	Inorganic Phosphate
CRC	Ca release channel
NMR	Nuclear magnetic resonance
SD	Sprague Dawley
WF	Wistar Furth
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β -amino-ethyl ether) N, N' - tetra-acetic acid
APD	Action potential duration
KB	Krebs - Henseleit
BSA	Bovine serum albumin
CICR	Calcium induced calcium release

CHAPTER 1

INTRODUCTION

1.1 The force - frequency relationship

The force-frequency (F-F) relationship relates to the force/tension/pressure generated by muscle (cardiac or skeletal) in response to increasing stimulation frequency.^{1,2} These (stimulation frequencies) vary, and the relationship is usually studied over a wide range of frequencies. Most mammalian cardiac tissues respond to this intervention with an increase in contractile force.³ That is, as pacing frequency is increased, developed tension increases, whereas decreasing pacing frequency results in a decrease in tension. This phenomenon has come to be known as the Bowditch effect, “treppe” or positive staircase.² The rat heart however is thought to exhibit an anomalous behavior in this regard. Several investigators have found that in rat cardiac tissue, as pacing frequency is increased, contractile force decreases.^{4,5} This phenomenon has been called the negative staircase. In general, it is thought that mammalian species with high physiological heart rates, such as the rat and mouse, demonstrate this kind of relationship. Another instance in which the negative staircase manifests itself is in hypertrophy and heart failure. Several studies have found that in the

failing heart, independent of species, the positive staircase is either flattened or is actually negative. Studies comparing the F-F response between failing and non-failing human myocardium have demonstrated a reduced ability of the failing heart to increase force at increasing stimulation rates.⁶⁻⁹ In fact, this behavior is so consistent that clinical researchers routinely use the F-F response of the heart, to characterize whether tissue is of the failing or normal type.¹⁰

The reasons advanced for the treppe are numerous and varied. One of the most popular ones is the “sodium lag” hypothesis. Using ion selective microelectrodes several investigators have demonstrated that an increase in pacing frequency results in an increased influx of Na^+ via the sodium channels.^{11,12} Measurements of $[\text{Na}]_i$ by ^{23}Na NMR experiments have also revealed a frequency dependent increase in intracellular sodium levels.¹³ Intracellular Na^+ in cardiac tissue is of the order of 10 mM.¹³ The increased influx of Na^+ causes this amount to rise to about 13 mM.¹³ Langer¹⁴ hypothesized that with an increasing rate of stimulation, the $\text{Na}^+\text{-K}^+$ pump “lags” behind the increased rate of Na^+ entry, giving rise to a increase in $[\text{Na}^+]_i$. This delay in reaching a new steady state, during which $[\text{Na}^+]_i$ continuously increases, is termed the “ $\text{Na}^+\text{-K}^+$ pump lag”. Such an increase in $[\text{Na}^+]_i$ is thought to reduce diastolic Ca^{2+} efflux and enhance net cytosolic Ca^{2+} influx via the Na-Ca exchanger. In subsequent beats, this results in an increased cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$), an increased sarcoplasmic reticulum (SR) Ca^{2+} concentration, which ultimately leads to an increase in the size of the intracellular Ca^{2+} transient ($[\text{Ca}^{2+}]_i$). This would then result in a progressive frequency-dependent increase in contractile force or a positive-staircase.¹³

The other hypothesis involves a direct frequency modulation of the L-type Ca^{2+} channels. Using single ventricular cells perfused internally by the suction-pipette method, Lee found that the sarcolemmal Ca^{2+} current can be increased progressively by repeated stimulation.¹⁵ He hypothesized that the sarcolemmal Ca^{++} channel has two distinct open states: one can be opened rapidly by a single step depolarization; the other can be opened only in small increments by repeated depolarization. Given the fact that increased calcium current causes increased release of calcium from the SR and also an increased loading of the SR,¹⁵ Lee concluded that this additional channel opening could be responsible for the generation of the positive force staircase effect in mammalian myocardial cells in response to increased heart rate. The negative force-frequency of the rat myocardium on the other hand, was assumed to be a genuine property of that species.⁴

Finally, the reason behind the altered F-F relationship in hypertrophy and heart failure is not entirely clear. Factors such as disturbed excitation-contraction coupling with decreased calcium cycling at higher stimulation rates, have been commonly cited to explain the flattened or sometimes negative F-F response in heart failure.^{7,16} Another hypothesis that has gained some popularity is that at higher stimulation frequencies, especially in the failing heart, there is an intracellular accumulation of metabolites such as inorganic phosphate and adenosine, resulting in a desensitization of the myofilaments to calcium.¹⁶ Thus, although intracellular calcium transients may be normal, force production is drastically reduced.

1.2. Mechanical alternans

In 1872, Traube first brought attention to a clinical phenomenon called pulsus alternans,¹⁷ associated with patients in heart failure. Mechanical alternans (MA) in cardiac muscle, as it has now come to be known, is defined as alternating large and small amplitude of force (or pressure) at a steady heart rate.¹⁸ MA is often seen in patients with cardiac hypoxia or ischemia, tachycardia and increased ventricular loading.¹⁸ Recent advances in clinical technology have made possible the detection of alternans not only at the mechanical (level) but also at the electrical level. Surawicz et al.,¹⁹ have classified several types of electrical alternans including repolarization alternans which is frequently associated with a long QT interval or increased U wave amplitude, and ST segment alternans which increases the likelihood of fatal ventricular tachyarrhythmias. The phenomena of mechanical and electrical alternans have thus been of continuing interest to both physiologists and clinicians alike.

Mechanical alternans has reliably been introduced in the laboratory by a variety of interventions such as hypothermia, acidosis and a low bathing calcium.²⁰ Another commonly used method is to increase abruptly, the pacing frequency of the myocardial preparation. Spencer et al.,²¹ have successfully used this technique to generate alternans in papillary preparations. The rate threshold for alternation can be decreased and the degree of alternation can be increased by implementing a combination of the above mechanisms.²²

As with the rate staircases, a number of hypotheses have been advanced to elucidate the mechanism of alternans. Mouloupoulos proposed that alternans is a

manifestation of different ventricular fibers contracting differently,²³ whereas Verheugt et al., attributed it to an alternating atrial electromechanical dissociation.²⁴ Another frequently cited explanation for MA involves an alternation of hemodynamic variables. Wenckebach was one of the first to propose that pulsus alternans is a manifestation of concomitant alternations of end-diastolic pressure and volume.²⁵ Using a model of aortic impedance, McGaughey et al.,²⁶ showed that in this case, as a consequence of the Frank-Starling mechanism, stroke volume and systolic pressure are greater when end-diastolic volume is increased. Consequently, the heart empties more completely, which in turn results in lower end-diastolic volume, smaller stroke volume, lower systolic pressure and less complete ventricular emptying. The result is an increase in diastolic volume and the continuation of alternans. Finally, using a closed-chested canine model, Freeman²⁷ demonstrated that oscillations in end-diastolic volume precede pulsus alternans.

Yet another hypothesis involves the production of mechanical alternans by electrical alternans. Hogencamp et al.,²⁸ suggested that electrical alternation is primary to and is the cause of mechanical alternans. They hypothesized that at rapid stimulation rates, there is an alternation in action potential duration (APD), due to refractoriness of sarcolemmal channels and impaired Ca^{++} gating. The alternating action potentials then lead to alternations in intracellular calcium fluxes and consequently, alternations in force production. Working on similar lines, Hirata et al.,²⁹ reported that at high stimulation rates there is a hypoxia / acidosis induced alternation in APD, leading to MA.

Recently Orchard et al.,³⁰ demonstrated that mechanical alternans can be induced by acidosis in isolated myocytes during a train of voltage-clamp pulses. In this experiment, even though APDs were held constant, alternans was observed in the $[Ca^{2+}]_i$ transient. They reasoned that electrical alternans is secondary to mechanical alternans with the former being a feedback effect of the latter. With the advent of myocyte technology and the observation of MA in myocytes, the involvement of hemodynamic variables was also ruled out (myocytes being isolated single cells, are not under any hemodynamic influence). Thus, the more recent theories put forward to explain the alternating strength of the ventricular myocardium propose alternations in the intrinsic contractile state of the myocardium.

It is well known that calcium released from the SR binds to the myofilaments resulting in systole. During diastole, this calcium comes off the myofilaments and is taken back up by the SR. While it is tempting to suggest that mechanical alternans may be a manifestation of incomplete relaxation, experiments by Lab and Lee have conclusively shown that mechanical alternans can be accompanied by complete relaxation after every beat, ruling out any myofibrillar involvement in this phenomenon.¹⁸ Furthermore, using fluorescent dyes for monitoring intracellular calcium movements, several investigators have found that there is actually a beat to beat alternation in $[Ca^{2+}]_i$.^{18,22,30} It has thus been suggested that mechanical alternans is then caused by an alternation in the amount of $[Ca^{2+}]_i$ available for activation of the myofilaments. Prolongation of the time for recycling Ca^{2+} by the SR, i.e., depressed uptake function of the SR Ca^{2+} pump with concomitant slow transportation of Ca^{2+} from the uptake to the

release compartment in the SR, is suggested as a cause for the abnormal Ca^{2+} handling during mechanical alternans.²²

Finally Adler et al.,³¹ have proposed a theoretical model to elucidate the mechanism behind MA. In their model, SR calcium release governs both the rate threshold and the degree of alternation with the so-called release function depending upon the amount of calcium contained in the release terminal of the SR.

1.3 Mechanical restitution

While the force-frequency relationship is an excellent prognosticator of the extent of heart failure, the force-interval (F-I) relationship is also commonly used to assess myocardial performance. Alterations in the strength of cardiac contraction produced by brief changes in the stimulation pattern are described by the F-I relationship. The commonly used protocol involves the imposition of a test stimulus at a variable time interval, after a train of steady state stimuli. The response of the myocardium to such a protocol has been well documented and can be considered to derive from two properties: mechanical restitution (MR) and post-extrasystolic potentiation (PESP).³² MR is exemplified by the recovery of contractile strength of an extrasystole observed when the interval between a steady-state and subsequent extrasystolic stimulus is lengthened. PESP is the enhancement of contractile strength of a post-extrasystole produced by a shortening of the same interval.³²

Koch-Weser and Blinks were among the first to provide a mechanistic model for the F-I relationship.³³ They described this relationship in the “abstract” terms of a

positive and a negative inotropic effect of activation. The “effects” were assumed to be cumulative and to decay with different time constants. Over the years, this model has been extended by Schouten et al. to a “calcium compartment” model.³⁴ In this scheme, the SR is assumed to be made up of separate “uptake” and “release” compartments. The force of contraction is assumed to be proportional to the amount of Ca^{2+} released upon excitation from the release compartment. Furthermore, the release compartment empties completely with each excitation; with the events of excitation, Ca^{2+} release, contraction and Ca^{2+} uptake by the SR calcium pump, taking infinitesimal time. Their model assumes that the only time-dependent process is the transport of Ca^{2+} from the uptake to the release compartment within the lumen of the SR. Thus, in the event of an early stimulus, there is incomplete transfer of activator calcium from uptake to the release site, less calcium release and a smaller contraction. However, during the extrasystole, Ca^{2+} entering the cytoplasm via the sarcolemmal Ca^{2+} channels and perhaps via the Na / Ca exchanger, becomes available to the uptake site and is subsequently translocated to the release site. If the diastolic restitution period is sufficiently long, the increased amount of Ca^{2+} available for release (including the amount not released during the extrasystole) will become manifest as the PESP.

The same author later proposed a new hypothesis in which he postulated that the SR Ca^{2+} -ATPase is “activated” during each contraction and “inactivates” slowly.³⁵ Thus, after a short interval, a “normal” amount of calcium may be released; however the highly active pump sequesters much of the Ca^{2+} before it reaches the sarcomeres and the result is a contraction of smaller amplitude. As inactivation proceeds during long intervals, a

larger fraction of Ca^{2+} from the SR contributes directly to contraction. Other theories have included the possibility that during an early stimulus, APDs are shorter, invoking lesser amounts of calcium release.³⁶ Conversely longer stimulus intervals are accompanied by longer APDs, greater amounts of activator calcium release and greater force of contraction.

A more recent hypothesis³⁷ extends the earlier work of several investigators^{32,38} to suggest that the kinetics of the SR calcium release channels is the rate determining step in mechanical restitution. This model suggests that the SR calcium release channels (CRCs) enter a state of refractoriness immediately after calcium release during which time they are unresponsive to trigger calcium. Restitution then exemplifies the time dependent kinetics of the recovery of these release channels (from their state of inactivation).

Several studies have indicated that restitution is often depressed during heart failure.^{39,40} While the reasons for the above are not entirely clear, Gwathmey et al.,⁴¹ have suggested that uptake of calcium by the SR Ca^{2+} pump is the rate limiting step during restitution. Their study suggests that as a result of compromised SR pump function in heart failure, the diminished amplitude of the intracellular Ca^{2+} transient and accompanying contraction most likely reflect insufficient loading time of the SR for subsequent release. Thus while a number of hypotheses have been advanced to explain the force-interval effect, a cohesive hypothesis is still lacking.

1.4 The SHHF/Mcc-*fa*^{cp} rat model

A number of experimental models of hypertrophy and heart failure exist. These include the pressure and volume overload type, canine rapid pacing, the SHR rat model, etc.^{40,42-45} The SHHF/Mcc-*fa*^{cp} rat is a relatively new genetic model of congestive heart failure.⁴⁶ It originated from a cross between a Koletsky rat and a spontaneously hypertensive rat from the SHR/N colony at National Institutes of Health (NIH). The Koletsky rat carried a spontaneous mutation that produced obesity in animals homozygous for its *cp* or corpulent gene. Crossing the Koletsky and the SHR was originally intended to produce a genetic rat model with both hypertension and obesity - two afflictions that frequently coexist and produce myriad difficulties in man. This crossing was followed by seven backcrosses to SHRs (to partially eliminate the Koletsky's Sprague-Dawley genes) before inbreeding of the current SHHF/Mcc-*fa*^{cp} strain was begun. Further backcrosses were conducted at NIH to produce a truly congenic SHR/N-*cp* strain with virtual elimination of Sprague-Dawley (SD) genes: this resulted in the SHR/N-*fa*^{cp} rat. Since the SHHF rat is not congenic and possesses both SHR and Sprague-Dawley genes, neither the Wistar Kyoto nor the SD is a perfect normal control. The Wistar-Furth (WF) is most often used because of its similarities to the SHHF, but the prudent course is to examine several related normal strains.

The SHHF colony was originally designated SHR/N-mcc-*cp* to reflect its SHR/N origins, but because of 11 years of focused selective breeding to favor the early and reproducible development of heart failure and reduce the incidence of other pathologies, the Institute of Laboratory Animal Resources (ILAR), National Resource Council

(NRC), in 1990 replaced the term SHR with SHHF (spontaneous hypertension and heart failure).⁴⁷ Also, the recent matings of female SHHF rats heterozygous for the *cp* gene with male Zucker rats heterozygous for the *fa* or fatty gene produced ~ 25% grossly obese spotted offspring (SHHF rats are pure white). This established the fact that the *fa* and *cp* mutations are alleles. Accordingly, in 1992 the ILAR renamed the colony SHHF/Mcc-*fa^{cp}* to reflect this fact.

All SHHF rats are hypertensive. Those homozygous for the *fa^{cp}* gene are grossly obese while heterozygotes are phenotypically lean but slightly heavier than their true lean littermates. Obese males have non-insulin-dependent diabetes but obese females have only an altered glucose tolerance. All the SHHF rats possessing the *fa^{cp}* gene die well in advance of senescence, most from congestive heart failure. Those few animals that have been allowed to age but have died from other causes have succumbed to 1) cancer, 2) stroke, 3) infection (i.e. tooth abscess) or 4) unexplained sudden death-presumably cardiac arrhythmia. True lean colony members without the *fa^{cp}* gene also develop heart failure, but at a much later stage. These animals may be similar to members of the more failure-prone SHR colonies.^{48,49}

As with the SHR, male SHHF rats develop severe cardiac dysfunction at a younger age than do their female siblings. SHHF rats may be unique however in that all females as well as males eventually develop florid failure. The predictable occurrence of dilated cardiomyopathy in this strain of rats allows for a wide variety of longitudinal studies having to do with the transition from compensatory hypertrophy to decompensation and heart failure. The major limitation is that heart failure in this model

is multifactorial and the responsible genes have not yet been characterized. Furthermore, the other hypertensive rat strain that possesses the *fa^{CP}* gene, the SHR/N-*fa^{CP}*, does not reliably develop heart failure;^{46,50} thus, serendipity and selective breeding (as opposed to genetic engineering) have conspired to produce this unique animal model. It should be stressed that reproducible heart failure has now been maintained for 26 generations in the SHHF rat and there are no difficulties in the routine breeding of these animals.

1.5 Statement of Problems and Research Goal

Both the force-frequency and force-interval relationship in cardiac muscle have been well documented. That high rates of myocardial stimulation can lead to mechanical alternans, is well known. While it has been recognized that the above phenomena are manifestations of intracellular calcium dynamics, the exact mechanisms are still ambiguous. Three main hypotheses, giving a mechanistic explanation for MA and the F-I relationship, have gained popularity over the years: 1) inability of the SR Ca^{2+} pump to keep up with the stimulation frequency; 2) time-dependent transfer of luminal calcium from the uptake to the release sites within the SR; and 3) kinetics of SR calcium release as being the rate determining step in MR and in the production of MA.

This study characterizes the left ventricular performance of the SHHF rat with respect to the following: 1) response to increased pacing frequency; and 2) the force-interval relationship; the effects of a variety of agents such as β adrenergic agonists, calcium channel blockers, etc. on mechanical alternans is demonstrated; and finally, a model is proposed for the phenomenon of pacing induced mechanical alternans. The

performance of the SHHF rats were compared to young 3 month old Sprague Dawley and age-matched (~ 19 month old) normotensive Wistar Furth rats.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolated Langendorff perfused rat hearts

Methods are similar to those previously published.⁵¹ Hearts from young (300-400g) male Sprague-Dawley (SD) rats, phenotypically lean SHHF rats of either gender with end-stage heart failure, and 19 month-old male Wistar-Furth (WF) rats were used in the studies. The WF rats were purchased from Harlan (Indianapolis, Indiana), whereas the SD rats were from Zivic Miller (Pa, USA). SHHF rats were bred under the supervision of Dr. Sylvia McCune at The Ohio State University. All animals were on regular rat chow (Prolab 3000) and maintained on a 12 h light/dark cycle in a fully accredited facility.

For in vitro studies of left ventricular performance, rats were injected intraperitoneally with a lethal dose of pentobarbitol (150 mg/kg). An abdominal incision was made and heparin (0.7 mg) was injected into the inferior vena cava. The thorax was opened and hearts were rapidly excised and placed in ice-cold modified Krebs-Henseleit (K-B) buffer containing (in mM) NaCl 109, KCl 3.8, MgSO₄ 1.2, KH₂PO₄ 1.2,

NaHCO₃ 25, CaCl₂ 1.8, glucose 10, sodium pyruvate 10, EDTA 0.1 and insulin (0.1 unit/ml). Hearts were cannulated via the aorta and perfused retrograde in a non-circulating Langendorff mode at 20 ml/min with K-B buffer saturated with 95%O₂ / 5%CO₂ to achieve a pH of 7.4 at 37°C. In experiments where pyruvate was omitted, NaCl was increased to 119 mM. Also in experiments involving high [Ca²⁺]_o, a phosphate-free, 3 mM hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) buffer⁵² was used (NaHCO₃ was omitted from the medium). Consequently KCl was increased to 5 mM and 100% O₂ was bubbled through the medium. All reagents used in the procedure were purchased from Sigma (St. Louis, MO).

After a 10-15 min equilibration period, the atria were removed and the atrio-ventricular node crushed. This procedure slows down the intrinsic heart rate to about 120 beats/min. Hearts were immersed in a 10 ml beaker bath containing outflow perfusate to prevent drying and facilitate temperature control. A latex balloon was inserted into the left ventricle and the end-diastolic pressure was adjusted to $\sim 8 \pm 2$ mmHg by means of a fluid filled syringe. The balloon was connected via polyethylene tubing (Intramedic) to a pressure transducer (Viggo Spectramed) (**Fig 2.1 a**). The system was filled with water and care taken to ensure the absence of air bubbles.

Left ventricular pressure was monitored using a 486 IBM PC clone with data acquisition software from Coulbourn Instruments. Data were acquired at rates varying from 100 to 500 pts/sec. Hearts were paced using platinum electrodes, one of which was connected to the metal cannula and the other immersed in the beaker bath. Voltage was

set at twice threshold. An IBM-XT fitted with a CIO-DACO2 card (Computerboards Inc. Mansfield) and run using a TURBO-C program acted as the stimulator (**Fig 2.1 a**).

2.2 Pacing protocols

Two types of pacing protocols were used: 1) force-frequency (F-F) and 2) force-interval (F-I).

For the F-F relationship, hearts were paced at a basal cycle length of 333 msec (3 Hz). It was difficult to pace at a lower frequency due to the appearance of spontaneous beats. After the appearance of steady state pressure, a quick jump in pacing frequency was imposed in steps of 2 Hz, i.e., 3-5-7-9 Hz and finally to 10 Hz (**Fig 2.1 b**). Time was given for the heart to reach a steady state at each pacing frequency before switching to the next higher rate. For studies to determine the threshold frequency of sustained MA, the increases in pacing frequencies were carried out in steps of 1 Hz.

For the F-I relationship the hearts were again paced at a basal cycle length of 333 msec. However the temperature was maintained at 32°C to further reduce spontaneous activity. After a priming period of 100 beats (at 3 Hz), test beats were introduced at varying time intervals, from as early as 150 msec to as late at 600 msec as shown in **Fig 2.1 c**. Beyond 600 msec the hearts generally exhibited intrinsic activity.

Fig 2.1: Experimental set-up and pacing protocol. a) Schematic of the experimental set-up, showing an isolated heart with a balloon inserted to the left ventricle. The scheme also shows a pressure transducer, data acquisition board, output monitor and a stimulator. See text for details. b) Pacing protocol for mechanical alternans. Panel shows the pacing frequencies employed. c) Pacing protocol for the force-interval relationship. Panel shows the extrasystolic intervals (ESI) in msec. Basal pacing interval is 333 msec. The heart is primed for 100 beats before introduction of an ESI.

Figure 2.1

Figure 2.1 (continued)

b)

3 Hz	5 Hz	7 Hz	9 Hz	10 Hz
180 beats/min	300 beats/min	420 beats/min	540 beats/min	600 beats/min

c)

150 msec	180 msec	195 msec	210 msec	240 msec	270 msec	300 msec	333 msec	370 msec	400 msec	430 msec	460 msec	490 msec	600 msec
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Developed pressure during the extrasystole (F_1) was normalized to the developed pressure from the previous steady state beat (F_0). Restitution curves were generated by plotting (F_1 / F_0) vs. the extra-systolic interval (ESI). Post-extrasystolic potentiation curves were constructed by plotting (F_2 / F_0) vs. ESI.

2.3 ^{31}P NMR

Methods were identical to those described above except that a phosphate free, insulin free and glucose-free Krebs-Henseleit buffer was used. Pyruvate (10 mM) was the only external substrate. The hearts were electrically stimulated and a latex balloon inserted into the left ventricle was used to monitor left ventricular pressure. For NMR analysis, the heart was mounted in a custom built perfusion probe operating at 161 MHz (diameter = 28 mm; loaded $Q = 46$). ^{31}P NMR spectra were collected on a GE Omega 9.4 T / 89 mm spectrometer operating at 161 MHz for phosphorus observation. Each sample was shimmed for approximately 10 minutes on the water signal observable with the ^{31}P coil. ^{31}P NMR spectra were then acquired in 100 scans (4 minutes) using 90° , 2.5 sec repetition time, 1 K block size, and a 10 kHz sweep width.

Intracellular pH in this method is usually determined from the relative shift of the P_i resonance with respect to the PCr resonance.⁵³ However since the control hearts did not show a P_i resonance, 2-deoxy-glucose-6 phosphate (2DG6P) was used as the intracellular shift indicator.⁵²⁻⁵⁴ To accomplish this, 2-deoxy-glucose (2DG) was used as substrate. 2DG is taken up by the myocardium on the Glut-1 transporter. Once inside the myocardium, 2DG is phosphorylated to form 2-deoxy-glucose-6 phosphate (2DG6P), an

adduct sensitive to intracellular pH. The protocol consisted of initially acquiring a control spectra of hearts paced at 3 Hz. Hearts were then perfused with 0.5 mM 2-deoxyglucose (2DG) and (0.1 unit/ml) insulin for a 5 minute period. Once the 2DG6P resonance was present in the spectrum, perfusion medium was switched back to one without any 2DG. (This was deemed necessary because 2DG is a well known phosphate scavenger⁵³). Hearts were then paced at increasingly higher frequencies until mechanical alternans was observed. Spectra were collected before and during the period in which the hearts were in alternans.

2.4 Isolation of Cardiac myocytes

Myocytes were isolated as described previously.^{55,56} Adult male Sprague-Dawley rats (350-400 g) were anesthetized with pentobarbital sodium (150 mg/Kg). The chest cavity was opened and the heart was excised and placed in an ice-cold slush of Krebs-Henseleit buffer (in mM: NaCl 118, KCl 4.8, MgSO₄ 1.2, CaCl₂ 1 and 0.68 glutamine, pH = 7.3). The aorta was cannulated on a blunt end 15 gauge needle. The heart was then perfused in a non-recirculating mode for 14 min at 37°C. For the first 7 min of perfusion (washing out of remaining blood in the heart), the perfusate was 1 mM CaCl₂ K-B buffer supplemented with 11 mM glucose, 25 mM NaHCO₃, 5 mM pyruvic acid, vitamins and a complete set of amino acids. The second 7 min of non-recirculating perfusion was required to remove extracellular calcium using the same perfusion buffer without CaCl₂ (containing 40 µM EGTA). After 14 min, recirculation was begun and

the heart was perfused with 1 mg/ml collagenase (Worthington Class II) and 1 mg/ml bovine serum albumin (BSA) in the presence of 40 μ M EGTA. After 20 min perfusion with collagenase, calcium was added back incrementally over 15-20 minutes to bring the perfusate concentration to 1 mM. The flow rate was increased from 8 to 16 ml/min over a 10 min period after adding calcium back. The perfusion medium was gassed with 95%O₂-5%CO₂ throughout the perfusion period to maintain pH in the range of 7.3-7.4. When the connective tissue was fully digested, indicated by the absence of resistance to flow of buffer from the Langendorff apparatus through the heart, the heart was removed from the apparatus. The atria were removed and the remaining tissue was placed in 5 ml incubation buffer (in mM: NaCl 118, KCl 4.8, MgSO₄ 1.2, CaCl₂ 1, glutamine 0.68, glucose 11, pyruvate 5, HEPES 25, vitamins (100 X GIBCO stock), a complete amino acid supplement (100 X GIBCO stock) and 0.6 μ g/ml insulin with pH = 7.3) with 2 mg/ml collagenase and 2% (w/v) BSA. The tissue was teased apart and incubated under 100% O₂ at 37°C in an orbital shaking water bath at 120 cycles per min.

The cells were then dispersed with a nalgene pipette and filtered through two layers of cheese cloth. Myocytes were washed twice in incubation buffer 0.5% (w/v) BSA by centrifugation at 400 for 1 minute. The cells were resuspended in 5-10 ml of 4% (w/v) BSA and allowed to settle for 2 minutes at 37°C. The cells were finally suspended in incubation buffer containing 2% (w/v) BSA and stored at room temperature. Unless indicated otherwise, all incubations were carried out in this buffer.

2.5 Pressure - Volume relationship

Hearts were perfused in the Langendorff mode, as describe earlier. However, before inserting the balloon, a sharpened piece of polyethylene tubing (with the other end flanged) was advanced through the mitral orifice and the left ventricular apex was punctured. The tubing was left there to vent thebesian flow.⁵⁷ This was deemed necessary at higher preloads. The balloon volume was large enough so that a negligible pressure (<3-4 mmHg) resulted when the balloon alone was inflated to the maximum volume used in the study.^{58,59} Balloon volume was incremented in steps of 0.02 ml via syringe (maximal volume 0.14 ml). The hearts were allowed to reach a steady state before making further increments. During the entire procedure hearts were paced at a frequency of 4 Hz (temperature = 37°C). Developed pressure at every increment of preload was normalized to developed pressure at 0 preload. A plot of such normalized pressures vs. balloon volume was constructed.

2.6 $[Ca^{2+}]_i$ dependent Fura - 2 fluorescence ratio transients

Myocytes were loaded with 5 μ mol/L Fura - 2 AM for 3 minutes, postincubated for 1 hour at room temperature, superfused with bicarbonate-based K-B buffer, pH 7.4, at 37°C, field stimulated at 0.2 Hz with parallel platinum electrodes.⁶⁰ Fluorescence measurements, with excitation alternating between 340 and 380 nm, were obtained with a PTI filterscan. Because cells loaded with Fura - 2 AM accumulate Ca^{2+} sensitive dye in the mitochondria,⁶⁰ accurate calibration of the cytosolic signal is problematic. Data were therefore obtained as the ratios of fluorescence intensity at 340 and 380 nm excitation.

The amplitude and configuration of the Fura - 2 ratios are thought to reflect accurately the magnitude and time course of changes in cytosolic Ca^{2+} .⁶⁰

2.7 Analysis of Nucleotides, Nucleosides and Creatine

For analysis of total intracellular nucleotide content, hearts were perfused in the Langendorff mode as previously described. However there was no insertion of balloon or pacing in this case. After a 15 minute perfusion period, the hearts were freeze clamped using a pair of Wollenberger clamps cooled to the temperature of liquid N_2 .⁶¹ The hearts were then ground up with a mortar and pestle under liquid nitrogen until the tissue took the form of a fine powder.

Small amounts of tissue were then scooped into an eppendorff tube containing 1 ml of 0.6N PCA. After vigorous vortexing and centrifugation, 0.7 ml of the acid extract (supernatant) was mixed with 0.9 ml of Freon-Trioctylamine (1.1 part trioctylamine to 3.9 parts 1,1,2-trichlorotrifluoroethane). The mixture was vortexed for 30 seconds to neutralize the PCA. After centrifugation, 0.5 ml of the top aqueous phase containing the nucleotides and nucleosides was transferred to a microcentrifuge tube and stored at -20°C until further analysis.

The nucleotides and nucleosides were separated on a Whatman Partisil 10 SAX (strong anion exchange) column with a linear gradient of pH and potassium phosphate salt solution at a flow rate of 2ml/min and detected by UV spectroscopy at 254 nm.⁶² The separation conditions were 2 minutes at 100% buffer A (0.01 M H_3PO_4 , pH 2.65) followed by a linear gradient over 21 minutes to 63% buffer B (0.75% KH_2PO_4 , pH

4.5). Peaks were identified by comparison of their retention times with those of authentic standards and by co-chromatography with authentic standards. Quantification was based on peak area, using an Altex C-R1A integrating recorder and external standards. Total creatines were analyzed by a colorimetric method.⁶³

2.8 Protein Analysis

The protein pellets from the ground tissue were washed twice with acetone and dried before dissolving in 1.0 ml of 1N NaOH. This solution was then assayed for protein by the method of Lowry,⁶⁴ using bovine serum albumin.

CHAPTER 3

RESULTS

3.1 The SHHF rat model

Both male and female phenotypically lean SHHF rats, which ranged in age from 16-27 months, were taken for the study when they exhibited clinical signs of severe congestive heart failure, i.e., subcutaneous edema, dyspnea, cyanosis, lethargy, piloerection and cold tails.^{50,65} Body weights ranged from 360-420 g for males and 190-265 g for females. The SHHF hearts (weight range 1.9-3.2 g; mean heart to body weight ratio in mg/g = 7) revealed significantly thickened left and right ventricular free walls, dilated lumina, left and right atrial dilatation and well developed atrial thrombi. By contrast, the control hearts appeared normal (weight range 1.3 - 1.5 g; mean heart to body weight ratio again in mg/g = 3.45). The SHHF rats all had ascites, pulmonary edema and pleural effusion. None of these signs of congestive heart failure were seen in any of the controls. **Fig 3.1** is a comparison of the pressure-volume relationship in these hearts.

Fig 3.1: Pressure -volume relationship in SD, WF and SHHF rat hearts. Measurements were obtained at a pacing frequency of 4 Hz. Data are mean $\pm$ S.E.M. Left ventricular balloon volumes at an EDP of 8 mmHg for the SD, WF and SHHF hearts were (in ml) 0.04 ± 0.01 , 0.05 ± 0.0 and 0.08 ± 0.01 , respectively ($P < 0.01$, controls vs. failing)

3.2 The force - frequency relationship and mechanical alternans

The effects of a sudden increase in pacing frequency were examined in young Sprague-Dawley (SD), failing SHHF and age-matched Wistar-Furth (WF) rat hearts. As seen in **Fig 3.2 a and b** abrupt changes in pacing frequency from 3 to 5 Hz in the SD hearts, substantially reduced the amplitude of the next beat. This was followed by a steady increase in developed pressure that reached a plateau after approximately 5 seconds. At this point systolic pressures were, on the average, 30% greater than the 3 Hz steady state value (**Table 3.1**). Augmented pressure development was maintained for less than 20 seconds, however, and there was a slow decline to a new steady state, 20-25% lower than the 3 Hz value (**Table 3.1**). This pattern was very consistent and found to be independent of pyruvate concentration and end-diastolic pressure (EDP). A further increase in pacing frequency i.e., from 5 to 7 Hz, yielded similar results, although due to elevated EDP, increase in developed pressure was not as dramatic.

The initial phase of augmented left ventricular pressure development was absent in hearts from failing SHHF rats (**Table 3.1**). Instead a majority of these hearts exhibited mechanical alternans after a frequency jump from 3 to 5 Hz (**Fig 3.3 a and b**). This behavior was characterized by alternating big and small beats. The alternation between big and small beats could be sustained indefinitely in most cases but would disappear when pacing frequency was brought back to 3 Hz.

Alternans in SD rat hearts could be induced by increasing pacing frequency from 7 to 9 Hz. In the age-matched WF hearts, pacing frequencies above 6 Hz elicited transient MA, which would however disappear after a few beats. Sustained MA was

Fig 3.2: LVP recording from a SD heart in response to an increase in pacing frequency. a) Frequency was increased from 3 to 5 Hz at the arrow. Note the presence of an initial positive staircase followed by a decline in LVDP. b) Faster time base recording of the same experiment.

a)

Figure 3.2

Figure 3.2 (continued)

b)

	SD	WF	SHHF
LVDP (mmHg)			
3 Hz steady state	99 ± 9 (8)	99 ± 9 (5)	92 ± 8 (11)
5 Hz peak	128 ± 14 (8)	104 ± 7 (5)	80 ± 8* (11)
5 Hz steady state	77 ± 9 (12)	70 ± 11 (5)	56 ± 7* (6)

Table 3.1: Left ventricular pressure development in SD, WF and SHHF hearts. Data are mean ± S.E.M. The number of observations for each condition is given in parentheses. $P < 0.05$ v SD and WF hearts.

Fig 3.3: LVP recording from an SHHF heart in response to an increase in pacing frequency. a) Frequency was increased from 3 to 5 Hz at the arrow. Note the presence of mechanical alternans at 5 Hz and the absence of a positive staircase. Also relaxation is incomplete between contractions during alternans. b) Slower time base recording of the same experiment. Note the presence of a steady, stable alternans.

a)

34

Figure 3.3

Figure 3.3 (continued)

b)

however observed only at 8-9 Hz. Also, the WF hearts did not show as pronounced a positive staircase as the SD hearts, when pacing frequency was increased from 3 to 5 Hz.

In the majority of cases, alternans was characterized by incomplete relaxation after the bigger beat, but complete relaxation at the end of each small beat (as seen in Fig. 3.3). However, in many cases there was complete relaxation after both the big and small beats (Fig 3.4). Fig 3.5 charts the occurrence of sustained mechanical alternans in the three types of heart as a function of pacing frequency.

Fig 3.4: Fully relaxed mechanical alternans. LVP recording from a SD heart in mechanical alternans at 6 Hz. Note the complete relaxation between contractions during alternans.

Fig 3.5: A plot of the occurrence of sustained mechanical alternans as a function of pacing frequency in SD, WF and SHHF rat hearts. To determine this distribution pacing frequency was increased in steps of 1 Hz.

3.3 Modulation of MA by temperature

In most experiments the temperature of the perfusate was maintained between 35 - 37°C. However when temperature was held at 30°C, an earlier onset of alternans was observed (at 3 Hz in the failing and 5-6 Hz in the controls), in excellent agreement with Orchard et al.³⁰ Further increases in pacing frequency in the control hearts resulted in the exacerbation of MA. In other words, the ratio of small beat to big beat decreased. It was not possible to pace the failing hearts under these conditions (i.e. at frequencies greater than 5 Hz at 30°C). **Fig 3.6** is a representative plot of the ratio of small to big beat as a function of pacing frequency in a SD rat heart.

3.4 Effects of agents that disrupt SR function - caffeine, ryanodine and thapsigargin

To determine whether (in our pacing induced model of MA), and if so how exactly the SR is involved, agents that disrupt SR function were used. Alternans was abolished when the heart was challenged with 1 mM caffeine (a known promoter of SR calcium release), whereas washout of the caffeine restored the appearance of alternans after a subsequent 3 to 5 Hz frequency jump⁵¹(**Fig 3.7 a and b**). Similar results were obtained with 50 nM ryanodine (an agent, which at this concentration, has effects similar to that of caffeine).

Fig 3.6: The “degree” of alternans is exacerbated as pacing frequency is increased. A SD heart (temperature = 30°C) was subject to increasing pacing frequencies. As frequency is increased, the “degree” of alternans, expressed as the ratio between small and big beat, is exacerbated.

Fig 3.7: Effect of caffeine on alternans. a) LVP recording from an SHHF heart pretreated with 1 mM caffeine. Pacing frequency is increased from 3 to 5 Hz at the arrow. Note the absence of alternans in this case. Also EDP is elevated while LVDP is reduced. b) LVP recording from the same heart after caffeine washout. Increasing pacing frequency from 3 to 5 Hz results in the appearance of mechanical alternans.

a)

Figure 3.7

Figure 3.7 (continued)

b)

Thapsigargin is a known inhibitor of the SR Ca^{2+} pump.⁶⁶ When SD rat hearts were perfused with varying concentrations of thapsigargin (50-500 nM), SR function was clearly impaired as seen by the gradual increases in end-diastolic pressure, depressed force development and an increased time for relaxation (**Fig 3.8 a and b**). Incubation with thapsigargin however, failed to decrease the threshold (in terms of pacing frequency) for alternans in these hearts (**Fig 3.9 a and b**).

3.5 ^{31}P NMR and alternans

A number of authors have suggested intracellular acidosis as a possible causal factor of pacing induced MA.^{29,30} To examine if this was indeed the case, ^{31}P NMR studies were carried out. In vitro calibration curve of the 2-deoxy-glucose-6 phosphate (2DG6P) showed that the compound has a pKa of 6.17, in excellent agreement with Bailey et al. who reported a value of 6.16.⁵³ Simultaneous in vivo reports from P_i and 2DG6P, obtained by making the heart ischemic showed that the latter is a good reporter of intracellular pH (**Fig 3.10**).

(The following equations⁵³ were employed to calculate intracellular pH from the chemical shifts of P_i and 2dG6P

$$\text{pH}_i = \text{pKa}_{\text{P}_i} - \log [(\delta - X) / (Y - \delta)] \dots\dots\dots 1$$

$$\text{pH}_i = \text{pKa}_{2\text{dG6P}} - \log [(\delta - X) / (Y - \delta)] \dots\dots\dots 2$$

Fig 3.8: Effect of thapsigargin on LVP. a) LVP recording from a SD heart before the addition of thapsigargin. Pacing frequency is 3 Hz. b) LVP recording from the same heart 35 mins. after the addition of 500 nM thapsigargin. Note the elevated EDP and time to relax but reduced LVDP.

Figure 3.8

Fig 3.9: Effect of thapsigargin on mechanical alternans. Thapsigargin does not reduce the threshold frequency for alternans. LVP recording from a SD heart pretreated with 500 nM thapsigargin. a) Pacing frequency was increased from 3 to 5 Hz at the arrow. Note the elevated EDP but the absence of alternans. b) Pacing frequency has been increased from 5 to 7 Hz. Again, note the absence of alternans.

Figure 3.9

Figure 3.9 (continued)

b)

Fig 3.10: 2DG6P as a probe to monitor intracellular pH: Comparison with P_i . Results of a single experiment to monitor the efficacy of 2DG6P as an intracellular pH probe. To observe a P_i the heart was made ischemic. With increasing duration of ischemia, intracellular pH decreases. From the above plot, it is obvious that 2DG6P is a fairly accurate indicator of intracellular pH. See text for additional details.

where X is the chemical shift for free base, and Y is the chemical shift for free acid.⁶⁷ A pK_a of 6.72 was chosen for P_i ⁵³). **Fig 3.11 a** shows the ^{31}P NMR spectrum of a WF heart while in mechanical alternans. The PCr and ATP peaks are clearly visible. The resonance from the 2DG6P is seen to the left of the PCr peak. **Fig 3.11 b** shows a spectrum from an SHHF heart while in MA. As seen in the spectrum P_i is present in these failing hearts even under basal conditions.

Calculation of intracellular pH before and during MA in all three types of myocardial tissue, suggests that under our conditions there is negligible change in intracellular pH during MA (**Fig 3.12**). **Table 3.2** lists the nucleotide pools from the three types of myocardium.

3.6 Modulation of MA by β adrenergic activity

The effect of β adrenergic stimulation on MA was examined in greater detail by challenging the heart with β adrenergic agonists. **Fig 3.13** shows the effect of adding 300 nM isoproterenol (a non-selective β agonist) to a failing heart in alternans at 5 Hz. As seen in the figure, this results in a rapid increase of developed pressure with a concomitant resolution of alternans (within 30 seconds of the addition of this agent). The big and small beats increase in size (a phenomenon termed as double positive staircase) until they are both of equal magnitude. This effect of isoproterenol was seen in both control and failing hearts.

Fig 3.11: ^{31}P NMR spectra of WF and SHHF hearts during mechanical alternans.
a) Spectrum of a WF heart in alternans at 10 Hz. b) Spectrum of an SHHF heart in alternans at 5 Hz. 2DG6P was used as the intracellular pH probe in both cases. . The failing hearts showed a P_i resonance in addition to the PCr and ATP peaks.

a)

b)

Figure 3.11

Fig 3.12: **Intracellular pH before and during mechanical alternans.** $[pH]_i$ was determined at a basal pacing rate of 3 Hz and during mechanical alternans. There was no significant decline in $[pH]_i$ during alternans ($P \gg 0.05$ in each case).

Nucleotide / Cr content nmol / mg protein			
	SD	WF	SHHF
ATP	27.85 ± 1.43	29.97 ± 0.72	24.79 ± 0.73*
AN	33.26 ± 1.85	35.85 ± 0.73	30.26 ± 0.86*
Cr	102.17 ± 6.26	105.56 ± 2.95	47.16 ± 3.13*

Table 3.2: **Intracellular nucleotide pools and total creatine content.** Table lists intracellular ATP, AN and total creatine content from 3 month old SD, 18 month old WF and failing SHHF rat hearts. Separation and detection of nucleotides was done by HPLC. Total creatine content was assessed by a colorimetric assay following acid hydrolysis of phosphocreatine. Data are mean ± S.E.M. (n=6 in each case). Values for the SHHF hearts is significantly different from that of controls (P<0.05).

Fig 3.13: Effect of isoproterenol on mechanical alternans. LVP recording from an SHHF heart in alternans at 5 Hz. 300 nM isoproterenol was added at the arrow. Note the increase in developed pressure and the abolishment of alternans.

Norepinephrine, a β_1 agonist had a similar effect on alternans in all three types of myocardium. The kinetics of resolution of alternans was similar to that seen with isoproterenol. The β_2 specific agonist zinterol (300 nM) and the phosphodiesterase inhibitor IBMX (1 μ M) were also successful in abolishing the alternans.

3.7 Effect of trigger Ca^{++} on MA

In experiments where bath calcium was maintained at 1.8 mM, sustained alternans was observed in the rat hearts at the pacing frequencies seen in Fig 3.5. Decreasing bath calcium to 1 mM had an effect similar to that of decreasing temperature. The threshold frequency for alternans was reduced in all three types of rat heart, in agreement with Peterson et al.⁶⁸ **Fig 3.14** shows the effect of a 6-fold increase in calcium on MA. In this experiment, alternans was induced in a failing heart by pacing at 4 Hz (1 mM calcium). The perfusate was then switched to one containing 6 mM calcium. This resulted in a rapid increase in developed pressure and an abolishment of alternans. 100 nM BAY K 8644 (a calcium channel agonist) had a similar effect. Interestingly, in the presence of 100 nM verapamil (a calcium channel blocker), 300 nM isoproterenol could not abolish alternans. **Table 3.3** lists the various agents that abolished alternans and their respective concentrations.

Fig 3.14: **Effect of increasing $[Ca]_0$ on mechanical alternans.** LVP recording from an SHHF heart in alternans at 4 Hz. In this experiment $[Ca]_0$ was increased from 1 to 6 mM at the arrow. Note the increase in developed pressure and the abolishment of alternans.

	SD	WF	SHHF
caffeine (1 mM)	yes	-	yes
ryanodine (50 nM)	yes	-	yes
iso (300 nM)	yes	yes	yes
norepi (500 nM)	yes	yes	yes
zinterol (10 μ M)	yes	yes	yes
IBMX (100 μ M)	yes	-	-
[Ca ⁺⁺] ₀ (6 mM)	yes	yes	yes
BAYK 8644 (300 nM)	yes	-	-

Table 3.3: **Agents that abolish mechanical alternans.** The respective concentrations are in parentheses. Each “yes” is representative of 3 experiments. A “-” denotes that the agent was untested.

3.8 The force - interval relationship

To examine whether there is any effect of heart failure on restitution kinetics, mechanical restitution curves were generated from control and failing hearts. **Fig 3.15 a** shows the response of a SD heart to a test interval imposed at 150 msec (basal cycle length 333 msec). The accompanying extrasystole is of very small magnitude and rides atop the previous steady state beat. **Fig 3.16** shows the response of a failing heart to an identical protocol. As seen in the figure, there is negligible pressure developed. Measurable pressures were developed only when extra-systolic interval (ESI) was increased to 240 msec or above in the failing heart. The post extrasystolic beat however is potentiated in both control and failing hearts. That the above observations are properties of the intracellular Ca^{2+} transient ($[\text{Ca}^{2+}]_i$) as opposed to the contractile elements, is demonstrated in **Fig 3.17**, wherein actual Ca^{2+} transients have been recorded from isolated myocytes in response to a similar protocol.

As test interval was prolonged, extrasystolic developed pressures (F_1 s) increased whereas post-extrasystolic developed pressures (F_2 s) decreased (**Fig 3.15 b and c**). The one exception to this trend was in the age-matched WF hearts. Although these hearts responded well to the test stimulus at 150 msec, F_1 failed to increase till test intervals of 210 msec. Intervals greater than this (240 msec and more), elicited increases in extrasystolic developed pressure (**Fig 3.18 a, b and c**). As seen in **Fig 3.19**, the MR curve for the age matched WF controls ($T_{1/2} = 253 \pm 3$ msec, $n = 7$) and the failing hearts ($T_{1/2} = 266 \pm 4$ msec, $n = 7$) is right-shifted when compared to the younger SD hearts ($T_{1/2} = 227 \pm 3$ msec, $n = 7$). The curves for all three rat hearts however show two

Fig 3.15: Response of a SD heart to test stimuli after varying intervals. Basal pacing interval is 333 msec. After a priming period of 100 beats, a test stimulus is introduced, after which basal pacing frequency is resumed. The test stimulus is introduced after varying intervals known as extrasystolic intervals (ESI). a) ESI = 150 msec. Note the presence of the extrasystolic (arrow) as well as the post - extrasystolic contractions. b) ESI = 195 msec. c) ESI = 240 msec. As ESI is lengthened, extrasystolic force increases, whereas post - extrasystolic contraction decreases.

Figure 3.15

Figure 3.15 (continued)

Figure 3.15 (continued)

Fig 3.16: Response of an SHHF heart to a premature test stimulus. Basal pacing interval is 333 msec. ESI = 150 msec. Note the absence of a well - defined extrasystolic contraction (arrow). Post - extrasystolic contraction is potentiated.

Fig 3.17: $[Ca^{++}]_i$ and a premature test stimulus. Figure shows intracellular Ca^{++} transients monitored by Fura - 2 dye in a SD myocyte. Basal pacing interval was 1000 msec. Arrow shows the $[Ca^{++}]_i$ in response to an ESI = 500 msec. Also note the presence of the potentiated post-extrasystolic Ca^{++} transient.

Figure 3.17

Fig 3.18: Response of a WF heart to test stimuli after varying intervals. Basal pacing interval is 333 msec. a) ESI = 150 msec. Note the presence of a well defined extrasystolic response. b) ESI = 195 msec. Note the absence of a well defined extrasystolic response. c) ESI = 240 msec. Note the reappearance of a well defined extrasystole.

Figure 3.18

Figure 3.18 (continued)

Figure 3.18 (continued)

Fig 3.19: Mechanical Restitution curves for SD, WF and SHHF rat hearts. MR curves were constructed in response to the protocol in Fig 1.1 c. Responses have been normalized by their respective steady- state values. Seven animals were studied in each group. Inset: Half times for the MR curves ($T_{1/2 \text{ SD}} = 227 \pm 3$ msec, $T_{1/2 \text{ WF}} = 253 \pm 3$ msec and $T_{1/2 \text{ SHHF}} = 266 \pm 3$ msec).

Figure 3.19

distinct phases. Increasing the test interval from 150 to 333 msec resulted in a steep increase in F_1 / F_0 . This portion of the curve has been termed Phase A. At test intervals longer than 333 msec, viz. from 333 to 600 msec, F_1 / F_0 increased less dramatically; Phase B. Plots of F_2 / F_0 vs. ESI were also constructed (**Fig 3.20**)

3.9 Effect of thapsigargin on the F-I relationship

The effect of thapsigargin on the F-I relationship curve was investigated. **Fig 3.21** shows the response of a SD heart in the presence of 500 nM thapsigargin to an ESI imposed at 150 msec. As seen from the figure, the heart is able to respond to this ESI, with seemingly greater force than in the absence of thapsigargin. At longer test intervals (>333 msec) however, F_1 / F_0 is smaller than those obtained in the absence of thapsigargin (**Fig 3.22 a and b**). Interestingly, when the WF heart was perfused with thapsigargin, F_1 / F_0 increased as a function of ESI as seen in **Fig 3.23 a and b**.

Fig 3.20: Post-extrasystolic potentiation curves for SD, WF and SHHF rat hearts. PESP curves were constructed in response to the protocol in Fig 1.1c. Responses have been normalized by their respective steady- state values. Seven animals were studied in each group.

Fig 3.21: Thapsigargin does not prevent force production in response to a premature stimulus. LVP recording from a SD heart pretreated with thapsigargin (500 nM). Arrow shows response of the heart to a test stimulus at ESI = 150 msec.

Fig 3.22: Thapsigargin treatment results in post - rest depression. a) LVP recording from a SD heart in response to an ESI = 600 msec (arrow). Note the presence of a potentiated contraction. b) Same heart pretreated with thapsigargin (500 nM). Arrow shows response of the heart to an ESI = 600 msec. Thapsigargin treatment resulted in post - rest depression.

Figure 3.22

Figure 3.22 (continued)

Fig 3.23: Thapsigargin treatment corrects the anomalous trend seen in WF hearts. The heart was pretreated with 500 nM thapsigargin for 35 mins. a) ESI = 150 msec. b) ESI = 195 msec elicits a well defined extrasystole in this case, with amplitude greater than that in "a".

Figure 3.23

Figure 3.23 (continued)

CHAPTER 4

DISCUSSION

4.1 The SHHF rat model

Younger, non-failing SHHF rats exhibit pronounced left ventricular hypertrophy; however, right ventricular hypertrophy is observed only in those SHHF rats with overt heart failure.⁵⁰ During compensatory left ventricular hypertrophy, systolic pressures measured by the tail cuff method range from 145-210 mmHg, but during the transition to failure, blood pressures fall to normotensive levels (110-130 mmHg). Progressive changes in electrocardiograms⁶⁹ as well as echocardiograms⁶⁵ accompany the deterioration of cardiac function in SHHF rats.

A previous *in vivo* investigation of both male and female SHHF rats with comparable, albeit subjectively evaluated physical signs of failure, revealed significantly increased end-diastolic filling pressures and depressed contractility (+dP/dt) in both ventricles when compared to age-matched WF rats. Developed pressure, on the other hand, was similar to that of controls.⁷⁰ Maintenance of adequate ventricular pressure development in the presence of reduced contractility has been reported by other groups as

well.^{71,72} The increased intraventricular volume (indicative of a dilated left ventricle) as well as the depressed pressure-volume relationship (see Fig 3.1) are in excellent agreement with previous studies using hypertrophic rat hearts.^{8,58}

4.2 The force-frequency relationship in rat hearts

The force-frequency relationship in the mammalian myocardium has been well documented. While most species display a positive rate staircase, the rat heart was thought to be anomalous in this regard, since it demonstrated a negative force-frequency relationship.^{4,5} However, of late, the literature has been controversial in this regard.

Using isolated rat hearts, P.D. Henry observed a positive F-F relationship in the range from 4 to 8 Hz.⁷³ Further increases in pacing frequency yielded a negative staircase. More recently, using isolated rat ventricular myocytes, Borzak et al. observed that as stimulation frequencies were increased from rest to 1 Hz, the extent of unloaded shortening decreased.³ With further increases in the rate of stimulation (to 6 Hz) extent of shortening increased (positive staircase). The authors concluded that in rat ventricular cells, the rate staircase is biphasic; negative from rest to 1 Hz and positive from 1 to 6 Hz. Since most studies investigating the rat cardiac staircase start with resting myocytes and rarely involve pacing frequencies above 1 Hz, the dogma has been that the rat heart demonstrates a negative F-F relationship. It is well known that rat myocardium and isolated rat myocytes exhibit large rested state contractions.⁷⁴ Presumably, the rat cardiac SR accumulates calcium during rest while the SR of most other species becomes

depleted. Thus with extremely low pacing frequencies, and especially starting from the rested state, there is a clear negative treppe.

Schouten et al. have demonstrated that the observed rate staircase in rat myocardial preparations is a result of insufficient exchange of metabolites and oxygen between the bathing medium and the core of the muscle.⁷⁵ Using papillary muscles of varying thickness, the investigators found that in thick preparations (diameter 0.2-1.2 mm) an increase in stimulation frequency caused a reduction of peak force whereas in thin preparations (diameter < 0.2 mm) peak force was almost independent of stimulation frequency.

The sodium lag hypothesis (see introduction) was confirmed by Lotan et al., who used ^{23}Na NMR to demonstrate that increasing pacing frequency results in an increase in $[\text{Na}^+]_i$.¹³ Interestingly the authors were able to separate the male SD rat hearts used in their study, into two groups; Group I, which showed a positive staircase with increasing pacing frequency and Group II which displayed a negative staircase. The increase in $[\text{Na}^+]_i$ loading was more pronounced in Group I (although $[\text{Na}^+]_i$ increased in both groups with increasing pacing frequency). The authors concluded that there is a differential pressure response in rat hearts, which may reflect an enhanced sensitivity in some rats to the shortening of the restitution period of the SR, outweighing the positive inotropic response of the Na pump lag. Only in cases where each $[\text{Na}^+]_i$ increment outweighs the individual restitution deficit, would a complete positive inotropic effect be anticipated. Identical observations were made by another group, using isolated myocytes and $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ sensitive dyes.⁷⁶ Finally, Field et al have demonstrated a net

steady state negative force frequency relationship in isolated Langendorff perfused rat hearts⁷⁷.

Our findings with normal rat hearts, perfused at high flow rates and with high concentrations of glucose and pyruvate, i.e., an early force potentiation followed by a slow gradual decline, are nearly identical to those seen in the above publications. The early phase of force potentiation may be due to a combination of increased Na^+ loading and a direct potentiation of L-type Ca^{2+} channels.¹⁵ The subsequent gradual decline in developed pressure may relate to an acceleration of sodium pump activity and a reduction of i_{Ca} . At the highest frequencies (above 7 Hz), restitution kinetics of the SR may far outweigh any of the afore-mentioned processes. This may be especially true in the age-matched and failing rat hearts, where a negative or flattened staircase was observed at all frequencies tested.

Thus the rate staircase of the well perfused, well oxygenated rat myocardium is clearly multiphasic and depends upon the stimulation frequency, while it is flattened or even negative in aged and failing animals.^{7,8,16}

4.3 Mechanical alternans

Our results demonstrate that sustained mechanical alternans can be achieved by pacing the myocardium at high frequencies. Furthermore, the failing SHHF rat hearts are more predisposed to mechanical alternans, i.e., these hearts go into MA at much lower pacing rates, when compared to their normotensive counterparts. A unique feature of this model is that as the pacing frequency is increased, alternans is exacerbated, i.e. the ratio

of the small to big beat gets smaller (as seen in Fig 3.6). Decreasing pacing frequency results in the abolishment of alternans. This suggests that alternans is not an “all or none phenomenon”, but is better characterized by an “extent” or “degree”.

The majority of the studies so far have implicated reactions of the SR in alternans and several authors have recognized that there is a rate-determining step involved in the production and maintenance of alternans.^{18,22,30} However, it has been difficult to identify which (or a combination of which) of the following 3 factors is actually involved:

kinetics of

- 1) the SR $\text{Ca}^{++}\text{ATPase}$,
- 2) the transfer of calcium from the uptake to the release sites and
- 3) the processes governing SR calcium release

Most of the studies carried out so far, have utilized various interventions such as hypothermia and acidosis to induce alternans.^{21,22,30} It is well known that interventions such as the above have a deleterious effect on the SR $\text{Ca}^{++}\text{ATPase}$.⁵⁵ It was tacitly assumed however that alternans was a direct result of the SR $\text{Ca}^{++}\text{ATPase}$ not being able to pump a steady amount of calcium back into the SR with every beat. Insufficient loading of the SR between beats was then assumed to cause alternate amounts of calcium release.⁴¹ As for the pacing induced model, it has been suggested that the high rates of stimulation do not allow for energy supply to meet energy demand, leading to overall intracellular acidosis.²⁹ This model was thus thought to be an extension of the acidosis model.

In our study, direct measurement of intracellular pH by ^{31}P NMR did not reveal any significant intracellular acidosis in both control and failing hearts. This suggests that even at high heart rates, the myocardium is able to maintain a balance between energy supply and demand. Also, we have shown that thapsigargin, which inhibits the SR $\text{Ca}^{++}\text{ATPase}$, failed to reduce the threshold frequency for MA, even at moderately high doses (500 nM) ⁶⁶ (Fig 3.9 a and b). Fully relaxed alternans has been seen in several studies^{18,22,30} including our own (Fig 3.4). These results immediately imply that calcium sequestration by the SR Ca^{2+} pump is not the rate limiting step in inducing alternans.

Some investigators have suggested that there is a finite time interval involved in the transfer of calcium from the “uptake” to the “release” compartment within the SR.³⁴ Alternans at high pacing frequencies may then be explained by insufficient time for the transfer of activator calcium from these uptake to release sites.²² This would then lead to alternations in $[\text{Ca}^{2+}]_i$ and contractility. However, using caffeine induced contractures, Bassani et al. have demonstrated that during a twitch, only a fraction (35%) of the total SR calcium pool is released;⁷⁸ a process termed as “fractional release”. Thus, a majority of the calcium remains in the SR even after CICR. Also, evidence for distinct anatomical compartmentation within the SR is lacking.⁷⁹

Thus although our own study does not provide any direct evidence against the calcium compartmentation model, it would be safe to exclude a time dependent transfer of calcium from the uptake to the release sites as being the rate-limiting step in the production of alternans. The above considerations leave us with the possibility of alternans being linked to the behavioral dynamics of the SR calcium release channels.³¹

4.4 β adrenergic stimulation

Our data consistently show that increasing the phosphorylation status of the myocardium, results in the resolution of alternans. This was achieved by a variety of agents including β_1 and β_2 agonists as well as phosphodiesterase inhibitors (agents that inhibit breakdown of cAMP) (Table 3.3).

β - Adrenergic receptor stimulation has profound modulatory effects on cardiac contractility. It has been well documented that the β_1 adrenergic receptors are coupled to adenylate cyclase and that stimulation with a β_1 agonist leads to an overall increase in cellular cAMP content and subsequently to the activation of cAMP-dependent protein kinase (PKA).⁸⁰ The key target proteins for PKA include sarcolemmal voltage-gated L-type Ca^{2+} channels, phospholamban and TnI. Phosphorylation of the L-type calcium channels leads to an increase in the L-type Ca^{2+} current,⁸¹ which is the trigger for sarcoplasmic reticulum calcium release³⁸ and to increases in the cytosolic calcium transient and contraction following excitation. Phosphorylation of phospholamban and TnI accelerate the kinetics of relaxation.^{82,83} Recently it has been demonstrated by several groups that the cardiac SR calcium release channel (CRC) is also susceptible to phosphorylation by PKA.^{84,85} PKA dependent phosphorylation has profound modulatory effects on the CRCs, such as 1) directly activating the release channels,⁸⁶ 2) overcoming the Mg^{++} block of these channels⁸⁷⁻⁸⁹ and 3) increasing the amount of calcium released for a given concentration of trigger calcium.⁹⁰

Thus, the net effect of β_1 adrenergic stimulation is an increase in both the inotropic and lusitropic performance of the myocardium. **Fig 4.1** shows the signal cascade for β_1 adrenergic stimulation. β_2 adrenergic stimulation on the other hand increases L-type Ca^{++} current and therefore trigger Ca^{++} .⁸⁰

Fig 4.1: Signal cascade for β adrenergic agonists and key target proteins for phosphorylation by PKA. The net result of administering a β agonist such as isoproterenol is a positive inotropic and lusitropic effect.

Figure 4.1

4.5 A model for pacing induced mechanical alternans: Refractoriness of the SR Calcium release channels to CICR

Using skinned cardiac Purkinje fibers, Fabiato has shown that the process of calcium induced calcium release (CICR) is under temporal modulation.³⁸ This finding, since extended by Bers and now known as “refractoriness” of SR calcium release channels⁹¹ (following CICR), can be explained as follows: SR calcium release channels enter a refractory state immediately after calcium release, during which period it is not possible to elicit further release with trigger calcium. Although recovery from this refractoriness is time dependent, release of luminal calcium by other mechanisms and agents is still possible such as rapid cooling contractures and caffeine induced calcium release (once again demonstrating that the SR stores do not completely empty during a normal contraction).^{38,92} Based upon the above evidence, a model is now proposed for the phenomenon of pacing induced mechanical alternans.

It has been postulated that SR Ca release channel exists in and cycles between three states: 1) closed (ready for activation), 2) open (in the act of releasing calcium) and 3) refractory (unresponsive to further release) as shown in **Fig 4.2 a**.⁹³ In **Fig 4.2 b**, the vertical bar represents the channel in the closed conformation, ready for activation, with the black circle representing incoming trigger calcium. **Fig 4.2 c** represents the process of calcium induced calcium release with the “asterisks” denoting the calcium released from the SR. The release channel now moves from the open to the refractory state. If the next cycle of trigger calcium comes in at an interval T_i longer than or equal to the sum total of the open (T_o) and refractory (T_r) times of the SR release channel,

calcium release is again possible as depicted by **Fig 4.2 d** and **e**. This is because the release channel has recovered from its refractoriness and has moved back to the closed state. However, if pacing frequency is suddenly increased and the trigger calcium comes in at an interval T_p , shorter than $(T_o + T_r)$, then CICR is not possible since the release channel would not have recovered from its refractory state (**Fig 4.2 f**).

However mechanical alternans manifests itself as a normal contraction followed by a smaller contraction, rather than no contraction at all. This is because not all the release channels in a myocyte (would) have identical $(T_o + T_r)$'s. There is a distribution of these "times". Thus those release channels with shorter refractory periods would have recovered earlier and would therefore be able to open during the next cycle. In agreement with this scheme, alternans is exacerbated as pacing frequency is increased (or disappears as pacing frequency is decreased) as seen in Fig 3.6. With increasing pacing frequency, more and more of the release channels shut down and the percentage of channels able to fire with every beat decreases. In other words, we are titrating off the release channels using a temporal protocol.

Single channel studies of cardiac ryanodine receptors (or CRCs) reconstituted in lipid bilayers have been widely used to study the gating of these channels.^{88,94} While these channels are thought to behave rather differently under these conditions,⁵⁵ Valdivia et al. have conclusively shown that increasing the phosphorylation status of the CRCs, increases their sensitivity and accelerates the kinetics of adaptation, permitting faster availability of these receptors for subsequent

Fig 4.2: A model for pacing induced mechanical alternans. See text for details.

a)

b)

c)

Figure 4.2

Figure 4.2 (continued)

d)

$$(T_i > T_o + T_r)$$

e)

← * * * * * CICR possible

* * * *

● * * * *

o/r

f)

$$(T_i < T_o + T_r)$$

CICR not possible

triggers.⁹⁵ Increasing the bathing calcium had a similar effect, i.e., it increased the rate of opening of these channels. The net effect was an increase in the overall open probability (P_o) of the CRCs. In an in vivo situation, this would effectively translate into a shortening of the refractory period of the release channels. Our own experimental results suggest that increasing trigger calcium or the phosphorylation status of the myocardium abolishes the alternans (Table 3.3). Clearly, either of the above interventions results in the recruitment of an “identical” number of release channels with every beat. An equal amount of activator calcium is released during every cycle and alternans is abolished.

The basal phosphorylation status (i.e. cAMP levels) of the failing SHHF rat hearts and their normotensive counterparts used in this study has not yet been determined. However previous studies in a similar colony of rats, have shown that basal levels of cAMP are depressed in the hypertensive rat heart.⁹⁶ The present study suggests an increased refractory period in the failing rat heart following CICR, as manifested in the earlier onset of alternans. While the exact reason for this is not entirely clear, it may be hypothesized that in the failing heart, a lower phosphorylation status of the CRCs may result in an increased refractory period following CICR.

4.6 Mechanical restitution: A model based upon refractoriness of calcium release and SR calcium load

Our results demonstrate that the failing hearts have slower kinetics of contractile recovery when compared to the normotensive controls (Fig 3.19). This finding is in agreement with several other investigators.^{37,40,97} Interestingly, there is an age-dependent component to this recovery process, as exemplified by the individual half-times for the 3 types of myocardium ($T_{1/2 SD} = 227 \pm 3$ msec, $T_{1/2 WF} = 253 \pm 3$ msec and $T_{1/2 SHHF} = 266 \pm 3$ msec). This probably manifests itself in the form of the transient MA seen in the WF hearts at pacing frequencies above 6 Hz.

The very same hypotheses that were used to explain MA viz., calcium compartment model and slowed SR pump model, have been invoked to explain the phenomenon of MR.^{34,41} However, Prabhu et al. have recently demonstrated that the kinetics of recovery of the SR calcium release channel is the rate-determining step in the recovery of contractile function.^{37,40} Also, once again in our hands thapsigargin did not reduce the amount of calcium released in response to a premature stimulus (Fig 3.21). Based upon the existing literature and our own experimental evidence, a model is proposed to explain the restitution process.

The SR Ca release channels are more sensitive to cytosolic factors than luminal factors.⁹⁸ The effects of Mg^{++} , ATP, calmodulin, etc. on the CRC are well known.⁹⁹ However, once the CRCs are open, the amount of flux (i.e. Ca^{++} flow) through them is proportional to SR Ca load.⁹⁸ The mechanical restitution curve can then be explained by assuming that the force produced during a contraction is proportional to two factors:

1) the fraction of SR Ca release channels that are open during a beat and 2) the magnitude of the flux of Ca through these channels (a function of SR Ca load). During a premature test stimulus, only a fraction of the release channels open (in accordance with the refractoriness principle discussed above). This leads to a small amount of Ca release with the corresponding extrasystole being small. As ESI is increased, more and more channels recover from refractoriness and are able to fire. Thus extra-systolic contraction gets larger and larger until it equals previous steady state contraction (when ESI = basal pacing interval).

At intervals longer than basal pacing interval, SR Ca load comes into play. The SR has more time to re-sequester Ca from the cytosol and consequently is loaded to a greater extent. Also all the SR Ca release channels are able to open. Flux through these channels is greater and consequently the contraction is one of larger amplitude. Thus, during Phase A of the MR curve, the fraction of open SR Ca release channels plays an important role, whereas during Phase B of the curve, SR Ca load is the dominant factor. This model is not only in accordance with our thapsigargin results (thapsigargin does not influence the fraction of release channels that can open but influences SR Ca load (see Figs. 3.21 and 3.22 a and b)) but also in excellent agreement with the one proposed by Baudet et al.¹⁰⁰

Once again our studies indicate that in the failing heart the SR calcium release channels are refractory to calcium release. While it is not clear why the WF hearts behave in an anomalous manner (Fig. 3.18 a, b and c), it may be hypothesized that in these animals the SR pump may be highly active between 150 and 210 msec, sequestering most

of the released calcium before it reaches the myofilaments. A similar explanation was first proposed by Schouten.³⁵ Our own data with thapsigargin suggests that this may very well be the case (Fig. 3.23 a and b).

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