CALCIUM ACTIVATED NEUTRAL PROTEASE: DEFINING A PHYSIOLOGICAL ROLE IN THE DEVELOPMENT OF CARDIAC HYPERTROPHY.

by

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Abstract.

Calcium activated neutral protease, calpain, is thought to be activated where intracellular calcium levels are increased. Calpain substrates include myofibrillar, cytoskeletal, ion handling and cell signalling proteins, all of which are altered during cardiac hypertrophy. No assessment of calpain during hypertrophy has been undertaken, therefore my goal was to characterise any possible role and mechanism of calpain with physiological and pathological hypertrophy in Wistar rats. Calpain's involvement in intracellular alterations during hypertrophy was assessed using the calpain inhibitor E64c. Swim exercise and injection of the β-adrenergic agonist isoproterenol were predicted to increase calpain activity. Both models induced hypertrophy with E64c able to reduce the isoproterenol induced hypertrophy. Swim exercise decreased calpain activity, while isoproterenol injection increased activity of both calpain isoforms. E64c prevented isoproterenol induced calpain increases while increasing the activity of calpastatin, calpains endogenous inhibitor.

A comparison of calpain's response to swim or run exercise was carried out as these models both produce cardiac alterations. Running increased particulate fraction calpain activity of skeletal muscle and in both the soluble and particulate fractions in cardiac muscle. Swim exercise decreased cardiac and skeletal muscle calpain activities, demonstrating that striated muscle calpain responds differently to these exercise stimuli. Calpain does not seem to mediate the cardiac adaptations to swim exercise. Isoproterenol induces increased calpain activity and E64c's ability to inhibit this increase and to reduce its hypertrophy effects led me to focus on this model. Increased contractile function was demonstrated 72 hours after isoproterenol injection but not in E64c treated hearts, suggesting that underlying causes may be dependent upon calpain. Therefore, aspects of both Ca^{2+} homeostasis and myofibrilar composition were examined. Ca^{2+} uptake by the SR was significantly elevated 72 hours following isoproterenol with E64c actually increasing this effect. Certain key myofibrilar proteins were mobilized to a more cytosolic associated distribution following isoproterenol with E64c reducing this effect. Increased calpain activity may play a significant role in the development of isoproterenol induced hypertrophy. Further study of these calpain mediated processes in cardiac

hypertrophy could facilitate the development of treatments to reduce the deleterious adaptations that lead to heart failure.

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over 72 hours.

CHAPTER 1.

General Introduction, Hypothesis and Objectives.

Cardiac hypertrophy.

Cardiac hypertrophy can result from situations such as increased physical activity and thyroid hormone stimulation, which are considered physiological in nature. Converesly, hypertophy can also be pathological in nature such as with hypertension, valvular heart disease or in compensation for loss of heart tissue due to myocardial infarction. Both these types of hypertrophy reflect the ability of cardiac muscle to undergo continuous remodeling despite having to meet the acute and long-term demands placed upon it. A more comprehensive presentation on the regulation and control of cardiac hypertrophy is discussed in detail in Chapter 2 and elsewhere (110), (96), (155). Briefly, the heart responds to increased demands through functional and biochemical adaptation of various intracellular organelle systems. Although stimuli leading to hypertrophy are often associated with altered metabolic, ionic and hormonal states, the precise mechanisms responsible for hypertrophy have remained elusive. Selected physiological and biochemical processes/systems involved during cardiac hypertrophy are presented in Table 1. Despite recent advances in characterizing the factors regulating cellular/molecular processes such as gene expression (94), metabolic fluxes (226), ionic movements (59,98), ultrastructural organization (51,273) and contractile protein function (207,239,277) a description of the key process(es) contributing to cardiac adaptations are lacking in the literature.

A consistent observation accompanying hypertrophy is the alteration in Ca^{2+} handling within the myocardium. Indeed with most models of hypertrophy there are alterations in intracellular Ca^{2+} homeostasis which generally lead to a Ca^{2+} influx ('Ca^{2+} overload') in the cell (110). Changes in subcellular Ca^{2+} may well lead to some of the functional and biochemical changes accompanying the hypertrophy process (60,96). These include metabolic and ionic changes (2,110,226), re-organization of cytoskeletal proteins (273), increased Ca^{2+} sensitive protein degradation rates and modulation of selected ion channel activity (131). Clearly then Ca^{2+} is playing a role, either directly or indirectly in the development of hypertrophy, albeit through undefined mechanism(s). The experiments designed in Chapters 3-6 attempt to identify the nature of the interaction

between Ca^{2+} and cardiac adaptations during hypertrophy. Because the cellular changes noted with hypertrophy (Table 1) implicate Ca^{2+} dependent processes, the experiments focused on a Ca^{2+}-dependent neutral protease system, calpain, and its endogenous inhibitor calpastatin.

MYOCARDIAL ALTERATION	CHARACTERISTICS ASSOCIATED WITH HYPERTROPHY			
	Stimuli/Process	Physiological	Pathological	Reference
[ATP]i	Metabolic & Ionic changes	Improved ATP resynthesis	$\downarrow$[ATP] & [ATP]/[ADP]	(226) (144)
[Ca^{2+}]$_i$	Altered [Ca^{2+}]$_i$	Improved Ca^{2+} handling	Ca^{2+} overload. $\uparrow Ca^{2+}$ transients	(211) (59)
Contractile proteins	Altered cell structure	$\uparrow$ Function & performance	$\downarrow$Function & performance	(110)
Cytoskeletal proteins	Ultrastructural modifications	Acute initial disruptions	Disruption and prolonged re-organization	(273)

Table 1. Morphological, metabolic, and ionic characteristics of myocardium undergoing pathological or physiological cardiac hypertrophy.

Calpain (EC 3.4.22.17) is a Ca^{2+} activated neutral protease ubiquitously present in cells as both a low and high Ca^{2+} requiring isoform, (u- and m- calpain, also known as calpains 1 and 2 respectively) (167,202). More recently, the discovery of what appear to be novel or muscle specific isoforms (n-calpains) have been reported (167,234,236). Calpain has been assigned a regulatory role due to specific activation requirements coupled with the very precise and limited nature of the proteolytic cleavages it catalyses (167,202). Calpain is regulated not only by Ca^{2+}, but by an endogenous and specific inhibitor protein; calpastatin, which is also ubiquitously distributed. Although the biochemical nature of calpain and calpastatin is being elucidated (39,157), the nature of their physiological interactions and precise roles are not forthcoming in the literature.

Most of the current hypotheses concerning the physiological and pathological roles for the calpain-calpastatin system have been deduced from the susceptibility of

several proteins to in vitro proteolytic modification (271). Conclusions from in vitro studies of isolated proteins to date have associated the calpain system with modulation of cytoskeletal proteins such as intermediate filaments, membrane receptors, channels and/or ion carriers through selective and limited proteolysis (24,43). Calpain has also been suggested to play a role in intracellular transduction cascades regulated by Ca^{2+} (158). In addition, as a soluble intracellular protease, in vitro data suggests that calpain may be involved in initiating cell protein turnover as a starting point for further proteolytic degradation for abnormal or non-functional proteins (248,287). Limited observations from in vivo models also implicate calpain and calpastatin in regulating certain cellular processes. These include the rearrangement of membrane and cytoskeletal proteins in red blood cells, renal cells and glial cells, which serves to regulate specialized cell functions such as mitosis and movement of secretory granules (180,215). In cardiac muscle, calpain substrates such as troponin-I and desmin are vulnerable in pathological conditions (51,273) when calpain activity is increased such as during β-adrenergic stimulation (105), hypoxia (260) and stunning (142). Interestingly the administration of calpain inhibitors has shown cardio-protective capability suggesting that calpain actions may underlie certain cardiac pathologies (105,142). Whether or not calpain and calpastatin underlie the biochemical and functional alterations accompanying hypertrophy is unknown and because many of the changes accompanying hypertrophy are characteristic of calpain's actions, the overall hypothesis I set out to test in my research studies was:

O Calpain-calpastatin is the mechanism through which Ca^{2+} mediates biochemical and contractile responses in cardiac hypertrophy.

In order to test this hypothesis two different models of hypertrophy were employed (exercise training, chapters 3 & 4; and β-adrenergic administration, chapters 5 & 6) with the role of calpain and calpastatin being assessed by administration of an exogenous cysteine protease inhibitor.

The development of hypertrophy can be a beneficial adaptive process in order to meet the new increased load or transient increases in demand (compensated hypertrophy).

However if the demand is prolonged and the hypertrophy continues over an extended period then generally the process becomes deleterious to cardiac function and integrity. This ultimately results in failure of the heart to maintain its physiological function (decompensated hypertrophy). The hypertrophy response therefore depends very closely on the model or condition studied and its duration (110). By definition, an increase in mass of cardiac muscle occurs during development of hypertrophy, yet the associated adaptive changes to the myocardium can be varied. For example, pathologic hypertrophy is associated with increased cell size accompanied by decreased cardiac contractility (96,110,155). On the other hand, physiologic hypertrophy associated with exercise, anemia and thyroid hormone stimulation is accompanied by normal or improved cardiac performance (109,289). These two types of hypertrophy also lead to different membrane, morphological, protein degradation, cytoskeletal, and vascularization changes in the myocardium. See Chapter 2 for a more detailed review of cardiac hypertrophy.

Exercise induced cardiac hypertrophy.

Swim exercise is commonly used to elicit hypertrophy (153,208). Although physiologic hypertrophy induced by exercise does not alter the action potential, myocardium undergoing pathologic hypertrophy shows marked prolongation of the action potential and incomplete time to relaxation (110). These observations are related to changes in ionic transport across the sarcolemmal membrane, in particular alterations in Ca^{2+} homeostasis (9). This adaptive change in the myocardium allows for a greater Ca^{2+} influx into the heart, which is necessary for maintaining contraction in the larger hypertrophied heart (110). The increase in maximal functional capabilities of the heart caused by exercise training is due to a coordinated increment of the capacity of at least one (if not all) of the three major biochemical systems responsible for the functional characteristics of cardiac muscle. For example, changes in actomyosin ATPase activity (178), sarcoplasmic reticulum (SR) Ca^{2+} transport (173,180), sarcolemmal function (9), and ATP resynthesis systems (144), have all been reported with acute exercise (one session), indicative of a functional link between the acute demands placed on the heart and the overall adaptive response. The elevated SR Ca^{2+} pump activity after swim training, enhanced SR Ca^{2+} transport, and more effectively buffered intracellular Ca^{2+} results in augmented cardiac relaxation rates. Despite some characterization of swim

exercise in the literature the mechanisms underlying the adaptive responses of the heart are still unknown. Acute swimming is also associated with dramatic morphological changes such as mitochondrial damage, dilation of the SR and myofibril disruption (144) which accompany the biochemical changes in Ca^{2+} transport processes (178,218). Therefore it may be suggested that Ca^{2+} regulated processes are critical for the expression of these acute responses to swim exercise. No information exists in the literature in regard to whether acute swim exercise or physiological hypertrophy following prolonged swim exercise training is accompanied by or dependent upon activation of calpain. The experiments in Chapter 3 were designed to test the hypotheses that:

○ Calpain activity will increase following a single swim exercise session.

○ Increased calpain activity is necessary to promote cardiac functional and biochemical adaptations with repeated swim exercise.

To further assess the relationship between calpain activation and cardiac hypertrophy, the experiments in Chapter 4 were designed to compare two modes of increased metabolic activity; run versus swim exercise. In light of the data found in chapter 3 and because the calpain system is known to be activated with a single run to exhaustion (10,17) although repeated run sessions do not result in cardiac hypertrophy (210,211), a further hypothesis was that;

○ The response of calpain to a single run exercise session will be greater in the degree and/or pattern of activation than for a single swim exercise session.

It was anticipated that the experiments in Chapters 3 and 4 would provide a first description of the nature of the calpain proteolytic system under physiological conditions.

Pathological cardiac hypertrophy.
There is currently very little information available to determine if the calpain system is required for the development of pathological hypertrophy. Typical of conditions leading to the development of pathological hypertrophy are altered intracellular Ca^{2+} regulation

and associated structural and functional modifications (60,273). Pathological hypertrophy induced by β-adrenergic agonists is associated with chronic disturbances in ionic activity (i.e. increased $[Ca^{2+}]_i$) and metabolic status (decreased $[ATP]_i$) as well as cardiac cell growth. As mentioned previously, these ionic changes (110), re-organization of cytoskeletal proteins (118,273), increased Ca^{2+} sensitive protein degradation rates and modulation of selected ion channel activities (9) are all reminiscent of the actions of calpain, as described in table 1. Therefore it is reasonable to postulate that calpain is involved in the process of pathological hypertrophy. The functional and biochemical responses to pathological hypertrophy may indeed reflect disturbances in the calpain-calpastatin system and/or its mediated effects on various intracellular processes. Therefore the objective of Chapter 5 was to determine if calpain is involved in the induction of isoproterenol stimulated cardiac hypertrophy. The hypothesis tested was:

○ Isoproterenol induced cardiac hypertrophy is dependent upon the activation of calpain.

Of particular interest was whether or not cardiac functional changes were accompanied by activation of calpain as myocardial contractility is controlled by Ca^{2+}, and Ca^{2+} regulated processes such as the interaction of Ca^{2+} released from the SR with the regulatory proteins of the troponin complex. Protein substrates of calpain within the myocardium suggest that the influence of calpain could be to modify cardiac function in response to the hypertrophy or increased demand placed upon the heart. Cardiac troponin, specifically the inhibitory subunit (TnI) and tropomyosin have all been shown to be sensitive to calpain mediated degradation and are vulnerable during ischemia (260) and stunning (76,142). Proteins associated with the SR and Ca^{2+} regulation such as the Ca^{2+} release channel structure are also vulnerable to calpain modification (80). Therefore it is reasonable to propose that any activation of calpain would impact on the functional characteristics and responses of the heart through one or both of these systems. The experiments outlined in Chapter 6 were designed to assess the location underlying calpain and calpastatin interaction during pathological cardiac hypertrophy. The hypothesis tested was that:

○ Isoproterenol induced hypertrophy is dependent on contractile and sarcoplasmic reticulum proteins/processes that are mediated by the effects of calpain and calpastatin.

Despite recent advances in the biochemical characterization of tissue specific isoforms of calpains (234,237), the functional differences if any between the low Ca^{2+} requiring isoform, calpain-1, and the high Ca^{2+} requiring isoform, calpain-2, in substrate recognition and actions remain unclear. Therefore additional hypotheses for the experiments outlined in Chapter 6 were:

○ The activities of calpain-1 and calpain-2 will be increased to a similar degree following administration of isoproterenol.
○ Calpastatin activity will be reduced following the administration of isoproterenol.
○ Administration of an exogenous inhibitor (E64c) will decrease calpain activation and promote calpastatin activity.

List of hypotheses.

The hypotheses to be addressed in this study are presented here.

<u>Main hypothesis.</u>

○ Calpain-calpastatin is the mechanism through which Ca^{2+} mediates biochemical and contractile responses in cardiac hypertrophy.

<u>Exercise (swim / run) induced cardiac hypertrophy.</u>

○ Cardiac calpain activity will increase following acute swim exercise

○ Increased cardiac calpain activity is necessary to promote functional and biochemical adaptations with repeated swim exercise.

○ The response of cardiac calpain to a single swim exercise session will be greater in the degree and/or pattern of activation than for a single run exercise session.

<u>Isoproterenol induced cardiac hypertrophy.</u>

○ Isoproterenol induced cardiac hypertrophy is dependent upon the activation of calpain.

○ Isoproterenol induced cardiac hypertrophy is dependent on changes in contractile and sarcoplasmic reticulum proteins/processes that are mediated by the effects of calpain and calpastatin.

○ The activities of calpain-1 and calpain-2 are increased to a similar degree in response to isoproterenol injection.

○ Calpastatin activity will be reduced following the administration of isoproterenol.

○ Administration of an exogenous inhibitor (E64c) will decrease calpain activation and promote calpastatin activity.

CHAPTER 2.

A complete review of the organization and function of cardiac muscle is beyond the scope of this literature review. I will focus instead on the processes most relevant to the cardiac hypertrophy studied here and the biochemical and functional processes that are altered during the hypertrophy process. A review of the calpain proteolytic system is also presented with the aim of outlining current understanding of this Ca^{2+} activated protease and its endogenous inhibitor calpastatin.

Myocardial contractility.

Excitation-contraction coupling.
The process of muscle contraction and relaxation is dependent on the controlled cycling of Ca^{2+} which must be regulated very closely to maintain the contraction of the cardiac muscle while maintaining the approximately 1,000 to 10,000 fold difference in Ca^{2+} from the extracellular compartment to the intracellular environment (7). This requires considerable energy expenditure and specific controls within the muscle cell to maintain appropriate cycling and ionic balance. The key to this system or process is the closely regulated compartmentalization of Ca^{2+} both between the extracellular environment and the intracellular organelles that regulate Ca^{2+}. Control of Ca^{2+} fluxes in cardiac cells has been the focus of numerous reviews (7,64,79,117). I will give a brief description of the key features, highlight the importance of the precise control of Ca^{2+}, the integrity of the proteins responsible for transmitting the Ca^{2+} signal into contractile activity and therefore adequate contractile function, see figure1.

Contraction of cardiac muscle is initiated by an increase in the cytosolic concentration of Ca^{2+}. Ca^{2+} currents in normal cardiomyocytes are produced by the controlled movement of Ca^{2+} within the cell due to ion channels within the sarcolemma and internal membrane systems (64). Action potentials depolarize the sarcolemma, Ca^{2+} enters the cell and in turn triggers the release of stored Ca^{2+} from the SR in a process called Ca^{2+} induced Ca^{2+} release (CICR) (26,64). The cardiac cell contains at least two kinds of voltage activated Ca^{2+} channels. Long lasting or large (L-type) Ca^{2+} channels are found in all heart cells and produce the slow inward currents. L-type channels are multimeric protein complexes made up of five subunits called alpha-1, alpha-2, beta, gamma and delta, which are the products of different genes (102). Actual Ca^{2+} ion

conductance occurs through the alpha-1 subunit which contains sites that Ca^{2+} channel blockers can bind to while the other subunits have regulatory roles. This channel type opens at a potential of approximately -40mV, shows peak conductance of Ca^{2+} at approximately 0mV and inactivates relatively slowly, hence the name "long lasting". These channels are responsible for the majority of Ca^{2+} entry into the myocardium during the plateau phase, characteristic to the action potential of cardiac muscle cells. There are also other types of Ca^{2+} channels, the "transient" or "tiny" (T-type) channels. These activate and inactivate at more negative potentials and have faster kinetics than the L-type channels. The distribution of these T-type channels is fairly wide but they tend to be denser in rat and cat atrial cells and cells involved in pacemaker activity (280).

As mentioned previously, the release of Ca^{2+} from endoplasmic reticulum seems to be triggered by the entry of Ca^{2+} into the cell. The two main types of Ca^{2+} release mechanisms are the ryanodine receptor (RyR), and the inositol 1,4,5-triphosphate receptor (IP3R). The SR of cardiac muscle contains more RyR than IP3R and RyR play the major role in CICR (141). Recently the details of this release of Ca^{2+} into the cytoplasm have begun to emerge thanks to reports showing that there is a close physical relationship in the junctional SR between the sarcolemmal L-type Ca^{2+} channels, the Na^+/Ca^{2+} exchanger and the SR Ca^{2+} release channel. The RyR forms a tetrameric structure made up of four monomers which are stabilized together by a closely associated protein called the FK506 binding protein (FKBP). The close proximity of the structures involved in CICR has been demonstrated by laser scanning confocal microscopy (41), with RyR, dihydropyridine receptors (DHPR) and triadin all found to be localized together. The localized release of Ca^{2+} from the SR at the sites where the junctional SR and RyR are found closely together are called "Ca^{2+} sparks" and they represent the fundamental units of Ca^{2+} release from the SR (45). These localized releases of Ca^{2+} occur infrequently at rest, are inhibited by ryanodine and occur with greater frequency as the loading of the SR with Ca^{2+} is enhanced. More recently, the process of CICR has been shown to also occur in more intact experimental preparations with manipulations of external Ca^{2+}. There is a close correspondence between Ca^{2+} entry into the cell and release of Ca^{2+} from the SR, supporting the functional association of the two processes (38).

Another possible source of Ca^{2+}, which may be able to stimulate Ca^{2+} release from the SR is via the Na^+ - Ca^{2+} exchanger in response to cell de-polarization (38). Whether indeed this mode of Ca^{2+} entry is able to trigger Ca^{2+} release from the SR under normal physiological conditions is unclear and its relative contribution to normal cellular Ca^{2+} transient regulation remains unclear. In fact the physical organization of the structures involved are not closely matched, suggesting that this path of Ca^{2+} release is unlikely to be a key regulator of contraction. In addition, the over-expression of Na^+ / Ca^{2+} exchangers up to nine fold still results in smaller CICR through this system and measures of the Na^+ / Ca^{2+} exchange current itself show that this pathway is around 25% as efficient in its ability to stimulate Ca^{2+} release from the SR (227). On balance, therefore, the evidence argues against a major role for the Na^+ / Ca^{2+} exchangers in beat to beat cardiac Ca^{2+} regulation (227).

The regulation of cardiac muscle contraction also requires a signal to terminate the release of Ca^{2+}, yet the CICR system as described so far indicates a positive feedback mechanism where the release of Ca^{2+} will stimulate further release of Ca^{2+}. Obviously the characteristic small and localized Ca^{2+} sparks seen in cardiac muscle are not the results of uncontrolled releases or cascades of Ca^{2+}. Indeed, CICR is a graded response, which results in transient increases in cytosolic Ca^{2+} that reflect the depolarization of the cell. It has been shown in skinned muscle cells that following depolarization of the cell membrane and Ca^{2+} release there is an inactivation of Ca^{2+} release (67). However the same phenomena has not been adequately demonstrated in other more controlled systems. There is some evidence that the activity or amount of Ca^{2+} release is initially high and then is reduced despite the continued presence of Ca^{2+} (265). It is now recognized that the small co-localized groups of RyR receptors with their closely associated L-type channels represent the basic units of the Ca^{2+} signal or Ca^{2+} sparks. Increasing recruitment of these basic units can allow a graded release of Ca^{2+} into the cardiac cytosol depending on the number and size of the release sites induced (38). The SR membrane system spreads throughout the cardiac myofibrillar apparatus. The Ca^{2+} released across the SR membrane via the RyR acts to trigger the generation of active tension throughout the myocardium by binding to selected Ca^{2+} sensitive proteins

resulting in removal of the inhibitory influence in the myofibrils provided by the troponin-tropomyosin complex.
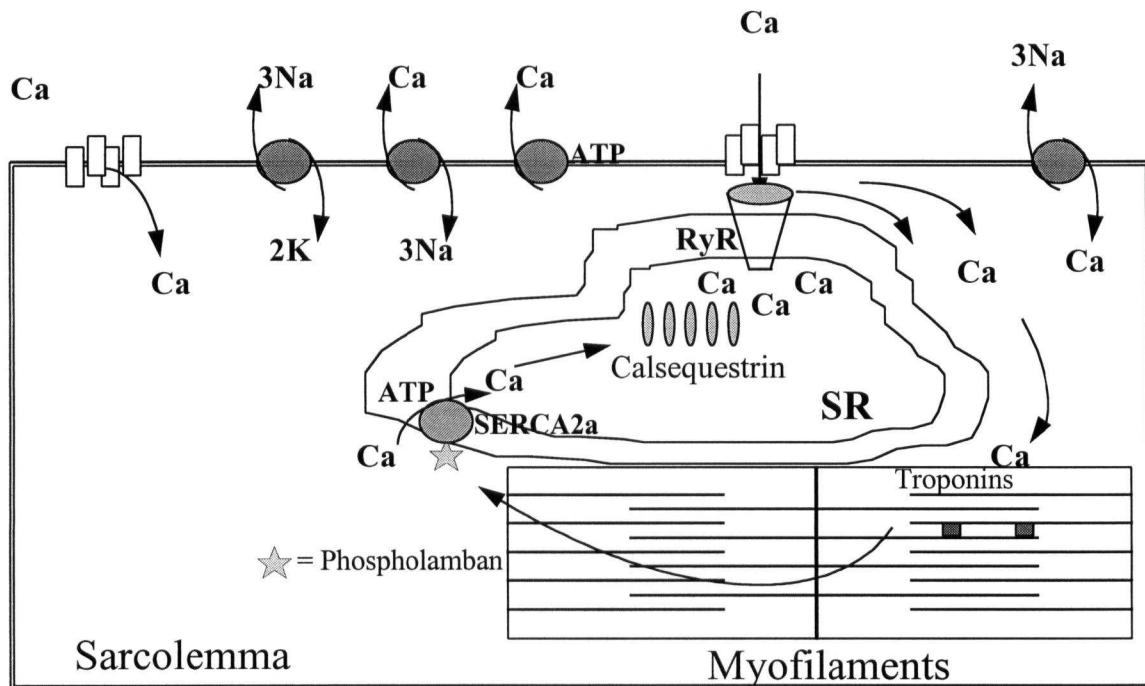
Figure1. Basic schematic of the main Ca^{2+} regulating protiens within the cardiac cell. Ca = Calcium. RyR = Ryanodine receptor. K = Potassium. Na = Sodium. SR = Sarcoplasmic reticulum. ATP = Adenosine Triphosphate. SERCA2a = Sarcoplasmic reticulum calcium ATPase 2a.

Myofilament proteins as regulators of the Ca^{2+} signal.
Increased intracellular Ca^{2+} resulting from action potential driven membrane potential changes is translated into active tension generation and shortening of the muscle through binding of Ca^{2+} to troponin-C (TnC) of the troponin-tropomyosin complex (230), figure 2. The ability of proteins to bind or interact with Ca^{2+} and thereby alter their structure and function depends upon select features that allow the muscle cell to couple Ca^{2+} release from the SR to actual contractile activity (228,277). The troponin-tropomyosin complex of proteins is composed of seven actin monomers with associated tropomyosin and a troponin complex. The troponin complex consists of an inhibitory unit, troponin I (TnI), a tropomyosin binding unit, troponin T (TnT), and the Ca^{2+} binding sub-unit, troponin C (TnC) (228). The tropomyosin (TM) protein lies in the groove formed between the actin helices of the thin filament. TM exists as a two stranded alpha-helical

coiled coil protein. Each TM makes contact with a neighbor in a head to tail overlap at the carboxyl terminal and amino terminal ends of the molecules. The area at around one third of the carboxyl end of the molecule is the site where the troponin complex is anchored to the TM. This binding occurs through close interaction with the TnT protein although TnI can interact with TM at this site also (230). When the levels of intracellular Ca^{2+} are low then TM inhibits actin and myosin interaction by its position on the actin filament. Actin-myosin cross bridges in the relaxed or contracting muscle can be described as being in either a weakly bound or a strongly bound state respectively (77,207). The key step in regulating which of these states prevails is the binding of Ca^{2+} to a regulatory site in the amino terminal domain on the TnC protein which produces altered interactions within the troponin complex. The actin-tropomyosin interaction is altered in response to Ca^{2+} such that myosin and actin can interact strongly and cross bridge cycling occurs (169). The TnC in cardiac muscle has one low affinity Ca^{2+} binding site that binds Ca^{2+} as the triggering event in removal of inhibition of the contractile process. The binding of Ca^{2+} results in an increased affinity of TnI for TnC, moving TnI away from actin-TM. The contractile protein complex is also controlled via phosphorylation of the TnI protein. This can occur through the action of PKA and by calmodulin dependent protein kinase. This phosphorylation results in decreased Ca^{2+} sensitivity of the TnI and increases the rate for dissociation of Ca^{2+} (50,277). The TnT protein has also shown to be phosphorylateable but the physiological significance of this is not clear (116). The maintenance of the integrity of the tropomyosin complex is therefore crucial to maintaining the normal function of the excitation contraction coupling process and ultimately the function of the heart (228,277). The contractile regulation is also subject to modification at the level of the interaction of actin and myosin at the level of the cross bridge cycle. The myosin protein contains six sub-units which include two so called heavy chains (MHC) and two pairs of so called myosin light chains (MLC). The 18kDa "regulatory light chains" are subject to phosphorylation by a Ca^{2+} and calmodulin dependent kinase called myosin light chain kinase (MLCK). Phosphorylation of MLC has been shown to modulate the contractile force generated by cardiac muscle fibres and includes a leftward shift in the force pCa relationship (253). The exact role that MLC and its phosphorylation plays is thought to fine tune contractile

activity however the role of abnormal MLC or its phosphorylation if any in pathophysiological conditions is unclear.
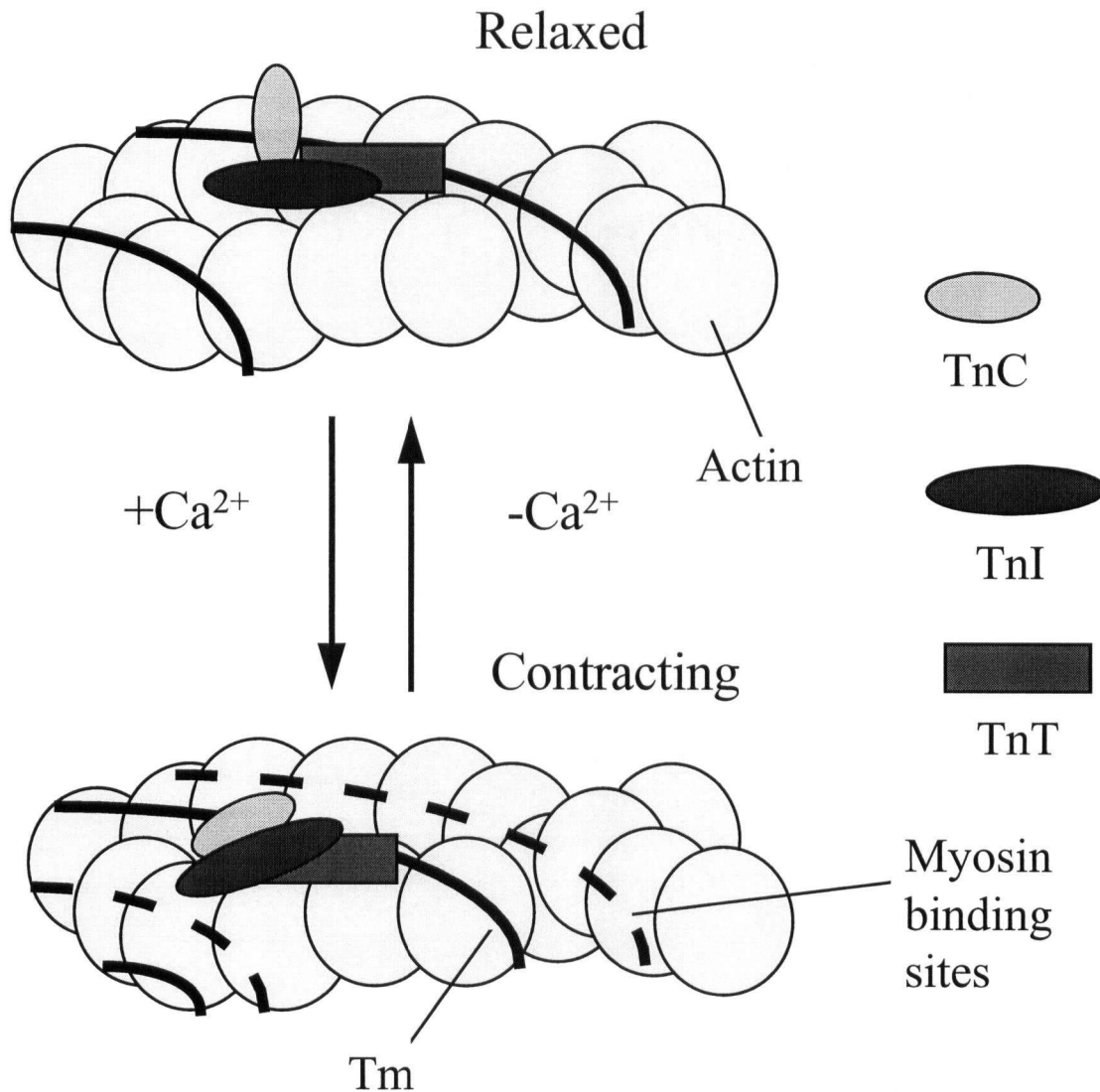
Figure 2. Basic schematic of the thin actin filament associated regulatory proteins and their alterations upon increased $[Ca^{2+}]_i$. TnC = troponin C, TnI = troponin I, TnT = Troponin T, Tm = tropomyosin.

Removal of Ca^{2+} and relaxation.
Muscle relaxation occurs when the flow of Ca^{2+} into the cytoplasmic space is turned off by closure of the L-type Ca^{2+} channels and ryanodine receptors. Ca^{2+} extrusion occurs from the cytoplasm through the Na^+ / Ca^{2+} exchanger and by sequestration into the SR by the Ca^{2+} ATPase pumps (present in cardiac muscle as the SERCA2a isoform) that are

integral parts of the SR membrane (26,78), figure 1. These pumps work to transport Ca^{2+} from an intracellular free concentration of around 0.1-10uM to a concentration of around 1mM in the SR or the extracellular space (136). Two Ca^{2+} ions are transported into the SR for every one molecule of ATP hydrolyzed. Thus the maintenance of Ca^{2+} uptake to facilitate relaxation of the muscle demands considerable energy supply which in skeletal muscle accounts for about 30% of cellular energy requirements during contraction. The SERCA2a activity in cardiac SR is regulated by another protein called phospholamban (PLB). This protein is closely associated with SERCA2a and can be reversibly phosphorylated (125). PLB inhibits the SERCA2a ATPase activity most likely through a direct interaction with the structure of the Ca^{2+} pump. Once PLB is phosphorylated the activity of the SERCA2a is increased primarily due to an increase in its affinity for Ca^{2+}. The importance of PLB is clear as it directs the rate of Ca^{2+} uptake by the SERCA2a ATPase pumps and therefore the rate of relaxation (277). The structure of PLB has not been fully revealed despite considerable research although it is known to exist mostly in a pentameric form made up of five small sub-units that form a 25kDa complex (275). The phosphorylation of phospholamban occurs via the action of cyclic adenosine monophosphate dependent protein kinase (PKA), (126), and Ca^{2+} - calmodulin dependent protein kinase (CaM kinase) (56). The phosphorylation of PLB results in an increase in the rate of Ca^{2+} uptake into the SR. This is thought to reflect increased activity or turnover rate of the ATPase and increased rate of Ca^{2+} transport while preserving the ratio of two moles of Ca^{2+} per mole of ATP hydrolysis (255). PLB phosphorylation is also thought to confer an increased flux of Ca^{2+} from the SR as shown by a reduced level of Ca^{2+} required to trigger Ca^{2+} release from the SR. How this is achieved is unknown but this process or effect has obvious implications for contractile regulation and highlights the diversity of the processes involved in regulating intracellular Ca^{2+} levels (31,188).

The lumen of the SR contains proteins that have low affinity but high capacity for binding Ca^{2+}. They facilitate and maintain the ionic gradient that allows the efficient removal of Ca^{2+} into the SR and subsequent release at the RyR. Calsequestrin is a 45kDa protein which is thought to bind Ca^{2+} at acidic residues located all along the molecule but especially concentrated towards its carboxy terminal. Calsequestrin can bind 20 to 40

moles of Ca^{2+} per mole of protein (136). It is found mostly at the terminal cisternae of the SR in close association with the junctional face of the SR and it may even exist in a crystalline form, especially when highly loaded with Ca^{2+} as this is known to allow a degree of cross linking between the molecules. The majority of SR Ca^{2+} is known to be associated with calsequestrin in these areas of SR and as such, calsequestrin accounts for most of the SR's Ca^{2+} storage capacity (136). This location of the Ca^{2+} store is ideal for the process of rapid release from the Ca^{2+} release channel (206). The sequestration from the SR lumen to a location at or near the junctional SR sites also reduces the concentration gradient against which the Ca^{2+}-ATPase must transport Ca^{2+} from the cytosol of the cell into the SR. The importance of calsequestrin has been demonstrated by its overexpression in mice. This overexpression resulted in cardiac hypertrophy accompanied by induction of a fetal type of gene expression and reduced contractile function associated with increased Ca^{2+} storage capacity but decreased CICR and an impairment of Ca^{2+} release (206). Another protein found in the lumen of the SR that binds Ca^{2+} is calreticulin, this 44 kDa protein is thought to be the non-muscle equivalent of calsequestrin (71). Despite a lack of obvious sequence homology with calsequestrin, calreticulin seems to be structurally and functionally similar. As with calsequestrin, the acidic residues more prevalent at the carboxyl terminal end are responsible for the Ca^{2+} binding and the molecule can accommodate around 25 moles of Ca^{2+} per mole of protein (136).

Other mechanisms of Ca^{2+} removal in the myocardium include the sarcolemmal Ca^{2+} pumps, but this contribution is small in comparison to that provided by the SR Ca^{2+}-ATPase and so these alternate systems probably do not play a significant role in beat to beat regulation of myocardial Ca^{2+} levels (130). Another sarcolemmal Ca^{2+} regulatory mechanism is the Na^{+}- Ca^{2+} exchanger. This mechanism contributes significantly to relaxation by extruding a single Ca^{2+} ion for each Na^{+} ion using the electrochemical gradient as a driving force and produces a net inward current. This system can also be reversed when the intracellular potential changes during an action potential (78).

Growth of cardiac muscle.

Development of skeletal and cardiac muscle begins with the proliferation of pre-myoblasts, which are non-differentiated precursor cells. These cells are derived from the precardiac splanchnic mesoderm and have no apparent identifiable characteristics that would set them apart from other types of proliferative cell types (104). During early myogenesis they become recognizable as muscle cells due to the appearance of cellular constituents characteristic of muscle. The cells elongate and the newly formed contractile proteins begin to provide the striated appearance typical of skeletal and cardiac muscle and allow contractile activity to occur. Cardiac myocytes retain mitotic activity at birth but rates of cell divisions begin to decline late in the embryonic period and continue post partum. Generally about only 2% of rat myocytes are still dividing in the newborn. In the first 3-4 weeks there is an increase in cell number and then because myocytes no longer retain an ability to proliferate the heart grows by enlargement of individual cells with typical myocyte diameter increasing from 5um at birth up to 14-18um in adult heart. Cardiac muscle does not have any myogenic precursor cells such as satellite cells that are found in skeletal muscle. The increase in size of the heart during the first several weeks after birth therefore reflects some hyperplasia but mostly hypertrophy of individual cells. It is generally thought that cardiac cells do retain some ability to produce DNA. The significance of this is not clear but in times of hypertrophy and growth of the heart there are examples where increased DNA synthesis occurs as in response to tumor promoting agents and growth factors (104).

Stimuli that induce growth of the heart are numerous and act through two general types of mechanism. The first mechanism acts in response to signals extrinsic in origin to the myocardium, these include vasoactive peptides, endothelin-1 and angiotensin-II, peptide growth factors such as insulin like growth factor-1, cytokines such as cardiotrophin and adrenergic agonists. The second type of hypertrophic mechanism can be classed intrinsic with hypertrophy occuring in response to signals originating within the myocardial cell. The main example of an intrinsic regulator of hypertrophy is intracellular Ca^{2+}. However, the process of Ca^{2+} mediated signaling is still unclear despite mechanisms such as calcineurin/calmodulin, PKC activation and other signals that result in increased protein synthesis. It is clear that extrinsic and intrinsic

mechanisms are not mutually exclusive. Stimuli that induce increased protein expression in the cardiac cell are numerous and the pathways or systems that translate the intracellular messages are even more diverse. Current articles dealing with the topic give a more detailed discussion than presented here (63,284). I will attempt to give a brief overview, but this is by no means meant to be an exhaustive review. I hope to summarize some of the more well characterized pathways involved and thus allow more meaningful reference to these systems during the discussion of the hypertrophy process as a whole. A general overview of the signaling systems used to alter protein synthesis is presented in figure 3.
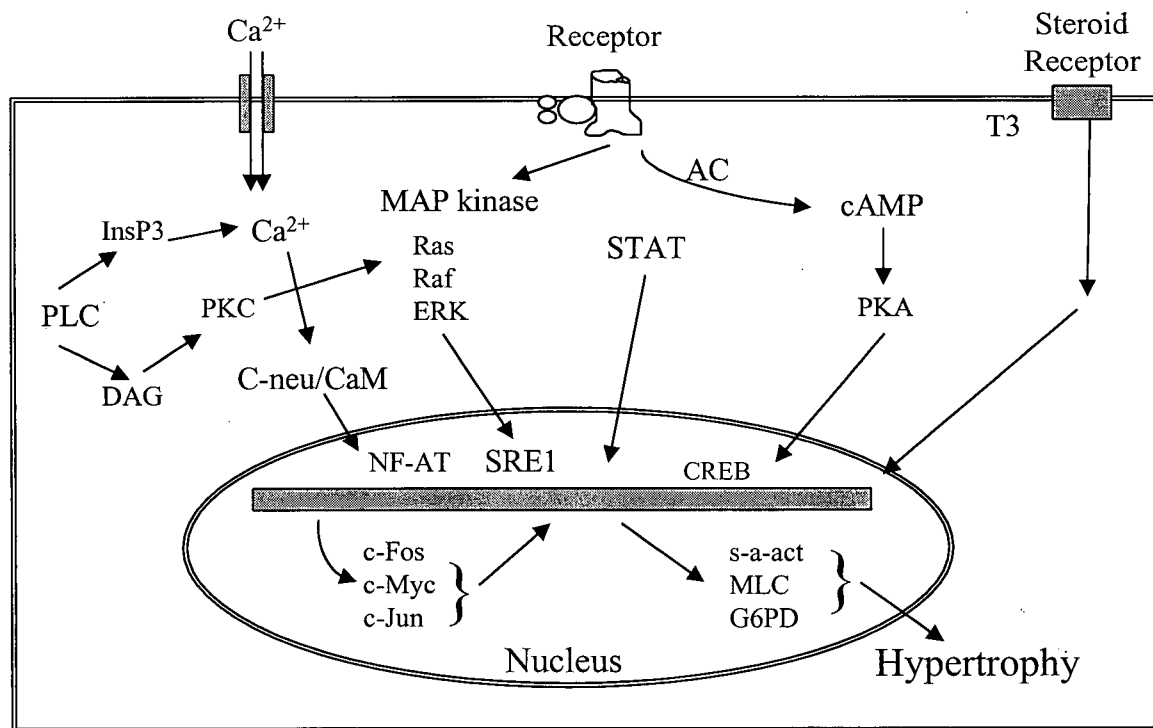
Figure 3: General summary of cardiac muscle signaling components involved in regulating protein synthesis. AC - adenylate cyclase, cAMP – cyclic adenosine monophosphate, PKA – protein kinase A, CREB – cAMP response protein binding element, T3 – thyroid hormone, MAP kinase – mitogen activated protein kinase, PLC – phospholipase C, InsP3 – Inositol triphosphate, DAG – diacyl glycerol, PKC – protein kinase C, C-neu/CaM – calcineurin/calmodulin, NF-AT – nuclear factor of activated T cells, STAT - signal transducers and activators of transcription, SRE-1 – serum response element 1, s-a-act – skeletal alpha actinin, MLC – myosin light chain, G6PD – glucose 6 phosphate dehydrogenase.

The response of the heart to hypertrophic stimuli does not simply involve a global increase in the amount of all constituent proteins of the cell. Cardiac cells respond with a defined pattern of gene expression depending on the circumstances encountered (216). Signals can activate transducing kinases that translocate to the nucleus of the cell where they act by altering the DNA-binding characters or transactivation functions. An example of this kind of pathway is the mitogen activated peptide (MAP) kinases (32). Membrane receptor or membrane associated kinases can also act to directly phosphorylate transcription factors that translocate to the nucleus leading to activation of selected genes. Examples of these are the signal transducers and activators of transcription (STAT) proteins (166, 241). Transcription factors can also be released from a cytoplasmic anchor or complexed proteins and translocate to the nucleus where they can activate gene expression by binding to specific target sequences. Examples of these types of mechanisms are nuclear factors kappa-B (NF-κB), and nuclear factors of activated T cells (NFAT's).

It is recognized that the current interest in the Ca^{2+} / calmodulin (CaM) dependent protein phosphatase, calcineurin is great and that focus on this pathway would be an extensive task. Therefore the reader is referd to two recent reviews that discuss the role this hypertrophy pathway is proposed to have and how it may interact with the other signalling pathways known to date (166, 241). This pathways is interesting in that it can provide a nice mechanistic link between elevated Ca^{2+} levels and the actual processes of increased protein synthesis. The focus of this thesis however is the calpain proteolytic system as an initiator and mediator of hypertrophy and muscle adaptation. It is recognised that these tow pathways or systems may well be interlinked and fact that calcineurin is a substrate for calpain at least in vitro is intriguing (270). Especially significant is the data showing that proteolysis of calcineurin in vitro results in an increase in its activity. The ability of calcineurin to dephosphorylate the NFAT proteins allows them access to the nucleus where they can interact with the AP-1 complex and influence hypertrophy effects. The end result of nuclear activity of such agents is an increase in protein synthesis coupled with a re-selection of genes specific to the hypertrophy model encountered determined by the increased expression of proteins initiated at the nuclear level (34,63,217). The transcription factors mentioned earlier,

such as c-fos are nuclear proto-oncogenes which activate genetic expression in the so called "early response" which determines the initial gene expression after stimuli as diverse as, neurotransmitters, phorbol esters, cytokines growth factors and Ca^{2+} ionophores. The control of this process is complex and involves cis-acting regulatory DNA elements upstream form the promoter sequence which include the serum response element (SRE) and the c-Sis (platelet derived growth factor)-inducable element (SIE). The SRE is a set of sites that bind transcription factors, SRE is found associated with a dimeric protein called a serum response factor (SRF). The SRE can bind several proteins of various oncogene families, one such protein that seems to play an important role in the function of gene regulation as part of the SRE-SRF complex is Elk-1. This complex seems to be the group of elements required by c-fos to modulate signals via the MAP kinase pathway (63). In comparison the stat-proteins are a more directly signaled group with few intermediates. Translocation of specific kinases to the nucleus no doubt presents interesting organizational and spatial challenges and most likely relies on nuclear localization signals, shuttle proteins or chaperones that assist with the translocation to the nucleus.

Obviously a large degree of cross talk is possible between intracellular kinases and phosphorylatable proteins (63). Selective expression of one or more cardiac proteins results from factors such as hormonal stimulation, altered patterns of innervation and changes in loading or contractile requirements (63). The responses or alterations of the myocardial cells are temporally quite characteristic of the conditions encountered with up, and down regulation of various genes. The initial reaction is a transient initial up regulation of the "immediate" or "early response genes" such as c-fos, c-jun, erg-1 and c-myc. Regulation of c-fos transcriptional regulation occurs very rapidly, peaks at around fifteen minutes and is quickly turned off. These early response transcription factors are thought to be important in regulating increased expression of myofibrilar proteins as shown in figure 3 (34). These genes include those coding for beta-myosin heavy chain (β-MHC) (109), skeletal alpha-actinin (216) and atrial natriuretic factor (ANF) (150). In the following 12-24 hours in models that cause "pathological" hypertrophy development, often the alterations in gene expression reflect a return to the patterns seen in the early stages of development (34). This indeed may reflect the re-expression of a fetal

"programme" of gene expression (29,34). From 24-48 hours, other genes are upregulated while some hypertrophy conditions result in down regulation of certain proteins such as the SR Ca^{2+} ATPase (58). The proteins recognized so far that are up regulated fit into three general categories, oncogenes that act as transcription factors, structural and functional proteins such as β-MHC, and extracellular signaling factors such as atrial natriuretic peptide (ANP), or transforming growth factor beta (TGF-β). Such growth factors are peptides that can regulate cell proliferation and differentiation (212). These factors such as endothelial growth supplement (EGS), thyroid hormone, epidermal growth factor (EGF), insulin, bFGF, and IGF, stimulate myocyte proliferation in vitro.

The alterations of the "late response genes" define the response of the heart in terms of functional alterations to the hypertrophy stimulus. It is also clear that specificty of the hypertrophy model can be expressed in terms of the timing, extent, and magnitude of protein expression changes. Therefore the variety in responses seen in response to hypertrophy inducing models while being somewhat stereotypic can also be quite different in terms of functional outcomes of the myocardium. For example, thyroid hormone will increase the expression of the α-α isoform of myosin heavy chain (V1), while pressure overload hypertrophy results in an increased expression of the β-β form (V3), (110). The response of the cell to β-adrenergic agonists involves G-protein mediated stimulation of adenylate cyclase. This produces cAMP and activates the protein kinase A (PKA) which then phosphorylates various transcription factors including the so called cAMP binding element (CREB) (81) and the activating transcription factor (ATF) family that are bound at the CREB's. Other signal pathways such as used by steroid hormones and thyroid hormones can pass freely through the nuclear membrane so they have no need for cytoplasmic messenger proteins and they act directly on nuclear receptors to initiate alterations in gene expression (205). The systems in place to regulate the responses of the cell to external stimuli are open to modulation at many levels by the intracellular environment and cross talk from different pathways. Thus the elucidation of the exact triggers and signaling components that drive the hypertrophy of cardiac muscle is far from simple but holds great potential for interventions that may alleviate and prevent cardiovascular disease states.

Also of interest is how these intracellular signals are turned off. Currently it seems attractive to have appropriate phosphatases induced in response to gene upregulation in order to de-phosphorylate the transcription factor and also the activating kinase in question thereby turning off the signal.

Mechanisms of cardiac hypertrophy.

The heart has the ability to sense an increased workload or demand, then via various signaling mechanisms, activates increased protein synthesis while continuing to meet the new increased workload or demand (as described above). However if the demand or stimulus is maintained then this initial adaptation can be continued into a state where the function of the heart is compromised leading eventually to failure. The growth of the heart in response to "hypertrophy stimuli" consists of an initial or primary overall growth that increases proportionately the mass of the heart. A secondary or related response seems to act in a gene specific nature resulting in selective expression of individual genes and ultimately protein expression profile and altered structure and function. The nature of these changes and the rate at which they occur also depends on the type of stress or demand placed on the heart. A significant challenge therefore is to characterize the response of the heart to the different types of "hypertrophy" experienced and ultimately characterizing the underlying causes. In order for the heart to grow there has to be a net increase in protein content therefore most likely an increased capacity for protein synthesis is required.

Protein synthesis capacity in the heart.

A common factor in all forms of cardiac hypertrophy by definition is increased protein accumulation. Generally this is the result of increased rates of protein synthesis while rates of protein degradation are not altered significantly. As approximately 90% of cardiac cellular ribosomes are thought to participate actively in normal protein synthesis and are thought to operate at or near full capacity, then it follows that any hypertrophy process requires increased expression of ribosomal complexes in order to produce the proteins required by the heart (94). Indeed the early increase in total cellular RNA in many forms of hypertrophy reflects mostly increases in ribosomal RNA as this form represents approximately 90% of cellular RNA (49). Even this increase in protein

synthesis capacity is far from understood as the molecular signals that regulate the rDNA transcription and the factors that constitute the complete ribosomal RNA polymerase complex are still not completely clear (94,95). As mentioned earlier, the response of the cardiac cell to hypertrophy is specifically regulated as demonstrated by the expression and re-expression of different isoforms of the same proteins.

Isoform expression in cardiac muscle.

The myofibrillar apparatus of the heart is composed of many proteins each with specific roles and functions. Many of these proteins of the myocardium can exist as more than one isoform (28), the regulation and functional importance of which is not well understood despite extensive study. This does however confer a great deal of plasticity to the myocardium in terms of adaptive responses where different isoforms of proteins can be preferentially expressed and the subsequent alterations result in an adaptive change that is meant to allow the heart to meet the new demand. The regulation of isoform expression in some cases reflects alternate gene expression (36) and in others is due to alternative splicing of the RNA (54). One protein that has been well characterized is myosin heavy chain, which can exist as either α or β forms associated as dimers. There are α-α (V1), α-β (V2), and β-β (V3) myosins with the α and β myosin proteins being the products of two different genes. The functional significance of these altered isoforms are evident when it is considered that V1 has higher myosin ATPase than V2 which itself has higher myosin ATPase activity than the V3 isozyme (277). The expression of myosin heavy chains vary with body size and animal species. Generally larger species studied express predominantly the β isoform homodimer (V3), and therefore have lower intrinsic ATPase activity. In very small mammals such as mice the α heavy chain is expressed, resulting in a predominant V1 myosin type. Between these two examples are animals such as rats and rabbits where the ratio of isoforms themselves can vary with young adult rats expressing mostly V1 and neonates or very old rats showing some expression of V2 or V3 (133,217). Different isoforms in the heart allow it a remarkable ability to change throughout various developmental stages and under conditions such as increased or altered demand and hormonal stimulation. In the rat, all three isoforms are present to some extent in the fetal heart but are mostly β-MHC. After birth V1 becomes

the dominant form. This coincides with increased plasma levels of thyroid hormone, however late in life in the rat the V3 isoform increases again despite continued normal adult levels of thyroid hormone. As we can see the regulation of myosin isoforms is complex and often correlates to hormonal levels or imposed workload resulting in alterations in aspects of myocardial functioning. The direction or suitability of the prolonged changes are usually deleterious in pathological models of hypertrophy. For example, the responses to conditions such as haemodynamic overload with aortic constriction and in spontaneously hypertensive rats is a change towards the V3 isoform with a decrease in V1. In contrast to this, the intermittent increase in demand placed upon the heart by physical exercise results in a shift towards V1 although only swim exercise induces this effect and not running (153). This is interesting as both kinds of exercise have been shown to normalize blood pressure in spontaneously hypertensive rats therefore exercise induced alterations of myosin type does not seem dependent on haemodynamic load or at least not exclusively. The degree of change seen is approximately a 10% increase in V1 with swim exercise and a 30% increase in V3 following aortic stenosis (110). This also reflects the fact that the original composition in rat cardiac muscle is mostly V1 and therefore the potential for change is greater in the models that cause a shift towards V3 myosin, this is called the "potential adaptive range" (110). Again this highlights the importance of the model studied when drawing conclusions from data such as with human ventricular myosin which contains a greater proportion of V3 than rat which contains more V1.

Myosin light chains also exist as different isoforms with different forms playing a role in determining the function of cardiac muscle (154,207). The two pairs of light chains present in cardiac muscle are the "essential" MLC-1, both atrial, ALC-1, and ventricular VLC-1 and "regulatory" MLC-2 light chains. The precise mechanism of light chain influence on contractile regulation is not clear but it is possible that interaction of the light chains with actin can influence the regulation of the thin filament associated proteins (190). In hypertrophy, ALC-1 partially replaces VLC-1 which confers faster cross-bridge kinetics and a greater Ca^{2+} sensitivity of force generation.

Proteins of the SR that handle Ca^{2+} and associated regulatory proteins also exist as different isoforms expressed across tissue types. The SR Ca^{2+} release channel exists as

three different isoforms. There is a skeletal muscle isoform, RyR1, a cardiac isoform, RyR2, and a third isoform that has functional properties different from both RyR1 and RyR2 (137). The SR Ca^{2+} ATPase pumps have been found to exist as 4 different isoforms. SERCA1a is found in adult fast twitch skeletal muscle, SERCA1b is found in neonatal skeletal muscle. SERCA2a is expressed in slow twitch skeletal and cardiac muscle and a SERCA3 isoform is found in a variety of muscle and non-muscle tissues (89). Only one isoform of the regulatory protein phospholamban that is associated with SERCA2a has been recognized and is found in cardiac and slow skeletal muscle (125). Calsequestrin, the main Ca^{2+} binding protein of the SR exists as a skeletal muscle isoform and a cardiac isoform (205).

The proteins of the troponin tropomyosin complex exist in various isoforms as well. The troponin-C protein exists as a skeletal muscle and a cardiac/smooth muscle isoform. The two are thought to differ in their Ca^{2+} sensitivity with the cardiac isoform being more sensitive to Ca^{2+} (90). The inhibitory member of the troponin complex, TnI, has been found as fetal and adult forms in cardiac muscle with only the adult form persisting into adulthood (200). Tn-I also exists as an adult skeletal fast isoform and a slow muscle isoform. The fetal isozyme shares identical immunoreactivity with the slow skeletal form. Adult cardiac Tn-I differs from both the skeletal forms in that it contains extra amino terminal residues that allow it to be phosphorylated. This decreases its sensitivity to Ca^{2+} (277). Phosphorylation occurs via a calmodulin-dependent and cAMP dependent protein kinases and the fact that fetal heart is fairly insensitive to β-adrenergic stimulation is likely due to the fact that the skeletal TnI lacks the phoshphorylateable residues of the adult isoform (200). Various TnT isoforms in cardiac muscle have been demonstrated but the functional significance of these is still not fully resolved (3). Alternative RNA splicing of a single gene regulates the expression of cardiac Tn-T isoforms (cTnT) (112). Four cTnT isoforms exist, of which two are expressed as adult forms and two are expressed as embryonic isoforms. The adult forms are smaller than the embryonic proteins, reflecting the additional NH2-terminal sequences present in the embryonic forms. These may also confer the increased Ca^{2+} sensitivity found with embryonic cTnT as this end of the cTnT protein is thought to interact with the other components of the troponin-tropomyosin complex (143). The significance of isozyme

alterations during hypertrophy are likely to become clearer as more advanced methods of isoform substitution and more sensitive measures of protein function are studied within different hypertrophy models.

Mechanical stretch in regulation of gene expression

Many genes are known to respond following application of mechanical stress in cardiomyocytes (124). These genes can be classified into two main groups that have been alluded to earlier, namely, those that respond early and are called the immediate early response genes (IEG's) and those who are altered with a slower time course and called the late response genes. For example by stretching rat cardiomyocytes on deformable dishes it was found that the IEG's, c-fos, c-myc, c-jun and Erg-1 were expressed in a length dependent manner (283). The expression of c-fos was evident within 15 minutes and peaked at 30 minutes. This is thought to be the earliest of the gene expression markers in response to mechanical deformation of the cardiomyocyte. Similar increases in these early response genes were found following isoproterenol injection (34) where levels of the IEG's were increased within 30 minutes and peaked at four hours into an isoproterenol infusion protocol. Among the late response genes induced are the MHC isoforms. The stretch of cultured cells results in V3 myosin expression and V1 to V3 transition of the myosin isoforms decreases the ATPase activity which increases the efficiency of contraction for a given work done. This change is thought to be a suitable adaptation to a chronic overload (129). Both the smooth and skeletal α-actin mRNA levels are increased in stretched cardiomyocytes. Atrial natriuretic factor (150), β-tropomyosin (109), and atrial myosin light chain-1 (198) are also up regulated and these changes mimick embryonic development. Some genes are down-regulated by haemodynamic overload including the SR associated protein phospholamban (PLB) and the Ca^{2+} ATPase pump of the SR (SERCA2a) (239). The SERCA2a seems to be decreased gradually in animal models of pressure overload. Some in vitro studies also show that expression of Ca^{2+} ATPase in cardiomyocytes is reduced after increasing mechanical stress. It has also been demonstrated that an initial response (1 day) after increased mechanical stress with pressure overload hypertrophy results in increased SERCA2a mRNA, however as hypertrophy becomes more pronounced the levels are then

reduced (69). The effect of reduced SERCA2a is typically seen in experimental settings as a decreased ability of cardiac muscle homogenate or isolated SR to take up Ca^{2+} (58,131).

Cytoskeletal changes.

There is a two set system of cytoskeletal intermediate elements, the exo- and endoskeleton. The exoskeleton component is composed of the intermediate filaments (IF's), desmin, vimentin, alpha-actinin and synemin and is important in maintaining the lateral register appearance of striated muscle cells (262). Desmin is a protein filament that is hypothesized to inter link myofibrils, whereas other filaments such as vimentin and synemin, form a honeycomb-like network within the plane of the Z-line structures. The filaments thus play a role in the maintenance of longitudinal Z-line spacing. These protein filaments continue their attachments with sub-sarcolemmal cytoskeletal networks, such as spectrin, to form a complete scaffold of cytoskeletal elements within striated muscle. The presence of sub-sarcolemmal actin filaments and their networks are functionally linked to endocytosis, receptor stabilization and cell membrane fusion (262). Cytoskeletal components, intermediate filaments (i.e. desmin) and microtubule filaments, (262) undergo a re-organization in response to pathologic cardiac hypertrophy. It has been noted that there is a greater degree of disorganization early on in the hypertrophy process, the functional significance of these changes has attracted attention but are still unclear (51,118,273). Immunoelectron microscope studies revealed that desmin is particularly vulnerable during the development of pathologic cardiac hypertrophy (273). In addition cardiac hypertrophy is associated with Z-line streaming, which has been postulated to be the result of alpha-actinin removal (22).

The role(s) of the intermediate filaments such as vimentin and desmin may also extend beyond a purely structural one. Intermediate filament (IF) proteins have been proposed to act like transcription factors and participate in the activation of nucleosomes (263). Indeed, these intermediate filament proteins often contain alpha helical dimerization interfaces and a basic DNA binding region and all non epithelial IF proteins tested (including desmin and vimentin) have been found to be nucleic acid binding proteins (263). Indeed the activation of such transcriptional ability of the IF's has been suggested to be achieved by limited truncation by proteases (160). It was hypothesized

that this may regulate the release of partially degraded subunit proteins from the insoluble IF's, which as soluble ssDNA binding proteins could enter the nucleus and regulate gene expression (160). Up to 5 proteins of varying molecular weights with growth inducing ativity in cultured myocytes were purified from the myocardium of spontaneously hypertensive rats (155). A soluble protein growth factor was also identified in spontaneously hypertensive rat myocardium and suggested to play an important role in the pathogenesis of cardiac hypertrophy (219). So the cytoskeleton of the cardiac cell is critical for maintenance of cell integrity, force transmission (240), and possibly acts to transduce mechanical signals into growth regulating intracellular signals.

Effects of hypertrophy on expression of Ca^{2+} handling proteins.

The importance of maintaining close regulation of intracellular Ca^{2+} signaling is of paramount importance to cardiac muscle (26,60,64). The fact that Ca^{2+} handling proteins, and the intracellular cycling characteristics of Ca^{2+} (245), are altered in hypertrophy and eventually heart failure necessitates a great deal of research interest (9). The duration of the intracellular Ca^{2+} transient is prolonged in cardiac muscle from heart failure patients and in animal models of hypertrophy (8). Despite many studies focusing on the role of Ca^{2+} channels in the hypertrophy process, there are few clear and accepted descriptions of the role of these channels during the development of cardiac hypertrophy. The mixed evidence so far includes examples of either increased or decreased densities of L-type channels depending on species and duration of hypertrophy development (9). Again we see that the adaptations or alterations vary according to model and duration. One constant however is that in terms of the electrophysiological signal produced with cell depolarization, then the L-type Ca^{2+} current seen in myocytes from most models of hypertrophy are prolonged. This supports indirectly the data that shows prolonged Ca^{2+} transients although the link between L-type Ca^{2+} current and Ca^{2+} release is not fully resolved.

The studies of whole cell Ca^{2+} levels means that transient and local Ca^{2+} changes are more likely to be overlooked despite the fact that they reflect the underlying mechanism(s) responsible. Some studies have demonstrated that it is likely to be the process of Ca^{2+} uptake by the SR that is compromised as crude SR fractions from SHR animals showed decreased uptake of Ca^{2+} which was more pronounced as the

hypertrophy progressed. There is also evidence of reduced SR Ca^{2+} uptake in heart tissue from patients with cardiomyopathies and decreased mRNA of the SR Ca^{2+} release channel from failing hearts. Generally however the Ca^{2+} transient as a whole are known to initially increase in amplitude in the initial stages of hypertrophy and as the hypertrophy is continued then typically the amplitude of the intracellular Ca^{2+} transients decrease (8). Finally, at or near heart failure it is not uncommon to record increased duration of these lower amplitude Ca^{2+} transients (8,176). It seems likely that the underlying cause(s) of the altered intracellular Ca^{2+} transients could be due to alterations in function of the SR associated Ca^{2+} handling proteins (9). The Ca^{2+} handling mechanisms of the SR are closely linked in terms of the release, uptake, capacity and content of the SR (59,206) with the importance of the appropriate intracellular Ca^{2+} fluxes reflected in the close control of Ca^{2+} levels within the myocardial cell (64). During hypertrophy alterations in Ca^{2+} homeostasis occur to different extents for different models of hypertrophy with the end result of the alterations in SR function and Ca^{2+} handling proteins causing a net redistribution of Ca^{2+} to the cytosol. Interestingly such disturbances in Ca^{2+} handling are not present or are much less prominent with models that lead to more "physiological" type hypertrophy such as are seen with thyroid hormone treatment or swim exercise. This is not the only subcellular difference found when comparing these two types of hypertrophy. There are other alterations that occur differently, the study of which may help define the key signal(s), stimuli, etc, that determine whether growth of the heart will increase functional capacity or will ultimately lead to reduced function and heart failure.

Experimental models of cardiac hypertrophy

Hypertrophy is typically associated with conditions such as chronic hypertension (110), valvular insufficiency (106) hyperthyroidism (123,205), and long term exercise training (211). The process of hypertrophy also occurs in situations where there are increased levels of circulating hormones, neurotransmitters etc (212), including conditions such as increased sympathetic stimulation (91), chronic stress and hypertension associated with high renin levels (247). The most common experimental models involve inducing hypertrophy in small mammals such as rats and guinea pigs and subsequent analyses of the myocardium's adaptive response then employ in situ preparations (111), isolated

heart preparations (2,225), or isolated trabecular strips (258). Alternatively, isolated myocytes may be subjected to hypertrophy inducing stimuli (94,152).

A commonly used model is induction of hypertrophy by a pressure overload. Pressure overload hypertrophy typically results in heart failure and in adult humans is responsible for the majority of cardiac related deaths (75). Pressure overload usually results in a fairly rapid hypertrophy and is dependent to some extent on the degree of aortic constriction. This constriction can be experimentally induced in several ways including physical restriction of the diameter of the aorta at either the ascending aorta (111), the abdominal aorta proximal to the renal artery (2), or the pulmonary artery. Most often a clip or suture is used requiring surgical procedures and possible confounding physiological disturbances. Hearts exposed to chronic pressure overload also have depressed energy metabolism with characteristic decreases in high energy phosphates (226). Decreased mechanical performance is also characteristic of this hypertrophy but not in all situations (110). It seems that the degree and duration of increased stress on the ventricular walls can produce different responses. An example is the increase in myosin ATPase found in mild constriction of the pulmonary artery while more severe stenosis leads to a decreased myosin ATPase activity. Another common hypertrophy model used is the induction of a volume overload on the heart (110).

Another model of hypertrophy commonly studied is induced by chronic elevations in blood pressure (46,110) which leads to pathological type hypertrophy and also often results in heart failure (110). The underlying cause of hypertension induced hypertrophy is subject to considerable debate as the signals, hormones, etc that are involved in the hypertensive condition also stimulate myocyte hypertrophy. Therefore the cause and effect relationship in hypertension remains unclear (83). Models that focus more specifically on the effects of increased stress or tension on the ventricular muscle are frequently studied because regression of pressure induced hypertrophy occurs quickly when the pressure is lowered (46). The mechanism whereby increased pressure or wall tension experienced in the myocardium is translated into a growth response has been studied using mostly isolated or specific models such as isolated myocardial preparations or cell cultures in order to control for influences such as circulating hormones etc (53,94). Some examples have been mentioned previously in regard to isoform expression in

cardiac muscle and include mechanical stress in cardiomyocytes (124) and stretching of rat cardiomyocytes on deformable dishes (129,283).

The responses to the physiological models of hypertrophy show intracellular alterations that do not result in failure or pathological growth. These models, such as exercise induced cardiac hypertrophy and thyroid hormone stimulation actually seem to influence the contractile function of the heart in a positive way. Models used for the study of exercise induced hypertrophy commonly use rats undertaking treadmill running (97), or swimming (210,211). Swimming is the most common form of exercise used, as other modes of exercise do not produce as pronounced a hypertrophy. In humans it remains controversial as to whether there is a significant hypertrophy although some degree of cardiac enlargement does seem to be associated with prolonged exercise training (175). Exercise induced hypertrophy shows a "natural" growth in proportion to the increased mass of the heart. The temporal response to a swim exercise programme was studied by Hickson et al who showed that total protein levels increased within two days of the exercise programme starting and this increase in total protein reached a plateau at 14 days (99). RNA concentration is increased at 24 hours and is detectable before any increased weight of the heart becomes apparent. There is also a slight increase in connective tissue content of the heart associated with swimming induced hypertrophy reflecting a more "natural" pattern of muscle growth. In terms of functional effects of exercise conditioning the data generally suggests that there is an adaptive increase in myocardial contractility although this is not always demonstrated (210). Increased contractile ability is often best demonstrated or becomes significant only under conditions of increased cardiac work (211). Repeated exercise or training is also known to increase the contractile protein ATPase activity in rats (27), which is in direct contrast to pathological types of cardiac hypertrophy where the myosin ATPase activity of the myofibrils are decreased (276). Another subcellular alteration that underlies the improved function and adaptation of the heart to increased demand is the modification of Ca^{2+} handling properties of the myocardial cell. Training with swimming exercise leads to an increased Ca^{2+} uptake and binding by the SR (172). This increase in Ca^{2+} uptake by the SR is found to a greater degree with swim exercise training as opposed to running training (171). The effects of exercise training on the Ca^{2+} handling ability of the

sarcolemmal membrane has also been studied and generally the evidence is not as striking as it is for the SR. However there have been slight increases demonstrated in sarcolemmal Ca^{2+} pump activity following swimming in rats (179), and a possible increase in Ca^{2+} binding capacity of the sarcolemmal membrane following training (259). These alterations in Ca^{2+} handling are thought to explain the enhanced relaxation rates demonstrated by cardiac muscle from exercise trained rats. Another physiological hypertrophy inducing stimuli is increased exposure to thyroid hormone.

Administration of thyroxine to rats increases their overall oxygen consumption, elevates cardiac function and heart rate (165). The accompanying cardiac hypertrophy is evident within four days (225). Most of the known effects of thyroid hormone are thought to be due to direct effects on nuclear receptors that either inhibit (47) or promote specific gene transcription (205). The metabolic state of these hearts is not compromised (226), with a growth that seems uniform with little connective tissue increases or myocardial stiffening. The functional alterations seen with this model of hypertrophy are positive, showing increases in contractile function as measured by in situ measures (110,289), isolated left ventricular muscle (110), and myosin ATPase (226). There is a corresponding increased proportion of the V1 isoform of myosin (110). This model of hypertrophy is a good example of "physiological cardiac hypertrophy". The adaptations seen ultimately enable the heart to meet increased demands yet still maintain its viability and avoid the deleterious effects associated with the other more pathological models of cardiac hypertrophy such as with β-adrenergic agonist induced hypertrophy.

One such model that induces a pathological type response when sustained is isoproterenol injection or infusion (34,155,289). A single initial injection of approximately 1-10mg per kg body weight will result in a drop in mean arterial pressure of around 40mmHg, which recovers to control values in about 5 hours. Myocardial ATP content is depressed following isoproterenol injection and higher doses of isoproterenol will induce myocardial lesions (197), therefore the remaining myocardium may have to work more to compensate for damaged tissue. Studies employing isoproterenol are numerous and yet the exact mechanisms of how this non-specific β-adrenergic agonist induces hypertrophy are still unclear (25,85,177,264). Typical responses to isoproterenol include peak hypertrophy effects at around three days after acute injections or into

perfusion studies (25,197). The mechanism of action usually attributed to causing hypertrophy is that of the interaction of isoproterenol with β-adrenergic receptors resulting in a signal transduction cascade through G-protein activated adenylate cyclase to produce cAMP which activates PKA which in turn phosphorylates various protein substrates within the cell (66). These substrates include proteins involved in contractile function (98), ionic regulation (254), and gene expression (34), which increases the expression of myocardial proteins typical of the hypertrophying cardiac cell. However this pathway is not the only way isoproterenol produces effects within the cardiac cell. An alternative pathway is thought to reflect the direct activation of L-type Ca^{2+} channels to increase Ca^{2+} influx into the cell (285). The β-adrenergic blocker propranolol is known to block many, but not all, of isoproterenol's effects. For example, propranolol was shown to block the effects of chronic isoproterenol infusion on decreasing SERCA protein levels in rats but did not prevent the increased expression of skeletal alpha-actinin (33). Further support for an alternate pathway of isoproterenol action comes from Bishopric et al who conclude that skeletal alpha-actin expression in response to isoproterenol requires Ca^{2+} entry and release from the SR independent of the PKA pathway (29). Hansen and Rupp also have suggested that a Ca^{2+} dependent pathway can account for the hypertrophy induced by isoproterenol and that PKA is not critical (96). The exact nature of this Ca^{2+} dependent pathway is unclear but holds significant interest for the elucidation of how the cardiac hypertrophy process is regulated.

It is clear that hypertrophy does not absolutely require increased work or elevated mechanical stimuli, and the heart also does not absolutely require hormonal or membrane receptor mediated stimulation for hypertrophy but it has been difficult to dissociate the importance of each in vivo. Often the intracellular pathways stimulated are quite similar and are no doubt shared to some extent. It is important to remember that the heart is made up of a variety of cell types such as vascular, neural, interstitial, and endothelial cells. Therefore the responses of even isolated whole hearts reflects effects of the cardiac system and not just purely the cardiomyocyte. Clearly cardiac hypertrophy reflects a complex process of myocyte enlargement and remodeling of the extracellular matrix. This is why some researchers advocate the use of cell cultures where control of the extracellular environment and the stresses placed on the cells are much easier to control

(240). Despite these varied responses to different models of hypertrophy there seems to be a general delineation between pathological and physiological types of hypertrophy. These classes of hypertrophy will be discussed briefly below.

Physiological & pathological hypertrophy.

Cardiac hypertrophy can result from situations considered physiological or pathological in nature as reported above. Pathological situations include hypertension, valvular heart disease or in compensation for loss of heart tissue due to myocardial infarction. Pathological correlates seen with prolonged or excessive pressure overload or chronic catecholamine stimulation include alterations in chamber geometry, decreased capillary to muscle ratio, increased connective tissue content, and depressed contractile function. Growth is detrimental where pathologic cardiac hypertrophy is accompanied by a decreased functional capacity (155). Knowledge of the fundamental mechanisms that regulate cardiac growth during normal function and those situations where hypertrophy occurs is essential if treatments for the deleterious effects of cardiac hypertrophy are to be developed.

Alterations in hypertrophied cardiac muscle fibers are not restricted to cell size but also numerous functional changes have been reported. Functional alterations of the surface membrane and SR membrane influence the action potential and excitation-contraction coupling characteristics of the muscle. While physiologic hypertrophy does not alter the action potential, the myocardium undergoing pathologic hypertrophy shows marked prolongation of the action potential and incomplete relaxation time (110). This adaptive change facilitates an increased influx of Ca^{2+} into the myocardium. This is necessary for maintaining contraction in the larger hypertrophied heart (9). On the other hand, physiological hypertrophy such as with swim exercise training is not accompanied by such an unattended elevation in intracellular Ca^{2+} for maintenance of contraction. In studying the effects of various hypertrophy models mentioned previously, the basic difference in the response to physiologic and pathologic cardiac hypertrophy is a pronounced elevation in Ca^{2+} influx into the cell during pathological hypertrophy.

Changes in the Ca^{2+} handling characteristics are likely involved in hypertrophy induced contractile changes such as decreased active tension development, decreased velocity of shortening and increased time to peak force. As the sarcolemmal membrane is

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ultimately responsible for the Ca^{2+} movements into and out of the cell then modifications of sarcolemmal structure or function could alter the Ca^{2+} levels in the cell and the force generation. Prolonged action potentials are found in pathologically hypertrophied hearts (176). In a survey of the literature available it seems that decreases in the abundance of the L-type Ca^{2+} channels are only significant in more advanced or pathological cases or examples of hypertrophy (156). Loss of Ca^{2+} homeostasis in severe hypertrophy or failure could be due to loss of integrity of the proteins associated with CICR and a loss of their ability to interact efficiently. Brooksby suggests that the prolonged action potentials seen in SHR cells results in an increase in the Ca^{2+} content of the SR in comparison to normal control myocytes (35). The effects of inducing a physiological hypertrophy generally show an increased ability of the myocardium to remove Ca^{2+} from the intracellular space. In physiological hypertrophy the main response seems to be one of increased Ca^{2+} uptake and removal as seen in the quicker rates of relaxation demonstrated in exercise trained myocardial cells (110). A change in extracellular Ca^{2+} delivery to exercise trained myocardium was suggested with increased passive Ca^{2+} binding by the sarcolemmal membrane along with an increased membrane phospholipid content from exercise trained myocardium (259). Sarcolemmal Ca^{2+} pump activity was found to be significantly increased by Pierce et al (177) in the myocardium from rats trained by swimming exercise. However, no changes in the sarcolemmal Na^+/K^+ ATPase or the Na^+/Ca^{2+} exchanger were found and no changes in the membrane phospholipid or passive Ca^{2+} binding ability of the membranes were demonstrated (179). This highlights the caution that must be used when comparing data from studies that have employed different models to induce hypertrophy.

The increase in maximal functional capabilities of the heart caused by exercise training is due to a coordinated increment of the functional abilities of the ionic transport systems, myosin ATPase and ATP resynthesis systems (144), and more effectively buffered intracellular Ca^{2+} which results in augmented cardiac relaxation rates (179). Furthermore the mechanical responsiveness to catecholamine stimulation after swim exercise training is related to intracellular events, i.e. increased myosin V1 enzyme which does not necessitate a large Ca^{2+} influx into the cardiac cell to maintain adequate contractile function. The changes noted for excitation contraction coupling with

pathologic cardiac hypertrophy are accompanied by decreases in the Ca^{2+} activated ATPase activity and Ca^{2+} handling ability of the SR (60). In contrast 8-10 weeks of exercise training results in an improved handling of the intracellular Ca^{2+} (153).

Thus the basic difference in the response to physiologic and pathologic cardiac hypertrophy is a pronounced elevation in Ca^{2+} influx with pathologic hypertrophy. Of interest to the exercise biochemist is the fact that swim exercise which can induce a "physiological" hypertrophy in rats can indeed reverse some of the deleterious aspects of the "pathological" hypertrophy induced by chronic hypertension (209). The clinical significance of the processes that differentiate between these two classes of hypertrophy are great. If the underlying mechanisms are related, as the evidence just presented suggests, then the study of possible regulatory processes will yield valuable information in elucidating the best methods of treatment and prevention of pathological hypertrophy.

Calcium activated neutral protease.

Structure of calpain.

The Ca^{2+} activated neutral protease, (calpain, EC 3.4.22.17), is a non-lysosomal cysteine protease resident in mammalian cells. This protease, which shows an absolute requirement for Ca^{2+} in order to be activated was discovered by Guroff in 1964 in the soluble fraction of rat brain homogenate, (92). The demonstration of both low and a high Ca^{2+} requiring forms of the protease demonstrated that this system was likely to be fairly complex and closely regulated. An endogenous inhibitor protein called calpastatin was found to be inhibitory toward both isoforms, the micromolar Ca^{2+} requiring calpain, (u-calpain, uCL, or calpain-1) and the millimolar Ca^{2+} requiring calpain (m-calpain, mCL, or calpain-2) (159). Structural analysis of the protease revealed it to be a multi-domain protein comprised of a catalytic 80 kDa subunit and a regulatory 30 kDa subunit associated together as a heterodimer. The primary structure was elucidated for the 80kDa protein and it was shown to have four main domains, (164) while the 30kDa subunit consists of two domains (figure 1). The larger subunits of the calpains are genetically different proteins while the small subunits are derived from a single gene (249). Calpain-1 and calpain-2 have the same fundamental structures and share 50% sequence identity.
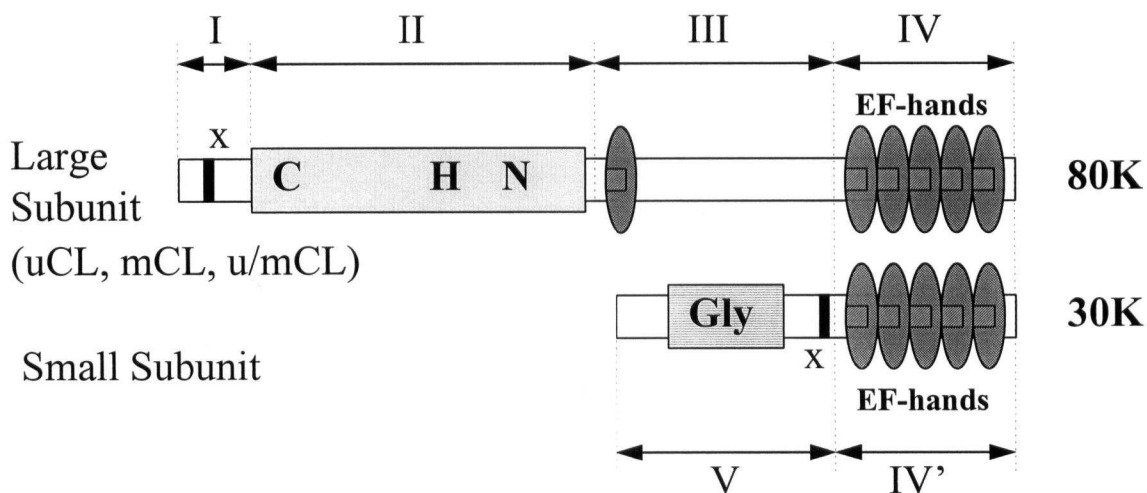
Figure 4. Representation of calpain. Large (80 kDa) and small (30 kDa) subunits are shown with characteristic features of each domain highlighted. C = catalytic cysteine residue, H = histidine and N = asparagine residues. Gly = glycine rich sequence (adapted from Ohno et al 1998), x = sites of autolysis. Hatched boxes within the Domain

IV and IV' domains represent EF hand motifs. uCL = micro calpain or calpain-1, mCL = milli calpain or calpain-2.

Domain I of the large 80kDa subunit shows little sequence homology to any known protein and its function remains unclear, however the N-terminal region may allow interaction with plasma membranes and is removed during the process of autocatalytic activation (see activation section below). Domain II, the second domain from the N-terminus, (residues 81-330), is responsible for the protease activity with the catalytic site containing a cysteine residue and its amino acid sequence being homologous to those of other cysteine proteases such as cathepsins B, H, L and papain (234). Recently the expression of rat calpain II in *Escherichia Coli* was achieved (86), and site directed mutagenesis revealed that the domain II residues Cys^{105}, His^{262} and Asn^{286} are responsible for proteolytic activity (6). Domain III function is unclear but it is most likely involved with calpains interaction with calpastatin. The C-terminal domains IV and IV' are calmodulin like Ca^{2+}-binding domains with five 'EF hand' structures (82). The three dimensional structure of the 30kDa subunit domain IV was recently defined and demonstrates that Ca^{2+} is bound at sites EF1, EF2, EF3, and EF4. The characteristics of these sites differ in that EF1 and EF2 make up a calmodulin like "open pair" whereas E3 and E4 have a closed conformation rather like the C-terminal EF-hand structures present in troponin-C (234). The EF5 site in both the large and small subunit are thought to allow or facilitate the association of the sub-units into their heterodimeric form as a IV' mutant lacking 24 C-terminal residues of EF5 no longer interacts with the 80kDa subunit (151). It is thought that the two subunits remain associated through their respective Ca^{2+} binding domains (161). A recent model of calpain activation that incorporates many features of the calpain system has been proposed by Suzuki and Sorimachi (252).

Participation of calmodulin binding domains have been demonstrated in a number of Ca^{2+} and calmodulin sensitive enzymes. Domain III of the calpain molecule is thought to act as a calmodulin binding region and to interact with the Ca^{2+} bound domain IV to render domain II free and active. The calpain I, and II, 80kDa subunits appear to have essentially the same domain II protease, however they do differ in domain IV, which is the domain that defines the Ca^{2+} sensitivity of the protease. Domain I can also inhibit the

intrinsic protease activity but is removed during autolytic activation. Despite being extensively studied, calpain structure and function are still not fully understood. Yet it is becoming clear that there is a large family of calpains that demonstrate molecular diversity representing tissue and organism specific isoforms of the protease expanding the complexity of an already enigmatic protease (167,234,252).

Name	Features	Structure
uCL, mCL, u/mCL	Ubiquitous, dimer with 30K	As in figure 1
p94 (nCL-1)	Skeletal muscle specific	Contains 3 extra insertions
nCL-2	Stomach specific	As in figure 1
nCL-2'		Truncated
nCl-4	Digestive organ specific	As in figure 1
Lp82	Lens specific (p94)	As in figure 1
CANP6 (CANPX)	Placenta specific	No Cys

Table 2: Selected calpain homologues demonstrating some of the increasing examples of tissue specific members of the calpain family.

Tissue specific calpains include those in skeletal muscle, also called p94 (120,232,235) or stomach, also known as nCL-1 (233) and nCL-4 which is digestive organ specific (234). The large subunit of skeletal muscle specific nCL-1 was recently identified and the encoded protein (94 kDa) shown to have the same fundamental domain structure as calpain I and II large subunits but contains two insertion sequences, IS1 within domain II, and IS2 between domains III and IV (251). A unique sequence also exists in the NH_2-terminal of domain I (designated the NS region). n-Calpain is activated and expresses its proteolytic activity in the cytosol, where the Ca^{2+} concentration is typically between 10 and 1000 nM. As will be discussed in the section on calpain regulation, this is the first example among calpain species of proteolytic activity at physiological intracellular Ca^{2+} concentrations. The expression of p94 mRNA is at least an order of magnitude higher than that of the conventional calpains in skeletal muscle (120,233,237) however the corresponding protein is detectable in only very small amounts. This is because it undergoes rapid autolysis immediately after translation, resulting in its disappearance. Removal of the IS2 region prevents this rapid autocatalytic degradation. Since autolysis is one of the mechanisms by which various proteases regulate their activity, extensive autolysis of the IS2 region may be involved in the regulation of p94 activity and function (236). The continued characterization of calpain species should ultimately assist the

process of assigning a role to the ubiquitous isozymes as the tissue specific calpains show distinct features that give clues as to how the protease is regulated in general.

The Ca^{2+} activated proteolytic system is therefore ubiquitously distributed yet also exists as isozymes specific to certain tissues. Structurally the isoforms are very similar and show conserved features that confer their Ca^{2+} sensitivity and proteolytic capabilities. It is likely that other features of their structure allow localization and substrate targeting as well as interaction with controlling factors. The specific endogenous inhibitor protein calpastatin is likely to be a key determinant of calpain regulation and function in vivo.

Calpastatin structure and function.

A specific inhibitor of calpain was discovered in the late seventies (161) suggesting that the calpain system was likely to be closely and specifically regulated within the cell. Calpastatin is equally effective in inhibiting both calpains 1 and 2 (82) and prevents both activation and the expression of catalytic activity. Calpastatin exists as two types, a 48kDa calpastatin found in erythrocytes and the more prominent 73kDa protein found in most other tissues. The specificity of calpastatin towards calpain is highlighted by the fact that it shows no apparent homology to the reactive sites of any other known protease inhibitors. More specifically, the 4 mutually homologous domains (140 amino acid residues) are composed of subdomains A, B, and C, and a consensus sequence of TIPPXYR (119). Subdomains A and C do not seem essential but do still potentiate calpastatin function which seems to be mainly due to subdomain B (directly inhibits calpain) (257) and the consensus sequence, which are essential for calpain inhibition. This distinct structure-function relationship, when combined with the unique physio-chemical properties inherent to calpastatin (i.e. high resistance to denaturants and lack of secondary structures) present an interesting proteolytic inhibition system. The tripartite structures of calpastatin and calpain provide the basis for a strong and close interaction (i.e. subdomains A, B, C and calpain's catalytic and two Ca^{2+} binding domains) (257).

Although the specific mechanism of calpain inhibition by calpastatin is slowly becoming clearer it is still far from being totally described and the precise relationship shared by the protease and its inhibitor are still unknown due to currently unresolveable features of their interaction. In the presence of Ca^{2+}, and possibly upon phosphorylation, calpastatin (1) complexes with calpain (with high affinity), inhibiting the protease at Ca^{2+}

concentrations lower than those necessary for autolysis or proteolytic activity. The mode of action of calpastatin presents a challenge in explaining how calpain can be active in vivo (114). Clearly, cells must possess some mechanism to reduce the Ca^{2+} requirement for proteolytic activity of calpain and to regulate inhibition by calpastatin (82). This could possibly involve regulation by specific phosphorylation of the calpastatin (1). Immunolocalization studies also suggest that calpain and calpastatin are co-localized within the cell (128). Indeed calpastatin seems to be a substrate for calpain and, at least in skeletal muscle, its degradation is suggested to reflect the fact that the inhibitor cannot bind tightly to the slightly different structure of n-calpain and is therefore vulnerable to proteolysis (232,235). Obviously then the regulation of calpain with calpastatin and the physiological importance of their interactions are far from resolved. A more detailed understanding and continued discussion of how these proteins interact in models of physiological calpain activity will likely help reveal the physiological role(s) for calpain (252).

Regulation of calpain.
We can see that the structure of this proteolytic family reflects very specific means of activation and regulation and these mechanisms are likely to vary from tissue to tissue. A summary of the evidence available and suggested methods of calpain activation and regulation follow.

Ever since its discovery, the question of how calpain is regulated in terms of its Ca^{2+} dependence within the cell has been foremost in calpain research and despite much interest in calpain in the last decade this question is still for the most part unanswered. A major question that is still unresolved is the fact that unphysiologically high Ca^{2+} levels are required for calpain activation in vitro. Incubation of calpain in the presence of Ca^{2+} and a caesin substrate initially results in modifications of both the calpain subunits. This modification of the subunits occurs before any proteolysis of the substrate occurs. During the activation process, a 76kDa fragment is formed from the large 80kDa sub-unit and an 18kDa fragment is formed from the small 30 kDa sub-unit. Calpain is therefore an inactive pro-enzyme, (201). The N-terminal regions of both the sub-units have been shown to change during this period of autolysis with the large sub-unit being modified

first and modification of the small sub-unit following just prior to the appearance of proteolytic activity, figure 4. Activation of calpain is most likely an intramolecular event rather than an intermolecular one as the rate of calpain activation has been shown to be independent of the concentration of the protease studied (107). The N-terminal region of the 80kDa sub-unit must be modified before proteolytic activity occurs. Modification of the small sub-unit apparently has no effect on the proteolytic ability of the calpain but seems essential for its interaction with phospholipids (249). Ca^{2+} is required at high levels for autolysis and for proteolytic activity: once autolysed, calpain requires lower Ca^{2+} for proteolytic activity and therefore may be active at near physiological Ca^{2+} levels.

The question is then: how does calpain become active in vivo if it requires higher than physiological Ca^{2+} levels for activation in vitro? Coolican and Hathaway in 1984 first reported that phospholipids could significantly lower the Ca^{2+} concentration required for autolytic activation of calpain (52). This possible mechanism for reducing the Ca^{2+} required for calpain activation was further investigated, (201,249) and biological disulfides were reported to reduce the Ca^{2+} required for calpain activation. This was suggested as a possible physiological mechanism that could play a role in the regulation of calpain activation. Acidic phosholipids especially were found to lower the Ca^{2+} concentration required for autocatalytic activation with the Ca^{2+} required being progressively lower as the number of phosphate groups on the phospholipid increased. With phospatidylinositol-4-phosphate (PIP) or phosphotidylinositol-4,5-bisphosphate (PIP$_2$), autocatalytic activation occurred at 10^{-6} to 10^{-7} M Ca^{2+}, a more plausible physiological Ca^{2+} concentration. Therefore the phospholipids PIP$_3$ or PIP$_4$, recognized as precursors or storage forms of 2nd messenger, may be more effective in assisting the activation of calpain. The amount of PIP$_2$ shown to reduce the Ca^{2+} levels required were also found to be within the levels normally found at typical biological membranes. Pro-calpain has been shown to be able to translocate to the plasma membrane from the cytoplasm in the presence of uM Ca^{2+} where autocatalytic activation could then occur. This possible activation of calpain at biological membranes would suggest that calpain substrates could be membrane proteins. However it does not preclude the possible

release of activated calpain from the membrane to act on cytosolic protein substrates, or the fact that calpain could be activated at sites other than biological membranes (145,252).

There is evidence to suggest that calpastatin could be associated with the cell membrane and possibly the SR (145). Considering the suggested method of calpain activation at the membrane, then calpastatin localized to the membrane is an attractive idea as calpastatin would be ideally placed to modulate the autoactivation of the calpain and so possibly regulate its action on membrane bound substrates. When the calpain to calpastatin molar ratio is increased then calpastatin could be removed from its membrane sites, leaving the membrane bound protein substrates open to proteolytic modification by the calpain. Such a role of membrane bound calpastatin could therefore be to act as a protective buffer against any brief exposure to elevated Ca^{2+}. Intuitively it makes sense to have this intracellular proteolytic system under tight spatial control to ensure no unwanted cleavages of substrates within the cell. Such control is illustrated by the fact that calpain-1 can be translocated to both the plasma and granule membranes as inactive "pro-calpain" prior to activation at the membrane, an example is thrombin stimulation of platelet activity (127). Another example is the potentiation produced from repetitive stimulation of hippocampal neurons resulting from post-synaptic activation of calpain-1. A suggestion as to how this occurs is that the pre-synaptic release of glutamate opens post synaptic Ca^{2+} channels and the resulting Ca^{2+} influx causes activation of calpain-1 and then hydrolysis of the membrane cytoskeletal attachment protein fodrin. It is thought that this post-synaptic remodeling makes latent glutamate receptors expose themselves to subsequent release of neurotransmitters (135). Further evidence for a calpain involvement in this process is that the cysteine protease inhibitor leupeptin can prevent the increase in glutamate receptors (135). Another example of a membrane location for calpain activity is the calpain dependent activation of protein kinase C (PKC). Calpain has been shown to be able to cleave PKC and the rate of this cleavage is enhanced by the addition of phospholipid and diacylglycerol. The proteolysis of the 80kDa PKC produces an active 50kDa fragment no longer requiring Ca^{2+} or phospholipid for activity (149). The "membrane hypothesis" for calpain activation is an attractive one and has been given

general widespread acceptance however it does not accommodate some other experimental findings and cannot explain many current pieces of evidence (82).

No single model of calpain activation and regulation can easily explain all of the data to date. Except for the Ca^{2+} required for the proteolytic activity of calpain after autolysis then the Ca^{2+} levels required by calpain for autolysis and proteolysis are higher than the 100-800nM free Ca^{2+} found in vivo (0.1-0.8 uM). It may be possible to suggest mechanisms that allow physiological Ca^{2+} levels to initiate autolysis of calpain, which requires Ca^{2+} levels of 1-5uM in the presence of phospholipid. An example of this could be transient large increases in Ca^{2+} locally at sites such as Ca^{2+} channels. Immunolocalization studies show calpain and calpastatin are distributed throughout the interior of cells with no preferential location near the plasma membrane, (128). Several studies have shown that calpain also binds to proteins and not the phospholipids in membrane vesicles, (107,128). In addition to plasma membranes, calpain will bind to a whole variety of subcellular structures e.g.; myofibrils and Z-disks. It could be that some feature of the binding of calpain to its substrate is required to lower the Ca^{2+} required for autolysis (148). It is likely that calpain activity is regulated by the binding of Ca^{2+} to specific sites on the calpain molecule, with binding to each site eliciting a response (proteolytic activity, calpastatin binding, autolysis, etc) specific for that site (82). Just how the specific role of calpastatin is regulated is also unclear in the membrane activation hypothesis. Calpastatin completely inhibits both autolyzed and unautolyzed calpains and does so at Ca^{2+} concentrations below those required for either autolysis or proteolytic activity (114). Also puzzling is the fact that most cells contain enough calpastatin to inhibit all the calpain that studies show it to be co-localized with. Therefore cells must also have a means for regulating the inhibition of calpain by calpastatin if calpain is to have any role in the cell.

Unlike other protease inhibitors, calpastatin can be phosphorylated by PKA and PKC. This suggests a role for these kinases in regulation of calpastatin. It has been reported that dephosphorylation of calpastatin attenuates its inhibitory activity towards calpain-2 but actually will increase its inhibitory potency against calpain-1 both in vitro and in perfused heart (183,203). As mentioned previously, calpastatin itself is a substrate

for calpain and has been shown in cardiac muscle to be proteolyzed during ischemia (231).

A simple and direct explanation for the various contradictory pieces of evidence regarding the actual and required Ca^{2+} levels could be that there is some other piece to the calpain puzzle. This "piece" could take the form of an "activator" that would enhance the affinity of the calpain proteolysis binding sites so that Ca^{2+} can bind at physiological concentrations. Such a factor may be analogous to calmodulin e.g.; can bind Ca^{2+} at physiological Ca^{2+} levels then bind to calpain and change its Ca^{2+} affinity. A 40 kDa protein has been reported in neutrophils and muscle extracts that lowers the Ca^{2+} required by calpain-2 by 50 fold but does not affect the Ca^{2+} required by calpain-1. This protein also prevented the inhibition of calpain by calpastatin, (184). A "calpain activator protein" has been reported which seems to modify calpain activity (223). It was purified from human platelets and can double calpain activity without altering the Ca^{2+} sensitivity of calpain. Recently there has also been a so called calpain activator found in rat brain which binds to the catalytic domain of the large calpain subunit and promotes dissociation of the heterodimer, this activator is said to compete with calpastatin and be associated with the inner surface of the plasma membrane. It is proposed that where this activator is present, calpain could escape the negative control exerted by calpastatin (147). Further study of this activator has revealed it to be calpain-1 specific and to associate in a 1:1 complex that increases the Ca^{2+} sensitivity 10 times (146). Another activator protein acts like a Ca^{2+} binding protein and binds 1:1 with calpain-2 to lower the K_a value from 400uM Ca^{2+} to 15uM through a facilitation of autolysis. These two activator proteins both bind in a Ca^{2+} dependant manner to the plasma membrane suggesting a membrane located function. Despite such advances in understanding the regulation of calpain, the process of calpain activation and regulation has not yet been explained in a way that can encompass all the pieces of evidence to date. It is even possible that calpain activation and regulation differs depending upon the location and characteristics of the substrate being targeted.

As if the picture of calpain involvement in the signaling process was not complex enough, it seems that calpain itself is subject to control by some of the proteins that are

reported as possible substrates for calpain. Signal transduction events that alter the phosphorylation patterns of intracellular calpain substrates could influence their degradation rates and substrate susceptibility. A major question in calpain research is just how calpain targets and recognizes its substrates. It is possible that dynamic alterations such as phosphorylation, sulfhydryl group status and Ca^{2+} induced hydrophobic interactions (19), may be able to regulate protein susceptibility to calpain. For example, phosphorylation of the 20kDa myosin light chain kinase, by PKC results in a ten fold increase in its susceptibility to proteolysis by calpain in human neutrophils (181,182). The phosphorylation of this 20kDa protein is itself thought to be dependent on PKM, which is produced by proteolysis of PKC. This process leads to re-organization of cytoskeletal network proteins and may facilitate the access of granules to the plasma membrane and promote exocytosis (182). There is also evidence to show that susceptibility of some protein substrates to calpain changes after phosphorylation. Actin binding protein (ABP) undergoes proteolysis by calpain during platelet aggregation and ABP phosphorylation by PKA has been shown to protect it from proteolysis by calpain both in vitro and in vivo (288). Thus the process of PKA phosphorylation activation in response to prostaglandin's is suggested as transduction of an inhibitory signal that allows blockage of cytoskeletal reorganization (44). As we can see the control of calpain activity is not clear and may be regulated on many levels.

The physiological significance of calpastatin.

Calpastatin regulation and its interaction with calpain is likely to be a key determinant in processes where the close control of the protease directs any physiological event. Considerable interest has been focused on the importance of proteolytic control in muscle (140,12,239). Specifically, interest of calpain - calpastatin in meat production and meat quality has been significant (12,170). In skeletal muscle it is known that addition of β-adrenergic agonists can lead to skeletal muscle hypertrophy (238) which is thought to be due to a suppression of myofibrillar breakdown (12). For example the decrease in calpain-1 activity and increased calpastatin activity is thought to be consistent with a condition of reduced proteolytic breakdown where net protein accumulation results. Yet calpain-2 activity tends to be elevated, a finding which is hard to resolve with the

decreased proteolysis theory of hypertrophy. Others have also concluded that in skeletal muscle the effects of β-adrenergic stimulation to increase muscle mass is more likely due to increased levels of calpastatin in the muscle as opposed to any alteration of calpain levels (238). This is also thought to explain the increased "toughness" or loss of meat quality in these animals exposed to β-adrenergic agonist agents.

Calpain substrates.

The list of substrates for calpain has grown considerably in the last few years and the following section describes many of them with regards to possible physiological functions. Many substrates have been demonstrated in vitro and yet the physiological roles or outcomes of substrate proteolysis in vivo are still poorly understood. It is clear that calpain does not act just as a general protease, but has specific substrates which it cleaves in a controlled, specific, and usually limited fashion (167,202). It is common for the large protein fragments produced to retain their activity and in some cases to show increased activity due to the removal or separation of an intrinsic inhibitory site (11,73,149). There are some slight differences in substrate specificity between the two isozymes but generally calpain-1 and calpain-2 will proteolyze the same substrate proteins in vitro (271). Calpain is thought to prefer certain classes of amino acids at or close to the cleavage site on the substrate protein. These are typically residues with large aromatic or aliphatic side chains at positions 3, 2, and 1 on the N-terminal side and basic or aliphatic residues on the C-terminal site at position 1 (39). However, there does not seem to be an absolute requirement for these and therefore other substrate features must be recognized by the protease. Another feature that is thought generally to confer substrate characteristics is the presence of calmodulin-like domains. Calmodulin-like domains are present in both the calpain subunits (251) and also in many calpain substrates (13,271). Indeed calmodulin binding domain phosphorylation in plasma membrane Ca^{2+} ATPase results in decreased susceptibility of the protein to proteolysis (204).

In order to determine which features of proteins will render them as targets for intracellular modification by proteases, Rogers and co-workers examined the amino acid sequences of ten proteins with intracellular half lives of less than 2 hours. Common to

each protein was at least one region rich in proline (P), glutamic acid (E), serine (S) and threonine (T), now known as "PEST" regions (196). The analysis of another group of 35 longer lived proteins showed that only 15 of these contained these so called PEST regions. Two types of PEST regions have been defined, determined on how closely related they are to the above amino acid sequence. These scores can be greater than zero or "strong pest regions", or they can be between -5 and zero, or "weak pest regions". Of the group of 35 longer lived proteins, only 3 of them contained strong PEST regions. Rogers suggested that as well as conferring the property of rapid degradation to these proteins, the negatively charged and phosphorylatable PEST regions could bind Ca^{2+} and these localized Ca^{2+} rich areas could allow activation of calpain (196). PEST regions are very hydrophilic and are thought to form surface loops on the protein that would be easily accessible to calpain, which would then cleave the protein nearby. As small protein fragments are not good substrates for calpain it is likely that higher order structures confer substrate specificity (256). The fact that many calmodulin binding proteins are known to be substrates for calpain prompted a search for PEST like regions in these proteins (271). The substrates for calpain generally showed the presence of PEST like regions located at or near the site of cleavage by the calpain. These included plasma membrane Ca^{2+} ATPase, where the C-terminal end of the protein showed PEST like regions near the sites of calpain cleavage, (270). Calpain-1 and calpain-2 both selectively cleave alpha-fodrin (240kDa) selectively to produce a specific fragment (150kDa). It has been shown that location of the calpain cleavage site of fodrin is close to the only PEST like region in the protein. The calpain cleavage site on PKC has also been found to be near a PEST like sequence. Another substrate for calpain proteolysis is HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis which contains two PEST like sequences approximately 40 and 47kDa from the N-terminal, (47). The calpain cleavage sites of HMG-CoA reductase are found at approximately 35 and 49kDa from the N-terminus. The degradation rates of proteins may also depend on their association with cellular structures such as membranes or cytoskeletal filaments. Binding to intracellular sites could mask the protein substrates sites normally recognized by intracellular proteolytic agents. It is likely that calpain and substrate localization patterns are likely to prove important in the regulation and co-ordination of this proteolytic

system. Knowledge of which proteins are substrates for calpain and under which physiological or pathological conditions should help clarify the role(s) of the calpain system. Protein substrates for calpain are quite diverse in structure and function and can be classified into broad categories. They include myofibrillar proteins both serving contractile and structural functions and are known to be co-localized with the calpains therefore remodeling or metabolic turnover of these contractile proteins was thought to be one of calpains main physiological functions (191). Studies on myofibrillar proteins have shown that calpain-2 was able to rapidly cleave desmin and TnT. TnI, TM and C-protein were cleaved more slowly, while actin, myosin and TnC were resistant to modification by calpain, (192). Activation of calpain under physiological conditions during prolonged running exercise in rat skeletal muscle has been demonstrated with myofibrils from the exercised animals showing increased rates of calpain induced degradation of desmin, vimentin, C-protein, and alpha-actinin (17). This study showed that physiologically induced modifications of these calpain substrates due to exercise made them more susceptible to calpain cleavage (17). The calpains may also contribute to the disassembly of cell cytoskeleton, especially at sites where the cytoskeletal proteins are attached to the plasma membrane (65). This ability of the calpains to influence the cytoskeletal proteins has been well studied in platelets. The activation of platelets is accomplished by an increase in intracellular Ca^{2+} concentration and this is followed by the specific cleavage of filamin, talin and spectrin (14).

A number of enzymes are also substrates for proteolysis by calpain. Usually when a kinase or phosphatase is proteolysed by calpain the large protein fragments produced retain their intrinsic activity and in some cases show increased activity or no longer require certain co-factors to be active. As mentioned previously, PKC is cleaved from an 80kDa protein to a 50kDa protein that no longer needs co-factors for activity (149). Another substrate that calpain converts to a constitutively active form is myosin light chain kinase, which requires calmodulin and Ca^{2+} for activation prior to proteolysis by calpain (108). Other enzymes that are activated by calpain include the skeletal muscle isoform of creatine phosphokinase, which is proteolysed in vitro and shows increased activity (4), protein activated kinase II is also converted to an active form by calpain-1 (174). Calcineurin, a calmodulin dependent protein phosphatase was also found to be a

substrate for calpain to produces an active, calmodulin independent form. Its activation was concurrent with the formation of a 45kDa protein after cleavage of the original 60kDa protein by calpain-1 (271). Specific cleavage of tyrosine hydroxylase removes the amino-terminal end and the large 56kDa fragment produced from the 60kDa original shows a slight but significant increase in activity. The 565 kDa calcium release channel of striated muscle SR is also initially cleaved by calpain (189), with the formation of 410 and 150 kDa fragments, which reduces by about 20% the amount of threshold Ca^{2+} required to induce Ca^{2+} release (80). The list of enzymes that are substrates for calpain is growing, however the selected features that define them as calpain substrates and control their proteolytic modification in vivo are still mostly unclear.

Certain cytoskeletal proteins are also thought to be calpain substrates and it has been proposed that one of the major physiological functions of calpain is in the reshaping or metabolic turnover of cytoskeletal proteins (148). The calpains have also been attributed with the role of disassembly of cell cytoskeleton, especially at sites where the cytoskeletal proteins are attached to the sarcolemmal membrane, (65). This ability of the calpains to influence the cytoskeletal proteins has been well studied in platelets where activation is accomplished by an increase in intracellular Ca^{2+} concentration followed by the specific cleavage of filamin, talin and spectrin, (14). When incubated with purified platelet calpain, filamin and talin are cleaved to the same polypeptide fragments seen during platelet activation (72). The initial studies with porcine muscle myofibrillar / cytoskeletal preparations showed that calpain rapidly cleaves desmin, C-protein and removes the Z-line protein alpha-actinin (57). Subsequently it was shown that calpain rapidly degraded all intermediate filament proteins of the cytoskeletal system, including desmin and vimentin in skeletal muscle (160). Calpain has been suggested to mediate direct changes in receptor geometry at the cell membrane (201). This suggestion was made because of the fact that fodrin, a major lining protein beneath the plasma membrane, provides multiple attachment sites for different proteins involved in membrane signaling. Fodrin is known to be a good substrate for calpain (135) and its susceptibility to calpain proteolysis can be changed as a result of modifications by other proteins. It also acts as an anchor for membrane receptors and can be phosphorylated by cAMP dependent protein kinase, protein kinase C (PKC), and tyrosine kinases (270). The

significance of these cleavages and the resulting altered function or modification of the substrate remains unclear however. It has been hypothesized that calpain may regulate the release of partially degraded subunit proteins from the insoluble intermediate filaments, which as soluble DNA binding proteins may enter the nucleus and regulate gene expression (263).

Calpain also acts directly on nuclear substrates, has been localized to the nucleus, and shows co-localization with chromosomes during mitotic nuclear envelope disruption (215). Components of the nuclear matrix are also subject to calpain mediated proteolysis and because n-calpain possesses a potential nuclear localization signal, its involvement in muscular development and differentiation by modifying specific transcriptional factors in the nucleus also seems plausible (88). It has been suggested that calpain may play a regulatory function during meiosis because calpain rapidly cleaves pp39mos, the protein product of the proto-oncogene, c-mos, that is responsible for arresting the cell in M-phase of meiosis and activates characteristic early response genes in the initial stages of cardiac hypertrophy. The fact that calpain-2 was able to proteolyze nuclear matrix proteins at Ca^{2+} concentrations as low as 3uM was attributed to a lowering of the Ca^{2+} required by the presence of DNA. This effect was thought to be due to a concerted effect of calpain binding with substrate and DNA rather than any direct DNA effect on calpain itself (145). These kinds of reports provide exciting possibilities in explaining how calpain activity is regulated and strengthens the likelihood that the protease plays a significant physiological role in the nucleus. Further examples of substrate modification and calpain targeting are discussed below.

As mentioned previously the Ca^{2+} concentrations required to induce calpain activity in vitro are higher than those that are encountered within a viable cell. Therefore it is necessary to account for this fact if physiological or pathological roles for calpain are to be assigned. However certain modifications of substrates during physiological or pathological situations do appear to provide possible mechanisms whereby calpain activation might become feasible. Proteolysis of a particular protein substrate may be dependent on a number of intracellular factors such as the metabolic status, phosphorylation, and ionic environment within the cell. For example, TnI phosphorylation by PKA and PKC causes a conformational change and alters its

sensitivity to calpain (61). In fact an inverse relationship arises between the Ca^{2+} sensitivity of the myofibrillar Mg-ATPase and susceptibility of troponin to proteolytic attack (61). Certain conditions could also make calpain substrates resistant to proteolysis. Phosphorylation of actin binding protein by PKA increases its resistance to proteolysis by calpain, (288). Another example of PKA dependent phosphorylation regulating protein susceptibility to calpain was shown with the microtubule associated protein tau (see function section for discussion of calpain in Alzheimer's disease). ATP depletion has also been shown to stimulate calpain dependent protein breakdown in chick skeletal muscle, (68). Covalent modifications of substrates and their oxidation and reduction status are suggested to allow targeting by calpain where $[NADH/NAD^{+}]$ seems to determine proteolytic susceptibility of certain substrates, as does the oxidation of thiol groups and the presence of disulfides (195). Altered cellular carbohydrate metabolism also appears to play a role in calpain targeting, as diabetics show increased troponin and tropomyosin proteolysis by calpain (195) perhaps reflecting increased glycosylation of these proteins which renders them more susceptible.

The examples above represent some of the conditions and systems studied to try and elucidate the function of calpain. There are currently many different possible routes by which calpain might influence cellular function. Some of the more favoured roles of calpain will be discussed and highlighted in the next section.

Physiological functions of the calpain - calpastatin system.
Mode of calpain action. As we have seen, calpain is not considered to be a digestive enzyme because it cleaves its protein substrates in a limited fashion leaving large polypeptide fragments rather than degrading them to small peptides and amino acids (201). Characteristic of calpain proteolysis is the production of peptide fragments that retain their catalytic function (kinases), and are resistant to further proteolysis (intermediate filaments). Thus calpain has been assigned a role as a regulatory or processing protease (202), (251). These specific and limited calpain mediated alterations of substrates, especially those associated with the plasma membrane, have reinforced the significance of the calpain / calpastatin system in physiological systems. As reported above, calpain is associated with modulation of a range of substrate types such as cytoskeletal proteins, intermediate fibres, membrane receptors, channels and / or ion

carriers. Calpain has also been suggested to play a mediatory role in intracellular transduction cascades regulated by Ca^{2+} (250,251) and may be indirectly involved in cell protein turnover as an initiator for proteolytic degradation pathway for abnormal or non functional proteins that may recognize calpain substrates as targets for further breakdown. The limited in vivo models indicate that calpain / calpastatin, at least in red blood cells, renal cells and glial cells of the nervous system, is responsible for the rearrangement of membrane-cytoskeletal proteins which serve to regulate functions such as mitosis and movement of secretory granules (215).

The IS2 region of n-calpain, the skeletal muscle specific isozyme contains a potential nuclear localization signal and is thought to control the expression of short-lived regulatory proteins, including transcription factors. Indeed, n-calpain may change its localization according to the cell cycle and be involved in muscle differentiation by interacting with the MyoD family and the conventional calpains are thought to regulate transcription by controlling the levels of c-Jun and c-Fos (40). In addition, specific cleavage of a number of hormone receptors occur; including receptors for, EGF (42), glucocorticoids (65), and progesterone (267). The receptors functional ability to bind its hormone is not changed after proteolysis by calpain therefore the significance of these cleavages are unclear.

Calpain has also been implicated in signal transduction cascades as there are many likely calpain substrates and situations where Ca^{2+} plays a role in regulating intracellular signaling. Calpain has been suggested to be involved in the insulin signaling system where the insulin receptor protein phosphorylates tyrosine residues both on itself and on a number of target proteins called the insulin receptor substrates (IRS) (246). IRS-1 protein is down regulated under prolonged exposure of cells to insulin and this protein contains up to 11 PEST like sequences (196). IRS-1 has a half life of 12-24 hours which is reduced to 2-2.5 hours with prolonged insulin stimulation, the end result being a down regulation of insulin responsive glucose transport. This down regulation is associated with only a 20 percent decrease in mRNA for IRS-1, suggesting that increased proteolysis is the main factor in its down regulation. The fact that the regulatory sub-unit (R) of cAMP dependent protein kinase undergoes proteolysis by calpain also suggests that calpain may act as a signaling or processing protease in response to receptor

mediated events. Proteolysis of the R subunit produces a 37kDa fragment that is no longer able to inhibit the catalytic function. The R subunit is more sensitive to proteolysis when it is in the dissociated state and neonatal lung extracts contain more of these dissociated sub units than adult lung. This is proposed to prolong kinase activation in the neonatal lung and play a role in normal lung development (16). Calpain has also been found to act on the 150kDa membrane associated phosphoinositide-phospholipase C (PI-PLC) in human platelets. PI-PLC generates the second messengers inositol trisphosphate and diacyl-glycerol and exists in three major sub-types. PI-PLC is regulated by interactions with G-proteins through both α sub-units and with β/γ sub-units. Proteolysis produces a 100kDa fragment more sensitive to G-protein β-adrenergic activation than the original intact PI-PLC (11).

Recent evidence has shown platelets treated with 1mM EST to have altered phosphorylation patterns. Phosphatidylinositol hydrolysis and inositol-1-phosphate production which occurs after thrombin induced platelet activation is also inhibited by EST with PI-PLC-B and PKC being likely substrates for calpain (11,149,159). It has recently been shown that calpain can cleave the alpha sub-unit of the G protein heterotrimeric complex (87). Calpain rapidly proteolysed the alpha sub-units of G_o but did not affect β-sub-units. It is interesting that alpha subunits show differential susceptibility to proteolysis by calpain, with alpha (s) > (o) > (q) > (i). It would be interesting to search these sub-units for PEST like sequences and if they are present, determine if their susceptibility correlated to the PEST sequence scores. It is also known that lithium has no effect on alpha (o) bound to the dissociated GTP [γs] but it does increase the proteolysis of alpha (o) in the heterotrimer complex. In vivo administration of certain drugs such as lithium or tricyclic antidepressants or corticosterone can cause altered brain G-protein levels. Exactly how the G-protein levels are regulated is as yet unknown but the rapid loss of alpha subunits after stimulation suggests that it may be due to proteolysis. Calpain has the ability to be activated by signal transduction events associated with increased intracellular Ca^{2+} and to have access to G-proteins. Lithium is a well known treatment for mania and bipolar mood disorders, it may be able to reduce signal transduction by increasing the susceptibility of G protein alpha subunits to calpain mediated proteolysis (87). Another brain disorder linked to calpain activity is

Alzheimer's disease. Tau is known to be a major component of neurofibrillary tangles found in brain tissue from people with Alzheimer's disease (279). Phosphorylation of tau by PKA significantly inhibited its proteolysis by calpain compared to unphosphorylated tau. It is interesting that populations of abnormally phosphorylated tau are found in the brains of Alzheimer patients (279). Therefore in these patients tau is abnormally phosphorylated. This is further supported by the fact that cAMP may be elevated in Alzheimer's brain tissue and the presence of aluminum in brain tissue, which is thought by some to cause this disease has been shown to cause elevations of cAMP in mammalian brain tissue (113). Therefore the abnormal regulation of this inhibitory transduction resulting in increased resistance of tau to calpain proteolysis is proposed to have a role in the development of Alzheimer's disease (132). Calpain is also likely to be involved in conditions where demyelination of neurons occurs such as in multiple sclerosis (224). There are obviously many possible substrates and roles for calpain within the cell, however the actual physiological significance of each of these are difficult to completely characterize due to lack of complete explanations regarding how calpain is activated and regulated in vivo.

Calpain in striated muscle.
Possible physiological roles for calpain have only recently been studied in intact cells or organs using techniques such as cell culture models, isolated muscle perfusions and cardiac perfusion systems. In muscle tissue the importance of calpain has been focused on pathological involvement such as with muscular dystrophy (192) and muscle hypertrophy (30,238), but despite increased research on calpain in striated muscle the role of calpain remains elusive. These approaches used in conjunction with cell permeable inhibitors of calpain are helping to elucidate the possible role of calpain in conditions such as myocardial ischemia, stunning, myofibrillar disassembly, and responses to focal ischemic injury (15,142,260). Evidence based on the administration of calpain inhibitors being protective against the deleterious effects of various conditions is growing. Calpain has been assigned a role in dystrophic conditions where there are increased rates of protein degradation and increased levels of calpain activity (192). Recently a specific deficiency in p94, the skeletal muscle specific isozyme of calpain (120), was suggested to be responsible for the defect in development of limb girdle muscular dystrophy 2A (194).

Skeletal muscle calpain activity has been shown to be increased while the Ca^{2+} required for 50% of maximal activity (pCa_{50}), was reduced after prolonged running exercise (17). Calpain and calpastatin mediated effects on protein accretion in livestock have been studied in response to various growth promoting agents and the subsequent control of meat quality reflecting possible post mortem proteolysis (140,170). The calpain system is also known to be present in cardiac muscle and has attracted interest as a possible pathological or therapeutic target in conditions of cardiac dysfunction as will be discussed in more detail in the following section.

Cardiac calpain.

The calpain system is known to be present in cardiac muscle (158,203,260) and its activity has been documented to increase during conditions such as β-adrenergic stimulation and hypoxia (105) or prolonged running exercise (17). In cardiac muscle, alpha-actinin which is a structural protein at the Z-line is removed much more rapidly by calpain from myofibrils from the larger hearts of diabetic rats (20). However no other direct evaluations of calpain and increased cardiac mass have been reported. The fact that β-adrenergic inhibitors can block the increase in calpain activity that occurs during cardiac hypoxia (105) also suggests that the calpain system may be playing a role in direct response to β-agonist stimulation. Possible calpain substrates within cardiac muscle include signal transduction proteins (87), Ca^{2+} regulating proteins (80) and the contractile machinery (19). As discussed briefly earlier there is considerable interest in calpain and calpastatin in the control of muscle growth (12,238). The possible role of calpain in cardiac muscle growth specifically will be discussed below.

Calpain in cardiac hypertrophy.

As indicated earlier, the majority of substrate specificity of calpain has been deduced from in vitro studies and the role, if any, that calpain-calpastatin may play in physiological systems requires further study. From the evidence available to date there is reasonable certainty that the calpain-calpastatin system should be playing a role in most tissues, yet a clear appreciation of its relationship to physiological systems is not available in the literature. No studies have attempted to evaluate whether there is a such a role for the calpain-calpastatin system in cardiac hypertrophy. Therefore the use of

cardiac hypertrophy as a model to understand calpain-calpastatin would be valuable if the effects of calpain action could be verified in this model. Despite the unclear relationship of calpain-calpastatin in other physiologically relevant processes, cardiac hypertrophy in response to stress and exercise has been partly attributed to a Ca^{2+} dependent pathway whose precise regulation is unknown (96).

The changes noted for excitation-contraction coupling with pathologic cardiac hypertrophy are also accompanied by decreases in the Ca^{2+} activated ATPase activity, Ca^{2+} transport function and Ca^{2+} stores (60,171). In contrast 8-10, weeks of exercise training results in an increased function of the SR which has been observed to occur 7 days into an exercise programme (210,211) and is manifest as an improved handling of intracellular Ca^{2+}. Thus the basic difference in the response to physiologic and pathologic hypertrophy is a pronounced elevation in Ca^{2+} influx into the cardiac cell during pathological hypertrophy. Whether this differential response in Ca^{2+} loads between pathological and physiological hypertrophy influences calpain-calpastatin activity differently is unknown. Calpain activity may initially increase and then return to normal with physiological hypertrophy in contrast to pathological hypertrophy where increases in calpain activity are more likely to be sustained. Pathologic cardiac hypertrophy is associated with chronic disturbances in ionic activity (i.e. increased $[Ca^{2+}]_i$) and metabolic status (decreased $[ATP]_i$) as well as cardiac cell growth. Therefore, an interesting question is whether the relationship between calpain-calpastatin and cardiac hypertrophy is related exclusively to processes of cell growth or interactive with the major ionic and metabolic disturbances accompanying these conditions. In this respect, calpain-calpastatin could play a functional role in a cell or organs ability to adapt by allowing for a remodeling or alteration of selected proteins and their function. Of particular interest is the possible role of Ca^{2+} as a mediator in the development of the functional and structural adaptive changes during cardiac hypertrophy that may ultimately lead to heart failure. With pronounced or pathological hypertrophy these functional changes include decreased active tension development (155), decreased velocity of shortening (276), and increased time to peak force (110).

It may be concluded that cardiac hypertrophy is a reasonable and important model to study the relationship of calpain-calpastatin because of observations on cytoskeletal

and membrane proteins. During the development of cardiac hypertrophy, alterations of certain proteins in vivo are reminiscent of calpains actions in vitro. It is known that swim exercise and isoproterenol injections induce a physiologic and pathologic model of cardiac hypertrophy respectively. The depressed metabolic status and abnormal Ca^{2+} handling, in pathologic cardiac hypertrophy, contributes to the cardiac dysfunction, concomitantly with the apparent effects of calpain-calpastatin activation. However this relationship has never been tested experimentally. Furthermore, whether the increases in cardiac mass seen with exercise are independent of the metabolic and ionic imbalances common to pathological hypertrophy development is unknown.

Calpain inhibition.

The most specific inhibitory agent towards calpain is the endogenous inhibitor protein calpastatin. However the cell permeability of this protein is very low due to its large size which precludes it from being effective in settings other than where the preparation studied is fully permeable such as with skinned cardiac cells (76) or where it can be micro-injected into cultured cells (215). Incubation of trabeculae with calpastatin can prevent the reduction in Ca^{2+} sensitivity caused by exposure of the muscle to calpain-1 (76). The development and application of non-endogenous inhibitors of calpain reflects the growing interest of this proteolytic system. The use of calpain inhibitors is becoming prevalent in assessing possible therapeutic roles in situations such as neuronal disorders (15) and cardiac conditions such as the events associated with periods of ischemia (261), hypoxia (105), and stunning (76). Another calpain inhibitor, and probably the most widely used to date is the microbial peptide aldehyde; leupeptin (N-acetyl-Leu-Leuargininal) which a fermentation product of actinomycetes. This inhibitor acts to reversibly compete with substrate at the active site in the presence of Ca^{2+} (269). More recently an oxamide inhibitor molecule AK295 [benzyloxycarbonyl-leucylamino-butyric acid-CONH(CH2)3-morpholine], was developed and its derivatives have been used in conditions such as post brain injury in rats where it can reduce motor and cognitive defects (199). Calpain inhibitors-I (N-acetyl-Leu-Leu-norleucinal) and -II (N-acetyl-Leu-Leu-methioninal) are commercially available reversible inhibitors of calpain that act via neucleophilic attack of the active site thiol at the aldehyde carbonyl group to form a reversible hemithioacetal. Calpain inhibitors I and II are known to also inhibit other

proteases such as papain and cathepsin-B and interestingly neither of these two inhibitors, leupeptin, or the E64 derivatives will prevent the hydrolysis of p94, the skeletal muscle specific isozyme of calpain (238).

Synthetic inhibitors of calpain were first employed in an attempt to reduce the myofibrillar changes associated with muscular dystrophy. E64 was isolated from cultures of Aspergillus Japonicus and used as a papain inhibitor. Derivatives of E64 [N-[N-(1-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]agmatin], were used to try and prevent the increased protein degradation seen in dystrophic muscle (242). Derivatives of E64, E64d [N-[N-(1-3-trans-ethoxycarbonyloxyoxirane-2-carbonyl)-L-leucyl]isoamylamine], a membrane permeable derivative of E64c, and E64c [N-[N-(1-3-trans-ethoxycarbonyloxyoxirane-2-carbonyl)-L-leucyl]isoamylamine] have more recently been applied to investigate calpain's role in various conditions (101). One mole of E64 will inhibit one mole of protease acting in a non-competitive, irreversible manner by forming a thioether bond with the active thiol of the enzyme. Another calpain inhibitor, and probably the most widely used to date is the microbial peptide aldehyde; leupeptin (N-acetyl-Leu-Leuargininal) which a fermentation product of actinomycetes. This inhibitor acts to reversibly compete with substrate at the active site in the presence of Ca^{2+} (269). Specificity and cell permeability of calpain inhibitors are key determinants of which agent should be used in particular systems, tissues, or experimental conditions. For example, E64c, E64d, and leupeptin are not specific to calpain but can inhibit other cysteine proteases (234) and are not completely cell permeable (269).

These inhibitors of calpain all act by targeting the active site and the more recently developed inhibitors tend to target the Ca^{2+} binding site on the calpain molecule. The development of specific and fully cell permeable calpain inhibitors is foremost for many laboratories focusing on potential therapeutic strategies where calpain is thought to underlie cardiac pathologies. Thus the tools at the disposal of the researcher are improving and understanding calpains precise role(s) in cardiac muscle can only benefit from such developments. Currently the field of calpain function in muscle physiology is growing, however there are no studies that assess the possible role of calpain in the process of cardiac hypertrophy. It was my aim to utilise the inhibitor E64c in both pathological and physiological cardiac hypertrophy models to determine any possible role

for the calpain proteolytic system in the process of myocardial growth and functional alterations. The first of these models is presented in the next chapter.

CHAPTER 3.

Calpain in swim exercise and swim exercise induced hypertrophy.

Alterations and cellular adaptations during hypertrophy in response to swim exercise are in some respects reminiscent of the actions of the protease calpain. However no information exists in the literature in regard to whether acute swim exercise or physiological hypertrophy following prolonged swim exercise training is accompanied by or dependent upon activation of calpain.

Introduction.

The performance of the heart has been reported to improve following prolonged exercise training (110,153). In the rat, swim exercise training induces primary changes in cardiac function by altering the capacity of at least one (if not all) of the three major biochemical systems responsible for the functional characteristics of cardiac muscle. For example, changes in actomyosin ATPase activity (180), SR Ca^{2+} transport (173) and sarcolemmal function (179) have all been reported with acute exercise (one session), indicative of a functional link between the acute demands placed on the heart and the overall adaptive response. Despite some characterization of responses to swim exercise in the literature, the mechanism(s) underlying these changes are still unknown. Acute swimming is also associated with dramatic morphological changes such as mitochondrial damage, dilation of SR, myofibrillar disruption (121) and biochemical changes in Ca^{2+} transport processes (178). Therefore it may be suggested that Ca^{2+} regulated processes are critical for the expression of these acute responses to swim exercise.

Calpain is present in cardiac muscle and its actions in pathological conditions such as ischemia (260), and stunning (105) have attracted much recent attention. Calpain has been reported to be activated in hindlimb muscles during running exercise (10,17) and to target membrane and myofibrillar proteins known to be altered with exercise. Therefore calpain may be a causal agent underlying skeletal muscle morphological and biochemical changes. However no published information is available to determine whether swim exercise is accompanied by activation of cardiac calpain despite it being a potentially important feature of the hearts overall response(s) to both acute exercise and longer term adaptations seen with swim training. Therefore a pre-requisite for any role of

calpain in response to swim exercise would be the demonstration of increased calpain activity during single swim sessions or with repeated chronic swim exercise leading to cardiac hypertrophy.

Therefore the first hypothesis of this study was.

○ Calpain activity will increase following a single swim exercise session.

Also of particular interest was whether or not cardiac functional changes would be accompanied by or be dependent upon activation of calpain. Because myocardial contractility is controlled by Ca^{2+} and Ca^{2+}-regulated processes (i.e. troponin complex / SR), it may be hypothesized that any exercise induced activation of calpain would impact on the functional characteristics of the heart in response to exercise. Therefore in order to assess calpain's role in the functional responses of the heart to both acute and chronic swim exercise, rats were treated with the cell permeable cysteine protease inhibitor, E64c, prior to swim exercise. This inhibitor has previously been used in our lab administered by sub cutaneous injection with no apparent confounding effects on control animals and was found to prevent increased calpain activity seen with stimuli such as running exercise (10) and β-adrenergic agonist injections.

Typically with continued swim exercise (i.e. a physical training program), a significant degree of cardiac hypertrophy develops (210,211) and although some of the specific responses have been characterized (96), the precise mechanism(s) underlying the ventricular mass and functional changes are not defined. Interestingly, increased intracellular Ca^{2+} load (138), which occurs with repeated exercise bouts are accompanied by modified activity of selected ion channels, increased Ca^{2+} sensitive protein degradation and altered cytoskeletal structure (273). The development of cardiac hypertrophy also involves a re-organization of the cytoskeletal network manifested as myofibrillar disarray, Z-line smearing and selective degradation of cytoskeletal proteins such as desmin and vimentin (121,273). Because these hypertrophic changes are all reminiscent of calpain mediated effects, a secondary hypothesis of this chapter was.

○ Increased calpain activity is necessary to promote cardiac functional and biochemical adaptations with repeated exercise (i.e. chronic swim training).

As such it was necessary to determine if calpain activity was modified by six weeks of swim exercise sufficient to induce changes in cardiac mass and myocardial function. To

further assess calpain's role, rats were chronically treated with E64c during the swim training protocol. E64c is known to inhibit calpain activity increases in the rat with running exercise (10). Previous work in our lab had shown which dose range to be effective, therefore the following methods were used to determine any calpain mediated effects in the development of hypertrophy and selected functional and biochemical variables.

Methods.

Male Wistar rats (N=96), with initial body weight of 275-300g were randomly assigned to one of seven groups. (a) sedentary (n=12), (b) single swim (n=18), (c) single swim with E64c treatment (n=12), (d) sedentary (6 wk) (n=20), (e) sedentary (6 wk) with E64c treatment (n=6), (f) repeated swim* (6 wks) (n=18), and (g) repeated swim (6 wks) with E64c treatment (n=10). (* = groups swam two days then had one day off followed by two consecutive days then two days off and this weekly pattern was repeated 6 times in total).

The rats were housed in an animal care facility maintained at a constant temperature, and given standard rat chow and water ad libitum. Animals swam in a fiberglass tank approximately 36" by 18" and 24" deep. The tank was divided down the centre to create two swimming areas in which 5 animals swam at any one time. The water was maintained at $33\pm1^{\circ}C$. After every swim session the animals were toweled off and placed in cages under a warm heat lamp for thirty minutes and checked for signs of respiratory infections before being returned to the animal room. The same conditions were used for the acute swims except no weighting was used and the animals swam until they were seen to be having some trouble keeping their heads above the surface. The repeated swim protocol started with 30 minutes of swimming 4 days per week with 1% of body weight attached to the base of the tail. Duration and weighting was gradually increased until the animals swam for 75 minutes with 2% of their body weight attached. Single swim groups were divided into single swim and sedentary. Each group was further divided into treated (E64c injection) and untreated (PBS injection). All injections were given subcutaneously one hour prior to swimming. Chronic swim groups were divided into repeated swimmers and sedentary. In the repeated swim programme, all injections

were given 1 hour prior to the first and third swim sessions. That is on Monday and Thursday of each week and no injections were given before the Tuesday and Friday swims. The body weights of the animals were recorded on each of the swim days prior to swimming (in order to determine the tail weighting). Immediately after single swim sessions, and 24 hours after the last repeated swim session, exercised and control rats were administered an intraperitoneal injection of 0.75 mg/Kg pentobarbitone sodium prior to exsanguination and tissue extraction (within 4 minutes). All procedures were approved in accordance with guidelines established by the Canada Council on Animal Care and overseen by the University of British Columbia animal ethics committee.

Cardiac growth / hypertrophy.

Hearts were removed from the single swim animals immediately following swimming. Hearts from animals in the repeated swim groups were removed 24 hours after the last swim session. Immediately upon removal of the heart the left ventricles were trimmed free of any atrial material then blotted dry and weighed. The ventricular weights (g) were divided by the body weight (kg) for each animal and an increase in this ratio was taken to represent a ventricular hypertrophy (25).

Left ventricular function measurements.

In order to measure functional parameters from the left ventricle, isolated hearts were perfused according to a modification of Neely and co-workers original apparatus. The hearts were removed and immediately placed in ice cold Chenoweth-Koelle solution, the aortic stump was located and tied to a 15-gauge stainless steel aortic cannula. Perfusion with Chenoweth-Koelle buffer maintained at 37°C and bubbled with 95% O_2/5% CO_2 was initially in the retrograde manner through the aorta at a perfusion flow rate of 19ml/min. A 16-gauge stainless steel cannula was then inserted into and tied to the pulmonary vein. A 20-gauge needle attached to a pressure transducer was then inserted through the apex of the heart into the left ventricle to allow continual measurement of left ventricular pressure. Cardiac work was initiated by switching the perfusion system from the retrograde mode to the working heart mode. In the working heart mode the perfusate entered the heart via the left atrium and was pumped out through the aortic stump

by the left ventricle. The aortic outflow was subjected to an afterload of a 19cm (7.5 in) column of H_2O inside PE160 tubing. Stratham pressure transducers attached to side arms of the left atrial cannula and of the aortic outflow system were used to measure left atrial pressure and intraaortic pressure. Left ventricular pressure, left atrial pressure, intraaortic pressure, and the first derivative of left ventricular pressure were recorded. Hearts were paced at 300 beats per minute. The above preparation allowed measurement of cardiac function across a range of left atrial filling pressures by changing the pump speed and therefore the perfusion pressure. The maximal rate of rise of left ventricular developed pressure (+dP/dt), rate of decline of left ventricular developed pressure (-dP/dt), and the maximum left ventricular developed pressure (max DLVP) were recorded for analysis.

Total calpain like activity of cardiac muscle.

Caesinolytic (calpain like) activity of cardiac muscle whole cell homogenate was measured using a microplate assay procedure. Approximately 100mg of tissue was homogenized in buffer containing 100mM KCL, 20mM Tris (pH 7.5), 5mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) & 5mM DTT. 200ul of sample was added to 2mg/ml caesin, 20mM DTT, and 50mM Tris (pH 7.5) and incubated for 30 minutes at 37°C. The assay was carried out with and without Ca^{2+} (+EGTA). Minimal activity (<5%) was observed when calpastatin (calpain's specific endogenous inhibitor) was added to the assay, thus showing calpastatin inhibitable, or "calpain like" proteolytic activity. The amount of caesin degraded was measured with Coomassie brilliant blue where a 0.1 increase in absorbance at an optical density of 595nm represented one unit of calpain activity.

Ca^{2+} stimulated myofibrillar ATPase activity.

The ATPase activity of cardiac myofibrils was determined using a slight modification of a previously reported procedure (23) at 30°C in a medium containing 50 mM KCl, 20mM Tris, 1mM $MgCl_2$ and 0.5mg/ml of myofibrillar protein (pH 7.0). Following a 5 minute pre-incubation period, the reaction was started with the addition of 5mM Mg.ATP (pH 7.0). The reactions were terminated after 5 minutes with the addition of ice cold 12% tricholoroacetic acid. The inorganic phosphate liberated was determined in protein-free

supernatant and the ATPase activity corrected for non specific ATP hydrolysis. The enzymatic reactions are linear under the conditions employed here (23).

Cardiac cAMP levels.

In order to determine the ventricular cAMP levels from each group 10-20 mg of cardiac muscle tissue was homogenized (Polytron) in 2.0 mls of ice cold 6% TCA buffer for 20-30 seconds with the sample maintained on ice. The homogenates were then centrifuged after which the supernatant fractions were transferred to extraction tubes and each sample was extracted 4 times with 5ml aliquots of water saturated ether. Following extraction the uncapped sample tubes were placed in a water bath at 60°C for 10 minutes to evaporate the ether. The cAMP levels were measured using a cAMP[^{125}I] scintillation proximity assay (SPA) system (dual range) obtained from Amersham.

Statistical analysis.

All results are presented as mean values with the corresponding standard deviations. Ventricular weight to body weight ratios, calpain like activities and cAMP contents of cardiac muscle were compared within the single and repeated swimmers across the groups with a one way analysis of variance and a Tukeys post hoc test for individual group differences. The values for each of the three groups at each filling pressure in the functional measurements were compared using a one way analysis of variance with a Tukeys post hoc test to determine the group(s) responsible for any significant differences. An α level of $p<0.05$ was considered statistically significant.

Results.

Single swim sessions lasted 54 ± 5 minutes. Average attrition over the 6 week program across all the repeated swimming groups was 6% with no differences observed as a result of E64c administration.

Cardiac growth (body and left ventricular weights).

Upon completion of the 6 wk program left ventricular weight was significantly increased in the trained animals and the E64c treated swimmers compared to the controls, table 3. The E64c injected controls were not significantly different from normal controls (p>0.05). Swimming did not produce any significant changes in body weight, table 3. Therefore, the swimming groups showed increased left ventricular weight to body weight ratios from 2.41±0.20 in controls to 2.90±0.24 in the swim group (p<0.05). The E64c treated animals had a left ventricular to body weight ratio of 2.69±0.12 which is also greater than that of the control group (p<0.05). Full data tables are shown in appendix 2.

Repeated swim	n	Body Weight	LV Weight	LV Weight / Body Weight
Control	20	479±35	1.154±0.109	2.41±0.21
Swim	16	478±23	1.386±0.123*	2.90±0.24*
E64c Swim	9	469±25	1.262±0.101*	2.69±0.12*
E64c Control	6	497±24	1.163±0.098	2.34±0.16

Table 3: Body weight and left ventricular weight data of repeated swimming groups. The results are mean ± s.d. * denotes significantly different from control (p<0.05).

Calpain like activity of cardiac muscle. A single exhaustive swim decreased the total net cardiac calpain like activity from control levels of 27.26±4.64 (U/g wet wt) to 15.96±2.37 after swimming (p<0.05). The E64c treated animals showed a similar decrease to 16.66±2.99, figure 5. Cardiac calpain levels were not significantly altered across all of the repeated swim groups. The control group for the repeated swimmers showed slightly lower calpain like activity than the controls for the single swim animals with total net calpain like activity of 22.83±2.7. The repeated swimmers had levels of 20.49±6.33 and the E64c treated repeated swimmers were also not significantly different at 21.39±5.63 U/g wet weight (p>0.05).

Single swim   Repeated swim
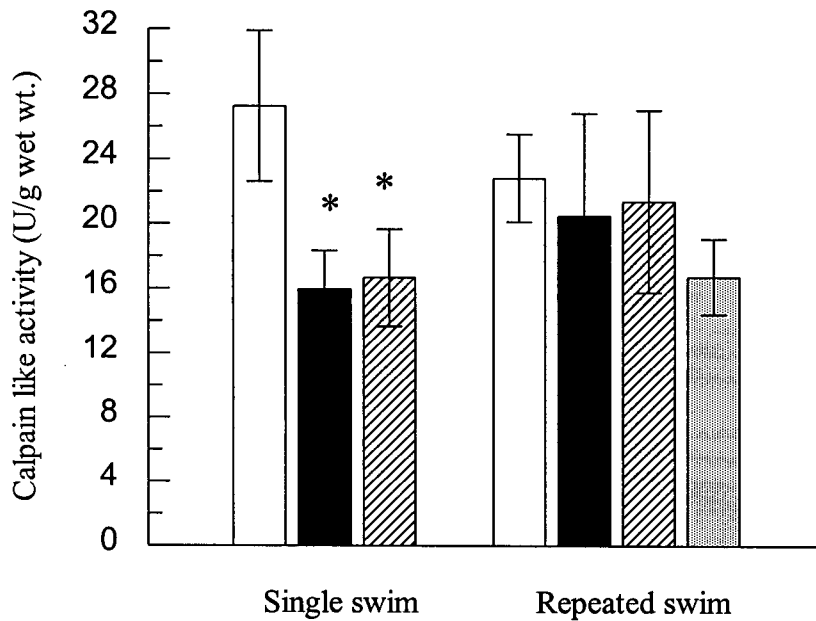
Figure 5. Ca^{2+} stimulated, calpastatin-inhibited protease activity of extracts prepared from cardiac muscle. (sedentary = open bars (n=5); swimmers = solid bars (n=5); E64c treated = cross hatched bars (n=6)) and 6 week repeated swimming group (sedentary = open bars (n=6)); swimmers = solid bars (n=7); E64c treated swimmers = cross hatched bars (n=4); E64c treatment only = speckled bar (n=4).

Left ventricular cAMP concentration. A single swim session resulted in a significant increase in the cAMP concentration from 606.03 ± 124.1 (pmoles/g wet wt) in controls to 987 ± 165.1 in swimmers (p<0.05). The left ventricular cAMP content from rats injected with E64c prior to the swim was 649.81 ± 234 which is not significantly different from controls, figure 3. The repeated swim exercise groups showed minimal differences from each other in left ventricular cAMP levels, figure 6.

Single swim   Repeated swim
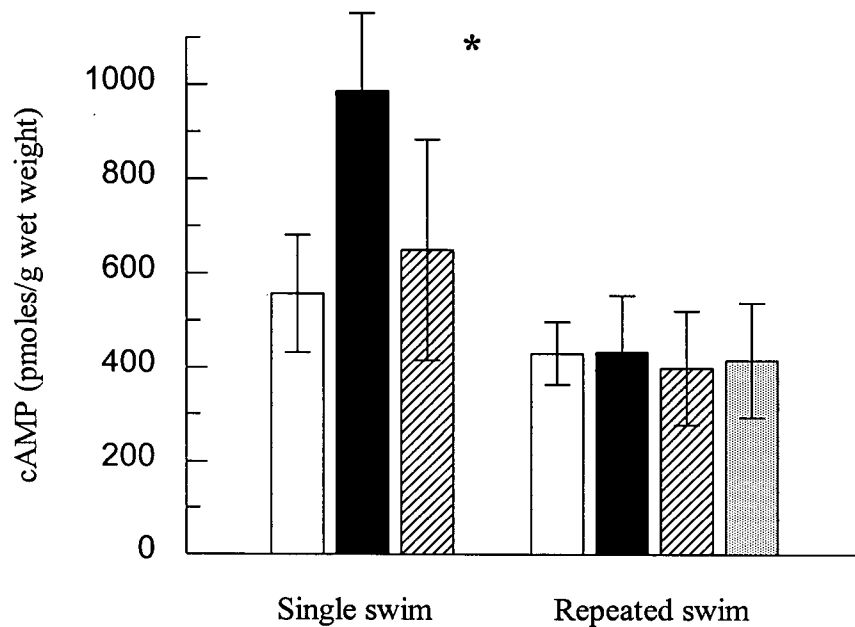
Figure 6. Left ventricular cAMP content (p/moles/g wet weight). Open bars = controls (single, n=4, repeated, n=5). Solid bars = swimmers (single, n=7, repeated, n=6). Hatched bars = E64c treated swimmers (single, n=4, repeated, n=4). Speckled bar = E64c treated control (n=5). Values shown are means ± s.d. * = significantly different from control, p<0.05.

Left ventricular function. The rate of rise of left ventricular pressure (+dP/dt) after a single swim was decreased across the range of left atrial filling pressures, at 8.1mmHg of left atrial filling pressure (LAFP) this was a 26.7% drop compared to control values (p<0.05). Hearts from the E64c injected animals also had lower +dP/dt values, with a 33.7% decrease at 8.1mmHg LAFP, figure 4. Hearts from repeated swimmers showed a similar depression from control levels of +dP/dt with a 26.7% decrease at 8.1mmHg LAFP (p<0.05), and those treated with E64c also showed depressed +dP/dt values to 33.7% (p<0.05), figure 7.
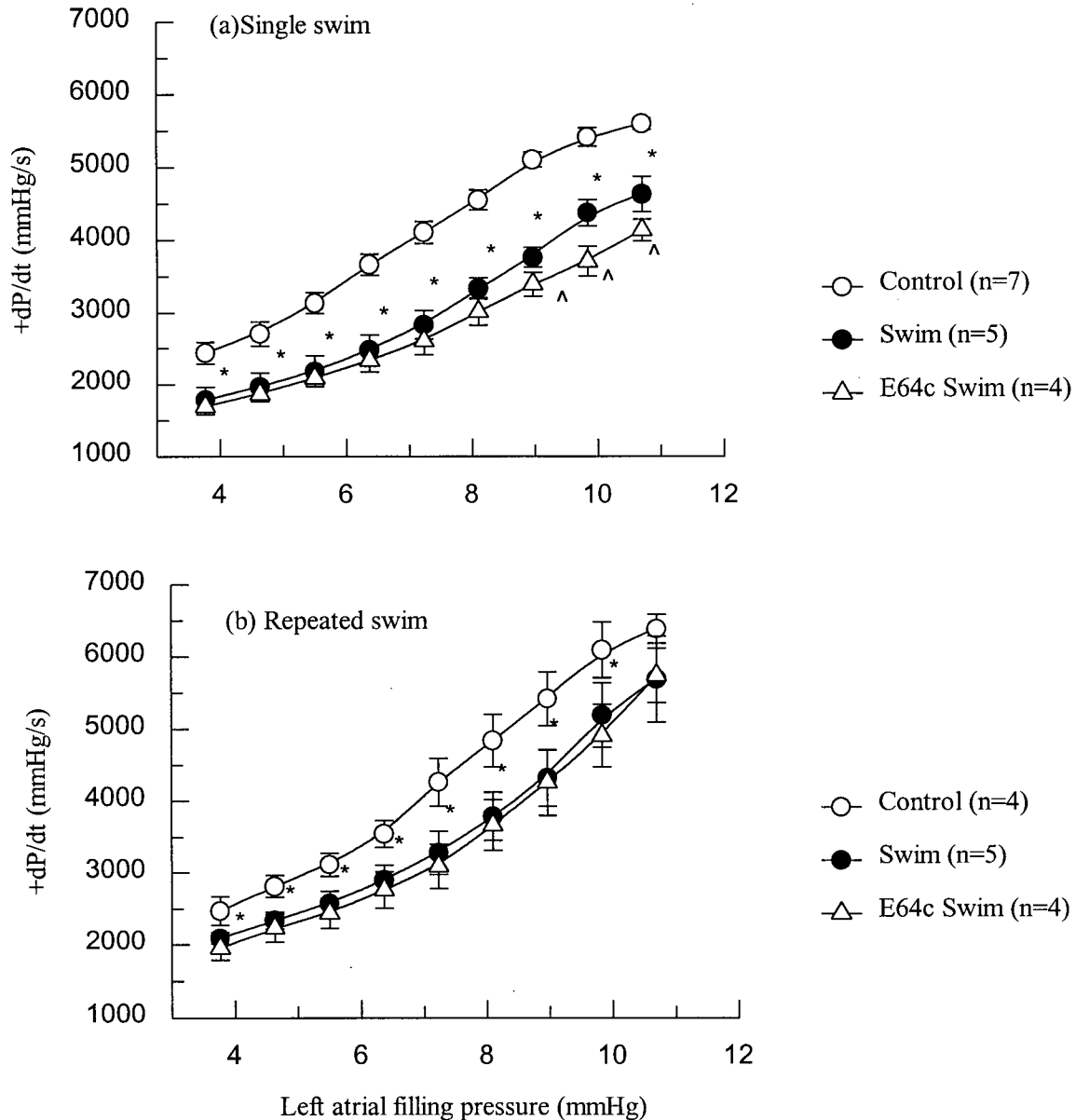
Figure 7. Rate of rise of left ventricular pressure; +dP/dt (mmHg/s). Values expressed are means and standard deviations. * = significantly different from control, ^ = significantly different from swimmers.

A single swim session also depressed the rate of relaxation for left ventricular pressure (-dP/dt) by 15.3% at 8.1mmHg LAFP and generally over the entire range of filling pressures measured, figure 8. The E64c treated rats showed a greater decrease in -dP/dt at 8.1mmHg LAFP of 33.4% from controls, this decrease was also significant when compared to swimmers (p<0.05). Swim training resulted in a lesser but still significant

decrease in -dP/dt of 18.6% (p<0.05), while training with E64c had an intermediate

11.4% decrease when compared to 6 wk controls (p<0.05).
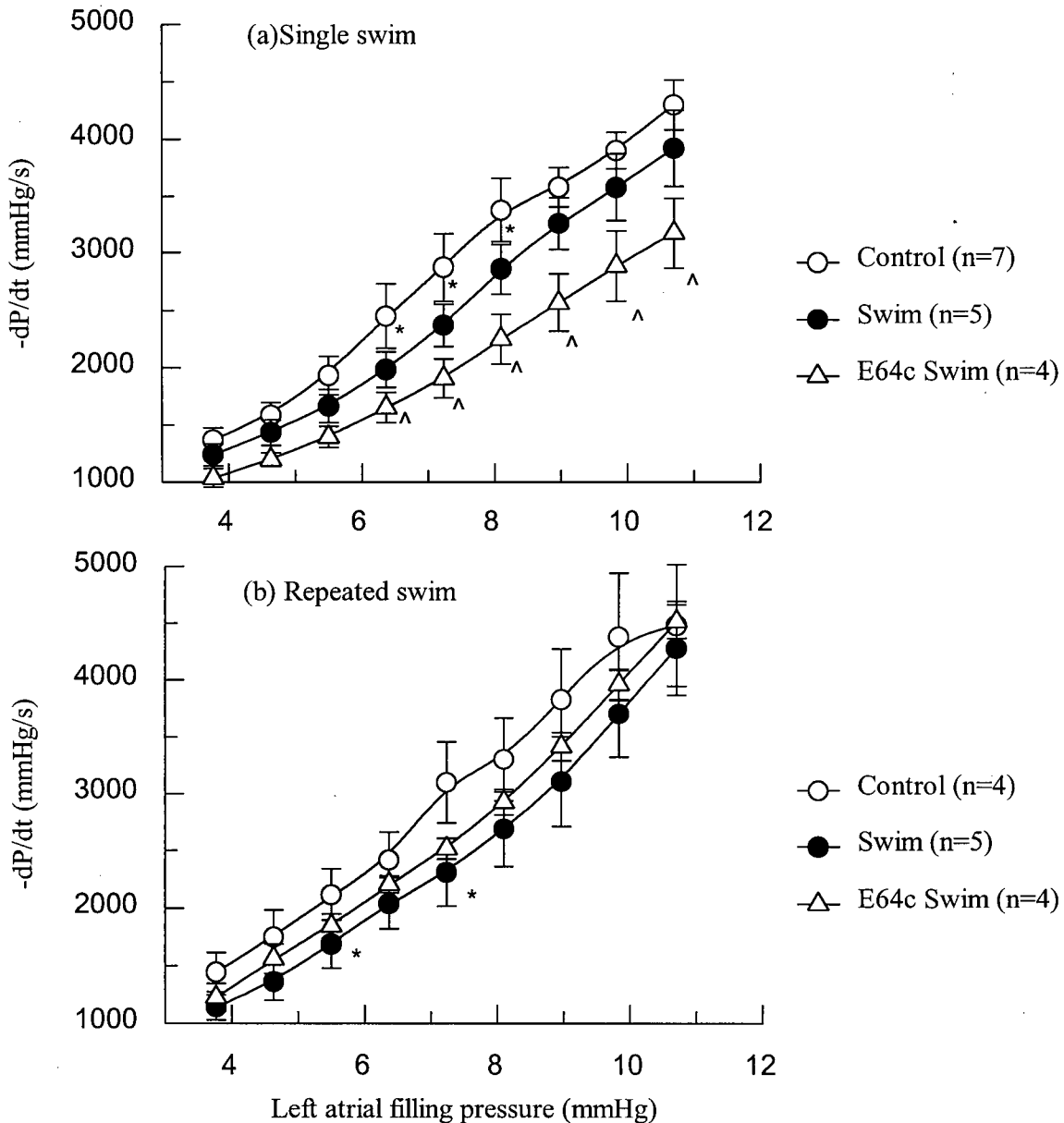
Figure 8: Rate of decline of left ventricular pressure; -dP/dt (mmHg/s). Values expressed are means and standard deviations. * = significantly different from control, ^ = significantly different from swimmers.

The only increase seen in any of the functional parameters was found for the maximum developed left ventricular pressure (max DLVP) following a single swim session. The max DLVP of the swimmers as compared to control animals being increased by 12.6% at 8.1mmHg LAFP (p<0.05), figure 9. E64c injected swimmers showed max DLVP 6.2%

higher than controls at 8.1mmH LAFP. Repeated swimming animals on the other hand showed decreased max DLVP by 15.1% at 8.1mmHg LAFP (p<0.05). This decrease was also found when repeated swimming was combined with E64c treatment, resulting in a 14.3 % decrease from controls (p<0.05) (Figure 9).
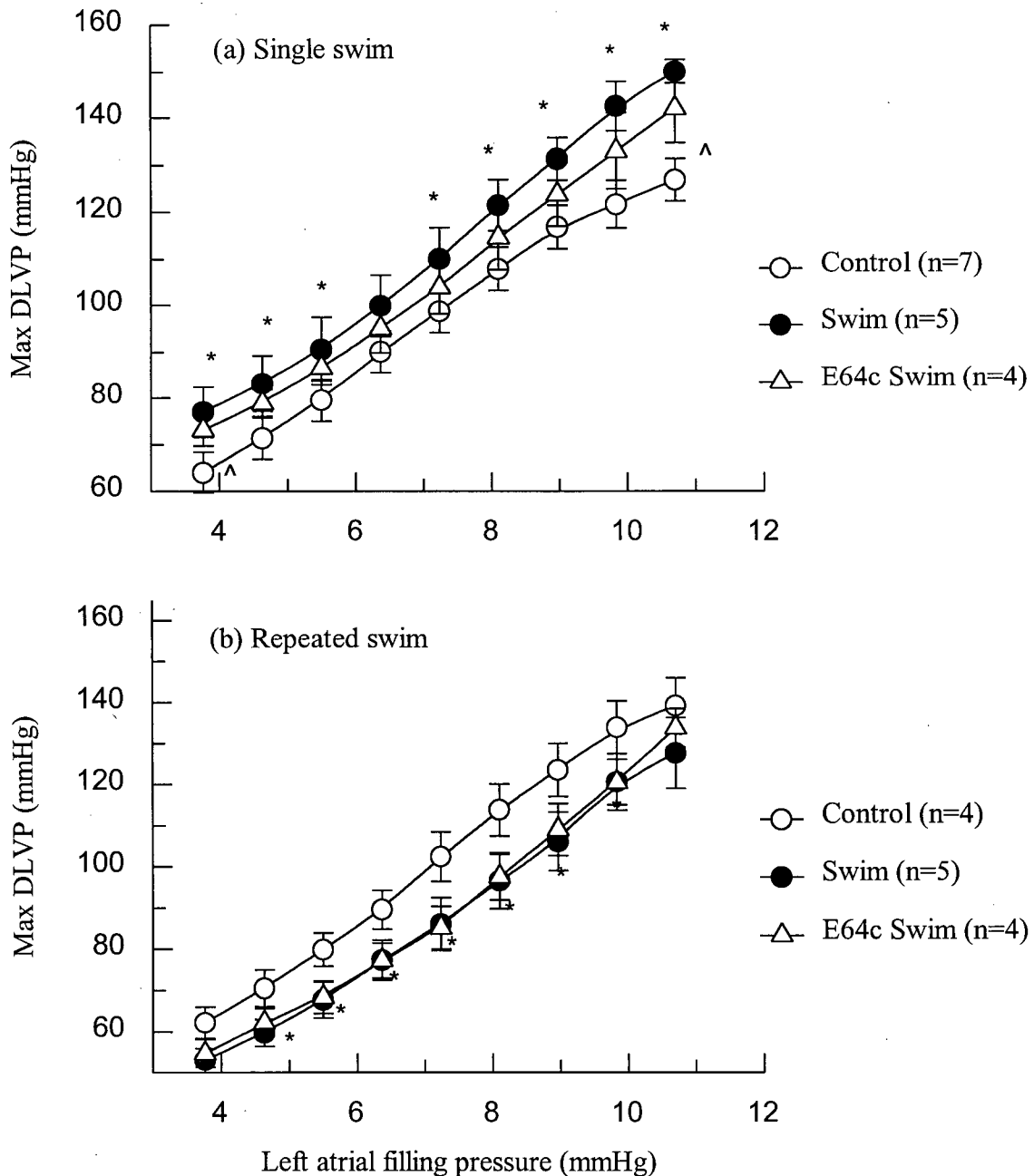
Figure 9: Maximum developed left ventricular pressure; Max DLVP (mmHg/s). Values expressed are means and standard deviations. * = significantly different from control, ^ = significantly different from swimmers.

Myofibrillar ATPase. The measurement of Ca^{2+} stimulated Mg^{2+} ATPase activity revealed no effect of either a single swim or repeated bouts of swimming on the cardiac myofibrils. E64c treatment did not effect the activity of any of the groups, table 4.

	Single swim	Repeated swimming
Control	0.180±0.04 (n=6)	0.170±0.02 (n=6)
Swim	0.167±0.02 (n=6)	0.167±0.03 (n=6)
E64c Swim	0.186±0.01 (n=2)	0.140±0.03 (n=6)
E64c control		0.178±0.02 (n=4)

Table 4. The Ca^{2+} activated Mg^{2+}-stimulated myofibrillar ATPase activity of cardiac muscle from single and repeated swimming groups. Data expressed as umol Pi/mg/minute. The results are mean ± s.d.

The effects of E64c injection alone were minimal and had no significant effects on any of the variables measured in this study ($p > 0.05$).

Discussion.

Cardiac hypertrophy in the rat is generally represented by increase in left ventricular to body weight ratio (153,178,211). It is accepted that the degree of hypertrophy resulting from exercise is not as great as that resulting from pathological hypertrophy (110). However the swim induced hypertrophy model likely represents a more natural adaptation to increased physical demand than the more rapidly induced chronic overload models that attempt to mimic pathological hypertrophies that lead to heart failure. The repeated swim protocol used in this study was sufficient to induce significant increases in left ventricular weight. The notable differences in hypertrophic response between swim exercise and treadmill exercise reported in the literature demonstrate how exercise induced cardiac adaptations are model specific (153,175,218). E64c administration (1mg/Kg body weight) 1 hour prior to swimming attenuated the cardiac mass gains induced with chronic swimming only slightly and not to a statistically significant degree. The dosage of E64c was chosen to maximize the inhibition of increased calpain activity as determined from previous observations in our lab. It seems that E64c acts to prevent increases in calpain activity but does not reduce basal activity levels. E64c combines

with the active thiol group to form an irrersible thioether, which is resistant to dialysis and competition with high concentrations of cysteine. Thus I am confident that partial purification does not remove E64c. Also of note is that in order to inhibit the calpain some kind of activating stimuli seems necessary. Pilot work had also shown E64c to be more effective when given 1 hour prior to whichever condition was being used to increase calpain activity (data not shown). Whether increasing the dosage and or mode/time of delivery would have further reduced the increase in cardiac mass cannot be determined from the results of this study. The ability of E64c to partly attenuate the swim induced cardiac mass increases suggests that further investigation is warranted, perhaps focusing on appropriate timing and dosage of E64c in the hypertrophy process. With a single swim session model it was hypothesized that total cardiac calpain like activity would be elevated, particularly since β-adrenergic stimulation (elevated cAMP levels) (105), ischemia (260), myocardial stunning (142), and treadmill running (17), all activate cardiac calpain. It was therefore surprising to observe acute swim exercise actually decreasing total net calpain like activity. In this study, 6 weeks of chronic swim training did not alter the resting levels of calpain in the heart. These results may reflect the differences in myocardial response or demand placed on the heart during swim exercise compared to running (70), for example, in the rat, the blood flow distribution to the active muscle mass results in a decrease in total peripheral resistance with running but not in swimming. Swimming also induces much less tachycardia than running (70). Such differences may be due to the vagal effects of partial or complete submersion and the fact that air breathing mammals experience bradycardia as a response to diving (185). Whether these different acute physiological responses underlie the opposite responses of cardiac calpain to exercise is uncertain. To date however, the effects of chronic physical training on the activation characteristics of calpain are not available in the literature so these results represent novel findings. An important consideration is also how this decreased net calpain activity with acute swimming is achieved, i.e. by lower calpain isoform activity or increases in the endogenous calpain inhibitor, calpastatin (82) or by some other regulatory factor(s). It is clear however, that the calpain system is not increased with acute swimming exercise and repeated or chronic swimming. The ability of acute swimming to reduce total calpain activity may be a useful model to study the

regulatory processes of calpain activity which are still not fully clear and so may prove useful in determining how calpain activity is regulated in vivo.

cAMP is known to be a candidate for regulating the hypertrophic response with β-adrenergic agonist stimulation (96,155) and swim exercise is known to increase cAMP in both skeletal and cardiac muscle (62,168). This increase in cAMP content after swim exercise is thought to be mediated by an adrenal hormone as exercise does not promote increased cAMP in animals that have been adrenalectomized and because the exercise induced increases can be blocked by the non-specific β-blocker propranolol (168). Therefore E64c's ability to prevent the increase in cAMP in this study is interesting. Whether this suggests a possible role of a calpain mediated effect upstream of adenylate cyclase, possibly at the level of the β-adrenergic receptor - G-protein - adenylate cyclase complex is an interesting question, as is the possible effect on phosphodiesterase activity to reduce cAMP (as with insulin). This is quite speculative and would benefit from some well designed experiments to determine any possible role for Ca^{2+} induced calpain mediated influence(s) at the sarcolemmal membrane where there are calpain substrates that participate in signal transduction pathways. Training studies have been shown to either increase or not affect cAMP (162), and the precise mechanism(s) underlying the cAMP changes also require further investigation. It is of interest however that despite elevated cAMP in the myocardium of the acutely exercised animals in this study there was actually a decrease in contractile function. Elevated cAMP is usually associated with improved contractile function due to PKA mediated influences on Ca^{2+} regulatory and contractile (62,243) proteins. In this case the deleterious effects of the exercise, possibly as depressed Ca^{2+} uptake (178) and lowered Ca^{2+} stores of the SR (60) may have depressed function despite PKA's inotropic actions. Also worthy of consideration is evidence that the "functional pool" of cAMP involved in contractile regulation may not be well represented by total cellular [cAMP] because not all pools of cAMP will alter Ca^{2+} dynamics or contractility of the heart (282). It is interesting also to note that PKA is itself also controlled by strict localization.

In regard to the functional responses of the myocardium, the single swim protocol of this study resulted in depressed +dP/dt and -dP/dt, which is in agreement with data showing, depressed function (138) and even acute endocardial ischemia with swimming

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(138). SR Ca^{2+} uptake has been shown to be depressed significantly following swimming to exhaustion in rats (218). This would suggest a depression in the absolute capacity of the SR to take up Ca^{2+}. On subsequent excitation a depression in Ca^{2+} loading would tend to lower Ca^{2+} stores and therefore depress SR Ca^{2+} transport and be associated with the impaired rate of tension development and relaxation (138).

Swim exercise training typically results in increased contractile function (110,153,210,211) although this is by no means a consistent finding. Data showing no improvement in functional parameters can be cited from run exercise studies (163), but generally swim studies result in some increases in cardiac performance. In this study, repeated swimming resulted in impaired contractile function as measured with a working heart preparation which is not typical of the data usually reported with swim exercise models. Our repeated swim groups also showed no significant changes in Ca^{2+} stimulated myofibrillar ATPase activity either with or without E64c treatment and suggests that the functional changes seen for +dP/dt and -dP/dt after single or repeated swims are likely due to factors other than myofibrillar ATPase. These could include alterations in muscle metabolism, altered myofibrillar elements, Ca^{2+} handling changes etc. The data from this study suggests that the adaptations induced by swimming are not dependent on activation of the calpain system and that calpain activity is not likely to be a major determinant of the hypertrophy response to swim exercise. It does provide some interesting comparisons in respect to the differences see in response to run as compared to swim exercise and this warrants some further investigation.

Conclusions.

This study assessed the effect of a cysteine protease inhibitor on the development of cardiac hypertrophy in response to swim exercise in the rat (including both biochemical and functional parameters). The hypotheses tested were

○ Calpain activity will increase following a single swim exercise session.

○ Increased calpain activity is necessary to promote cardiac functional and biochemical adaptations with repeated exercise (i.e. chronic swim training).

The absence of any well defined E64c effects on cardiac related parameters did not support an overall role for calpain in moderating the response of the myocardium to

single bouts of swim exercise and therefore also no role for the protease in long term swim exercise induced cardiac hypertrophy. It is interesting however that total cardiac calpain like activity was lowered after a single swim session, this effect also occurred in E64c treated animals, to my knowledge this is a novel finding. The increased maxDLVP, decreased +dP/dt and -dP/dt observed following a single swim were not prevented or altered by E64c administration suggesting also that calpain was not involved in mediating these functional changes. In contrast, E64c treatment did prevent the elevated cardiac homogenate levels of cAMP following a single swim bout. Cardiac hypertrophy observed after 6 weeks of repeated swim sessions was not significantly attenuated with E64c treatment, while resting [cAMP] and calpain like activities did not differ among any of the six week groups. MaxDLVP, +dP/dt and -dP/dt were reduced by repeated swim sessions when compared to sedentary animals, while E64c treatment did not change these responses, these results are somewhat different than were expected using this model of swim induced cardiac hypertrophy. Neither of the two hypotheses presented were supported by the results in this study.

In an attempt to verify that the calpain assay I was using was indeed sensitive to increases in calpain activity I proceeded to characterise the response of striated muscle to running exercise. A comparasin of running and swim exercise effects on calpain activity was not available in the literature. Partitioning calpain activity into soluble and particulate associated distributions was also attempted to gain insight into possible distribution changes associated with activity regulation.

CHAPTER 4.

Calpain distribution and activation with running exercise.

As developed in chapter 1, the possible relationship between calpain activity and swim exercise suggested that calpain effects could underlie the changes commonly seen with conditions leading to hypertrophy. Therefore an interesting question was why the running exercise which has been shown to increase calpain activity (10,17) does not induce cardiac hypertrophy when presented repeatedly as with a training program (210,211). Therefore a comparison was carried out to determine the possible differences of calpain activation with running as opposed to swimming exercise. It was my contention that the response of calpain would be different in magnitude, being greater with acute swimming compared to running and that the pattern of activation would differ also between the two exercise conditions. A significant question in terms of calpain activation in vivo was whether an alteration of intracellular localization was required (82,249), therefore analysis of the pattern of calpain activity during these two modes of activity was also a goal of this study.

Introduction.

A number of distinct morphological changes can be characterized in striated muscle after prolonged running exercise. Reports of post-exercise vacuolization of the SR and disruption of the normal sarcomeric register (22,74) primarily through weakening of the Z-line (74) are common. These exercise-induced morphological changes closely resemble the actions of calpain, which includes proteolysis of selected cytoskeletal elements in muscle (17,202). Indeed the susceptibility of certain myofibrillar proteins to calpain-mediated proteolysis increase as a result of prolonged exercise (17).

The mechanism(s) by which the activation of calpain might lead to ultrastructural changes are unclear, although the increased damage noted for sarcomeric, cytoskeletal and membrane proteins has led to the hypothesis that there is an increased translocation of active calpain from the cytosol to particulate structures with running exercise. Despite this qualitative evidence for localized calpain action no direct test of calpain localization exists in the literature. It is possible that calpain association with hydrophobic areas such

as membranes and Z-lines could be necessary to allow calpain to be exposed to high localized Ca^{2+} concentrations. The localized activated calpain, could then promote the selective degradation of bound proteins, thereby precipitating the damage response in striated muscle. That a translocation of calpain between cytosolic and bound fractions is an important event in calpain's action, concurrent with increased intracellular Ca^{2+} levels, has been reported in cultured cells (214). Changes in localization of calpain during activation have also been shown in red blood cells (127), and neurons (135). Because these in vitro changes in localization of calpain have been associated with its activation and regulation (145,248), this hypothesis seems tenable; however, no physiological in vivo assessment has been attempted.

Demonstrating calpain association with either soluble or particulate associated fractions of striated muscle is critical in support of its hypothesized role as an initiator of degradation pathways with running exercise. Moreover, protease activation, which occurs at the onset of contractile activity rather than being induced at its completion would be an essential feature of a regulated process for striated muscle. It is also important to determine the role of the model on calpain activation patterns and as such the comparison with swim exercise will provide valuable insight into the regulation of the protease. The overall aim of these studies was to elucidate the possible role of calpain in cardiac hypertrophy. Considering that swim exercise is known to induce cardiac hypertrophy and reduces calpain activity as shown in the swim study, coupled to the fact that run exercise does produce alterations condusive to calpain activation, led me to propose the hypothesis that.

O The response of calpain to a single run exercise session will be greater than the degree and/or pattern of activation than for single swim exercise session.

Therefore, the aim of this study was to investigate whether a time-dependent, exercise-induced redistribution of calpain activity occurs with prolonged running and whether the activation patterns of calpain are different to those found with swim exercise. Because the presence of calpain, as detected by immunoblots, may not be indicative of biological activity, we directly measured the enzymatic activity of calpain as Ca^{2+}-stimulated,

calpastatin-inhibited proteolysis on soluble and particulate fractions. This method allows us to assess the proportion of calpain activity associated with particulate or cytosolic fraction of the muscles studied. Rat striated muscle excised from animals after endurance running was used as the model in this study because damage-associated changes in SR reticulum morphology and myofibrillar composition have been reported for this type of activity (21,22). The calpain activity and its distribution between soluble and particulate associated fractions was measured in hindlimb skeletal and cardiac muscle from the animals studied in the previous chapter. However the cardiac muscle from the swim study had already been assessed using the whole cell homogenate technique and thus I could only compare the relative distributions of the remaining skeletal muscles from the swimmers with the muscle from this run study.

The adaptive responses to these modes of exercise are known to cause quite different effects in acute physiological responses associated with the exercise bout (70), and as such it was thought interesting to determine how the skeletal muscle calpain system responds with swim exercise. In addition, because exercise-induced muscle damage had been linked exclusively to force generation by striated muscle, a further aim of this study was to investigate the tissue specificity of the calpain redistribution to running exercise. To test the tissue-specific response, calpain-like activity was measured from heart, liver and skeletal muscle in response to a single session of treadmill running exercise.

Methods.

Male Sprague-Dawley rats (225 g) were randomly assigned to one of six groups: control or 2, 5, 15, 30 and 60 minutes of exercise. The animals were housed in a temperature-controlled environment (22-24 °C) and allowed food and water. Two days prior to the test day, animals were familiarized to running exercise on a motor-driven treadmill for 5 minutes at a speed of 15 m/min. The exercise protocol consisted of level running (0% grade) at a speed of 25 m/min for the appropriate time period. Skeletal muscle samples were taken from the animals in the single swim protocols reported in the previous chapter and treated identically to those of the run exercised animals for analysis. The running group animals were encouraged to continue running by noise and/or air

stimulation according to the guidelines of the Canada Council on Animal Care and approved by the University of British Columbia Ethics Committee.

Immediately after running, exercised and control rats were killed by administering 0.75 mg/g pentobarbitone sodium, i.p. Exsanguination and tissue extraction was completed within 4 minutes. Blood samples were collected and prepared for the measurement of plasma creatine phosphokinase activity (i.e. CPK activity) from the 60 min group only. A sub group of animals was allowed to recover for 24 hours post exercise prior to tissue analysis. The plantaris, cardiac and liver (60 min group only) were dissected out, quickly trimmed free of visible fat and connective tissue and frozen in liquid nitrogen using pre-cooled tongs. All samples were frozen within 4 min post-exercise to standardize post-mortem artifacts and then stored at -75°C.

Extraction and determination of Ca^{2+}-dependent, calpastatin-inhibited proteolytic activity (calpain-like activity) was accomplished on all tissue samples (80,185). Tissue samples (approximately 100 mg) were suspended with an Ultra-Turrax homogenizer (IKA Laboratories, model TR-10) for 20 sec at a setting of 65 in 10-15 volumes of a buffer containing 80 mM KCl, 20 mM Tris (pH 7.5), 5 mM EGTA and 2 mM DTT. The suspension was centrifuged (4°C) at 22,000 x g for 15 min (Hermle, model Z 233-M) and the supernatant (soluble fraction) decanted and stored in polypropylene tubes on ice (20 min) for subsequent assay of calpain-like activity. Following this centrifugation the particulate material was homogenized with a 2 ml Wheaton glass homogenizer (10 strokes) in 10-15 volumes of a similar buffer (as above) with the addition of 0.35% Triton-X 100 and re-centrifuged. The supernatant from the second centrifugation step (particulate fraction) was stored on ice and assayed for calpain-like activity. This procedure was judged to be adequate in removing the 80 kDa band from the first pellet, because the second pellet (following glass homogenization with Triton-X 100) contains negligible (if any) amounts of this protein as assessed previously by PAGE and immunoblotting (186).

The Ca^{2+}-dependent proteolytic activity of both the soluble and particulate fractions were determined in duplicate by a microplate assay using casein as the substrate (186). Briefly, 200 ul of soluble or particulate fractions were added to reaction mixtures final volume = 500 ul containing: 2 mg/ml casein, 50 mM Tris (pH 7.5) and 20 mM

DTT. Following a 5 min pre-incubation at 30°C, 5 mM total Ca^{2+}, 800 uM free Ca^{2+} (as determined by IONS software program) was added to one of the duplicates while the other contained 5 mM EGTA. After 30 min at 30°C, an aliquot (100 ul) of each sample was assayed for proteolysis in a total volume of 325 ul using a Bio-Rad protein dye reagent concentrate (Bio-Rad Laboratories). The protein dye reagent is composed of 0.05% (w/v) Coomassie brilliant blue G-250, 23.5% (w/v) ethanol and 42.5% (w/v) phosphoric acid. The enzyme activities (37) were determined from a casein - coomassie blue standard curve and expressed as caseinolytic activity, with a 0.1 change in absorbance at an optical density of 595 nm being equivalent to 1 unit of enzyme activity. The Ca^{2+}-dependent, caseinolytic activities of the soluble and particulate fractions are expressed as calpain-like activities because minimal activity (<5%) was observed when calpastatin (calpain's specific endogenous inhibitor) was added to the assay. The values for total calpain-like activity of rat skeletal muscle determined in this study are comparable to those reported for partially purified calpain (17).

Results.

All animals completed the appropriate time interval of exercise except for the 60 minute group, which completed an average of 58 ± 11 minutes of treadmill running. This kind of exhaustive running exercise protocol has been shown previously to result in significant reductions in glycogen of the hindlimb muscles including the plantaris (17) and also increases plasma CPK content immediately post exercise, these levels typically return to normal 24 hours post exercise (17).

Comparing total calpain-like activity for plantaris, cardiac and liver tissues from control animals revealed a tissue-dependent level of activity. The order of activities were: skeletal muscle (plantaris) < cardiac < liver (i.e. 13.2 ± 1.3, 26.9 ± 1.8 and 64.7 ± 14.7 U/g respectively) (p<0.05) figure 10.
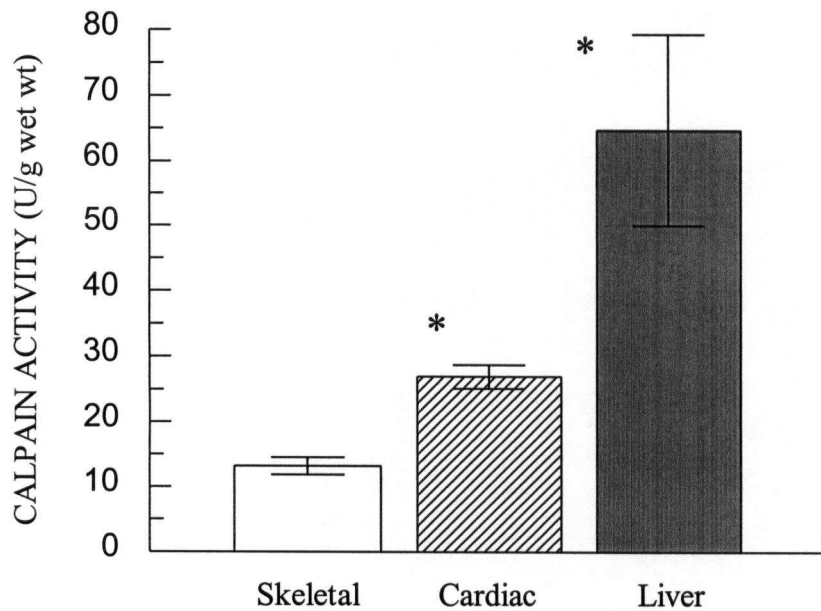
Figure 10. Control or non-exercise calpain like activities of skeletal muscle (open bar) n=5, cardiac muscle (cross hatched bar) n=5, and liver tissue (shaded bar) n=5. * denotes significantly different from skeletal muscle, p<0.05.

After 60 min of running exercise the total calpain-like activity of the plantaris muscle was increased by 35.6% over control (p<0.05) and was accompanied by a notable change in its distribution pattern, figure 11. The increase in total activity was primarily a result of an increased calpain-like activity for the particulate fraction (126.2% of control) post-exercise (p<0.05). The calpain-like activity in the soluble fraction was not different compared to the soluble fraction of control (p>0.05).
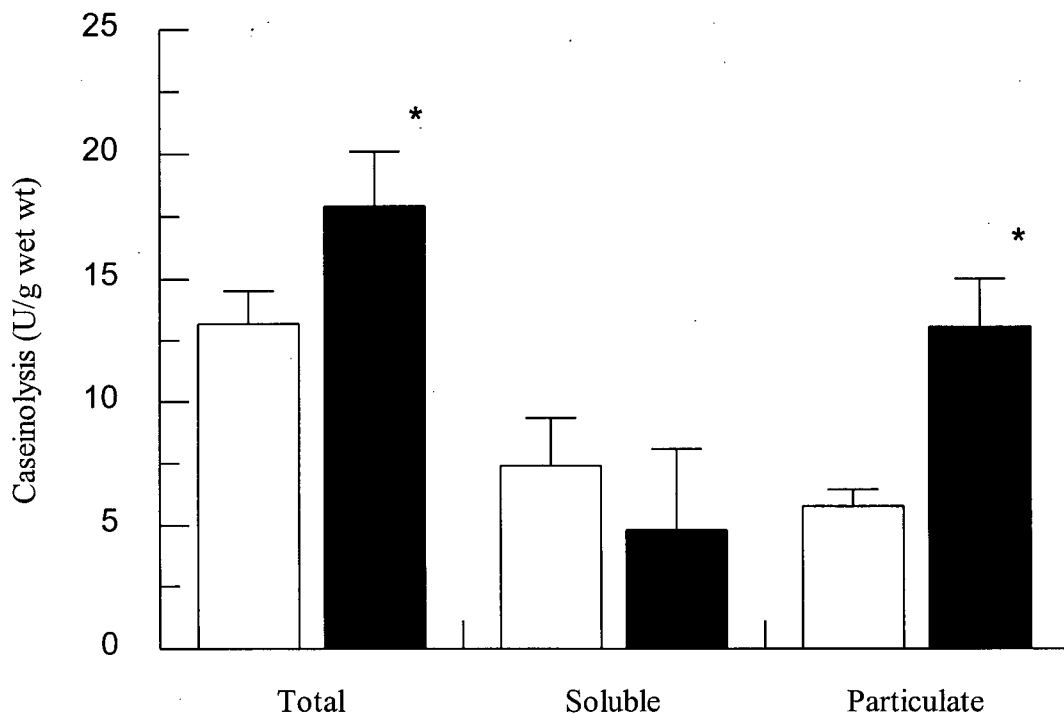
Figure 11: Ca^{2+}-stimulated, calpastatin-inhibited protease activity of extracts prepared from skeletal muscle of control (open bar) n=5, and run exercised (solid bar) n=5, groups. Animals ran for an average of 58 min on a motor-driven treadmill at 25 m/min, 0% grade. The calcium-dependent proteolytic activity of the supernatant (soluble) and pellet (particulate) fractions were determined by a microplate assay using casein as the substrate. * denotes significant differences ($p<0.05$) between control and exercise fractions.

The effect of a single swim on the total calpain activity of skeletal muscle was to reduce it by 37.4% which represents a significant decrease, 68.3%, in the soluble associated fraction of calpain, while the particulate associated activity was only slightly and not statistically significantly reduced by 12.4%, figure 12.
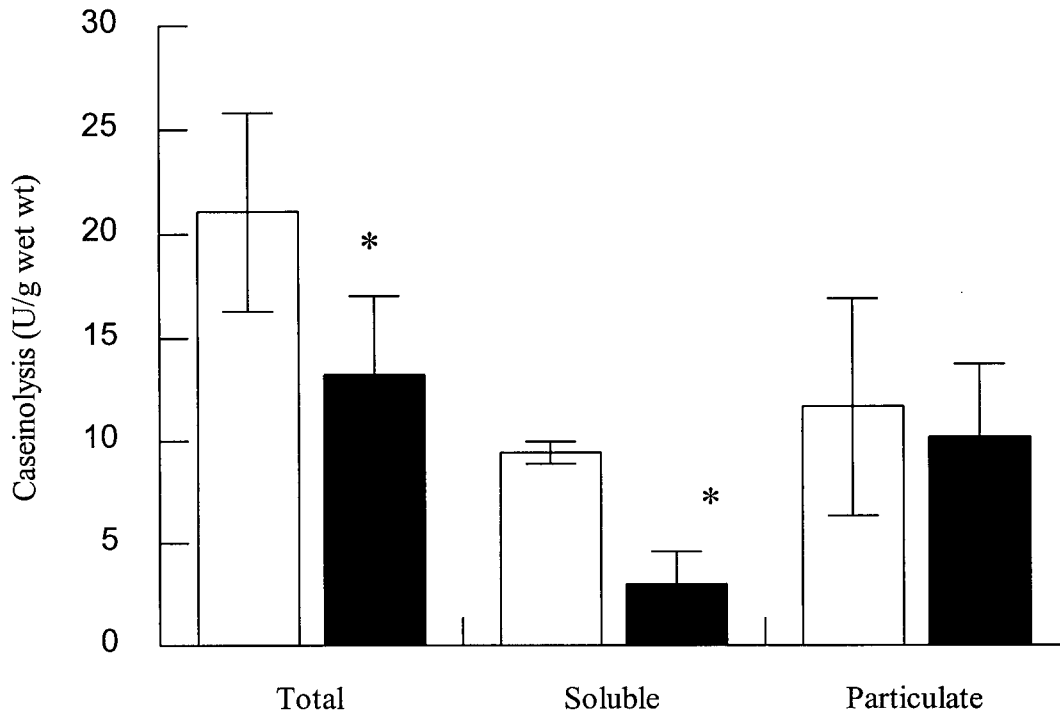
Figure 12: Ca^{2+}-stimulated, calpastatin-inhibited protease activity of extracts prepared from skeletal muscle of control (open bar) n=5, and swim exercised (solid bar) n=5, samples. * denotes significant differences (p<0.05) between control and exercise fractions.

The total calpain-like activity for cardiac muscle increased to 39.9 ± 11.6 U/g wet wt after run exercise, compared to 26.9 ± 1.8 U/g wet wt for rested controls (p<0.05) figure 13. This increase in total calpain-like activity reflects a 60.1% increase in the calpain-like activity of the soluble fraction (p<0.05) and a 42.4% increase in the particulate fraction (p<0.05) for the exercise group compared to the control group.
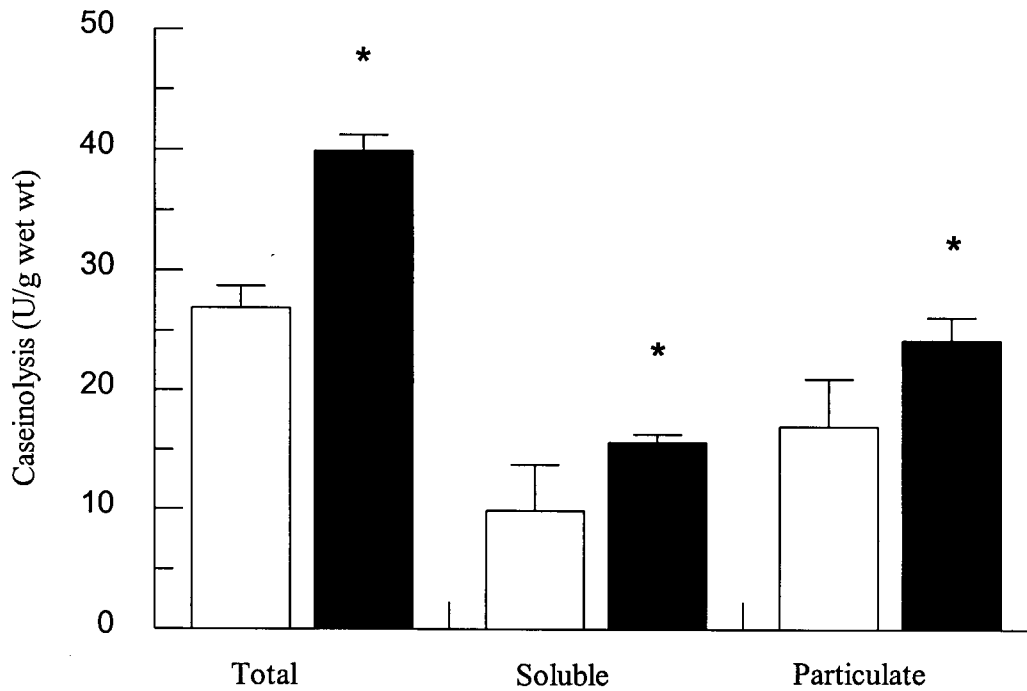
Figure 13: Ca^{2+}-stimulated, calpastatin-inhibited protease activity of extracts prepared from cardiac muscle of control (open bar) n=5, and run exercised (solid bar) n=5, samples. * denotes significant differences between control and exercise fractions at a significance level of p<0.05.
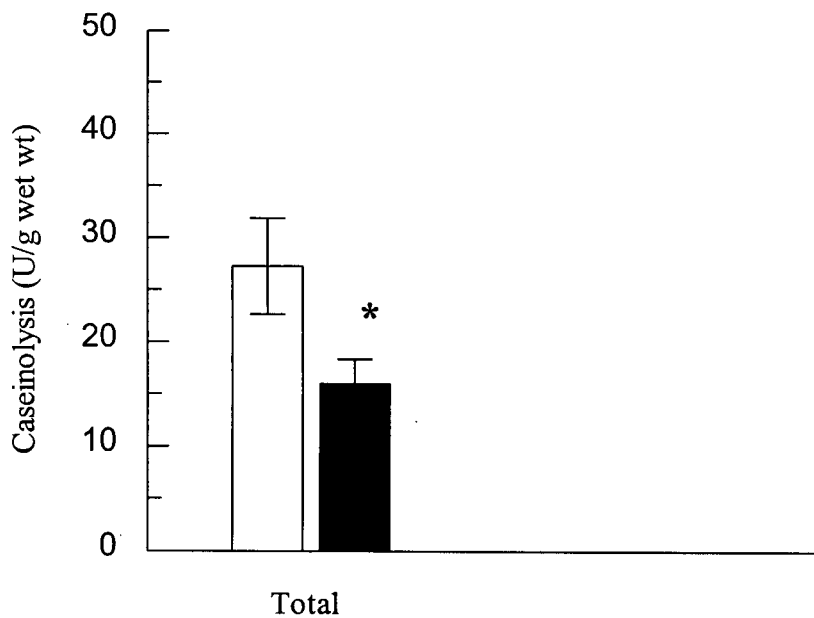
Figure 14: Ca^{2+}-stimulated, calpastatin-inhibited protease activity of extracts prepared from cardiac muscle of control (open bar) n=5, and swim exercised (solid bar) n=4,

samples. * denotes significant differences between control and exercise fractions at a significance level of p<0.05.

In contrast, 60 min of running exercise resulted in minimal changes for total calpain-like activity of the liver (control- 64.7 ± 14.7 U/g vs exercise- 69.2 ± 8.4 U/g) (p>0.05). Moreover, the values measured for the soluble (46.1 ± 7.6 U/g wet wt) and particular fractions (23.1 ± 2.8 U/g wet wt) were consistent with those noted for control tissues (p>0.05) figure 15.
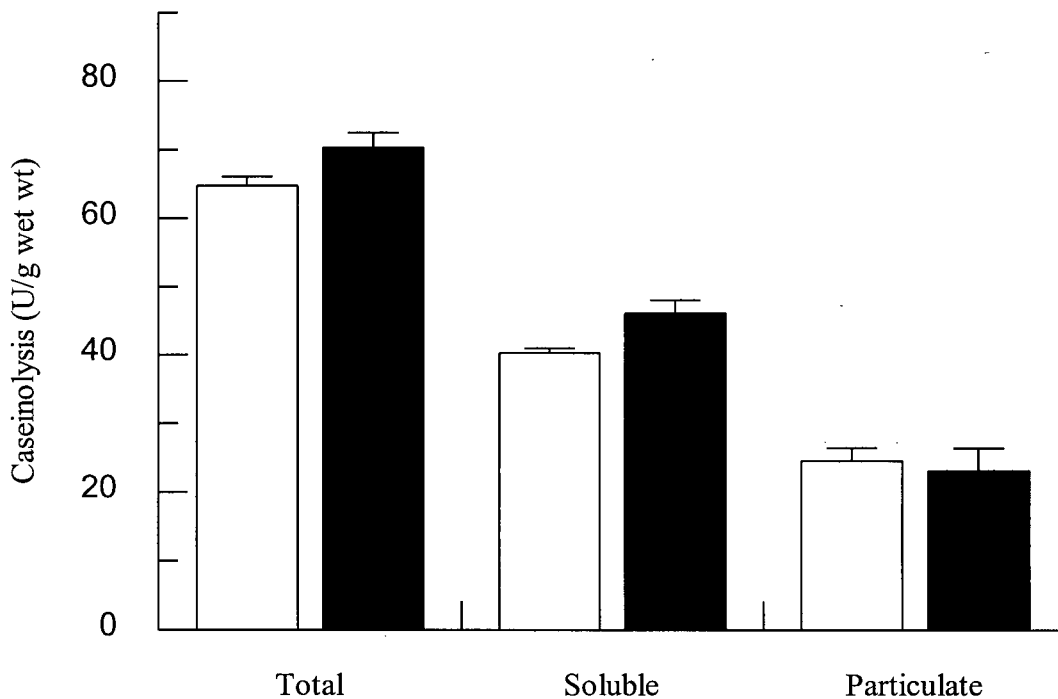
Figure 15: Ca^{2+}-stimulated, calpastatin-inhibited protease activity of extracts prepared from liver tissue of control (open bar) and exercise (solid bar) samples.

Although the increases reported for plantaris and cardiac samples were greatest for the 60 minute time point, there was clearly an early activation of activity accompanying exercise. For plantaris muscle a noticeable biphasic response occurred with a rapid peak at 2 min followed by a more gradual rise to 58 min, figure 16A. Cardiac muscle also showed a biphasic response in total calpain-like activity; however, the first peak occurred at 5 min of exercise, figure 17A. These increases were accompanied by a redistribution between soluble and particulate fractions, albeit to varying degrees for the plantaris and cardiac samples. The greatest increase was associated with the particulate fraction of

plantaris muscles occurred after 2 min, after which minimal change in distribution was measured, figure 16B). In contrast, the particulate fractions for cardiac samples were lowest at 2 min and then continued to increase, until by 58 min of exercise they were greater than those measured for control (p<0.05) (Figure 17B).
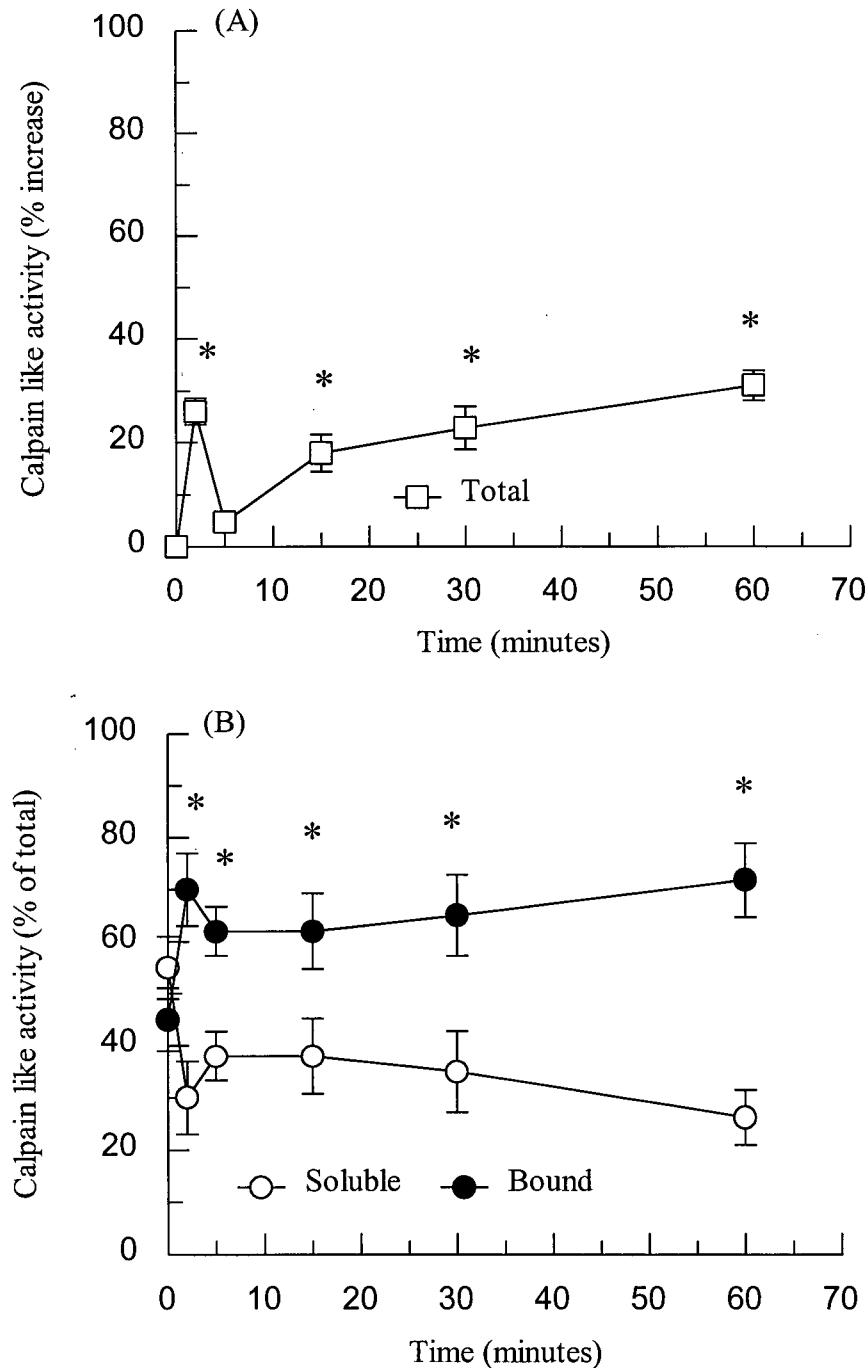
Figure 16. Time course of skeletal muscle calpain activity with increased contractile activity associated with run exercise. (A) Response of total protease activity expressed as

a percent increase over resting control values from 2 to 58 minutes. (B) The distribution
of protease activity between the soluble (free) and particulate (bound) fractions expressed
as a percentage of total activity at each time point. * denotes a significant difference
(p<0.05) for control and exercise groups.
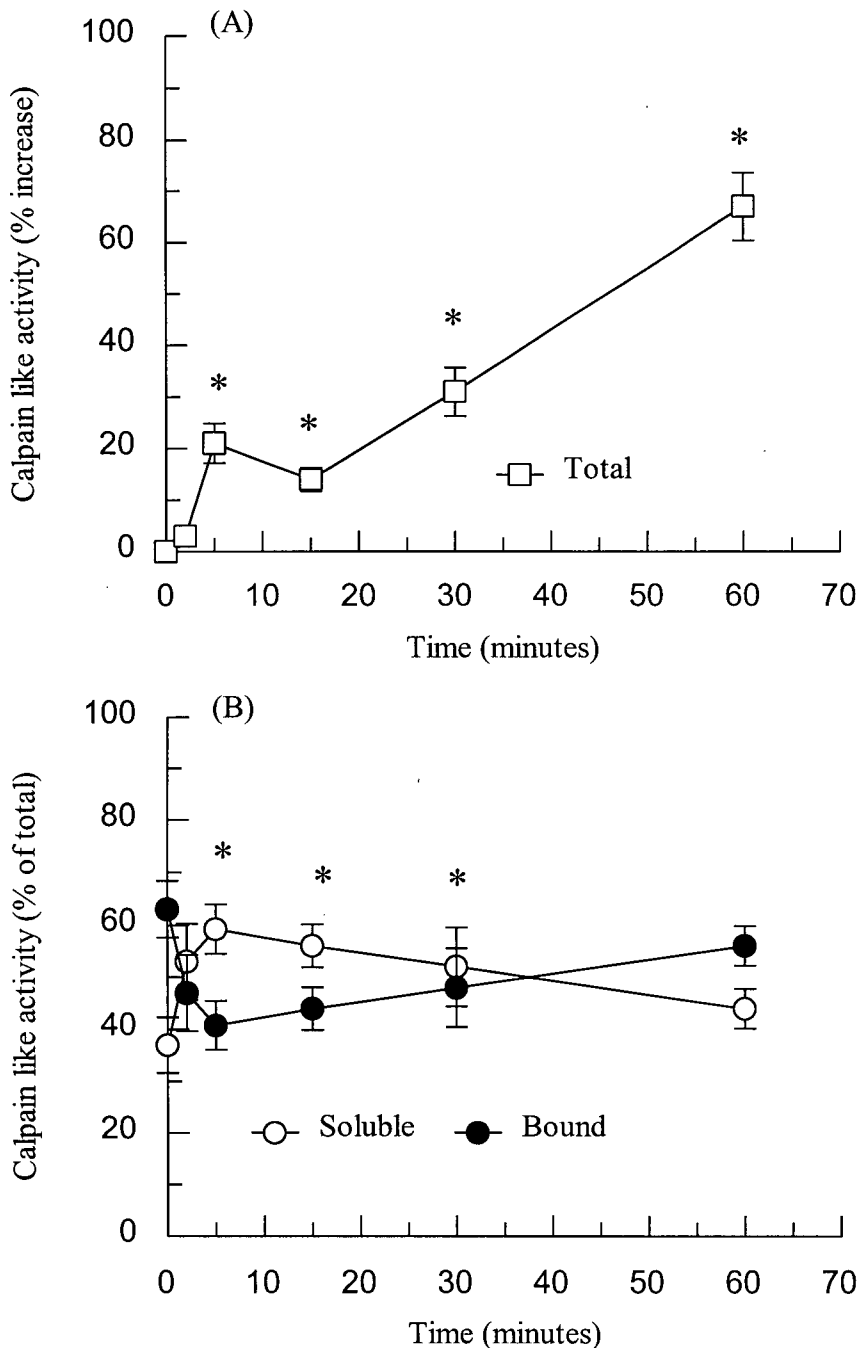
Figure 17. Time course of cardiac muscle calpain activity with increased contractile
activity associated with run exercise. (A) Response of total protease activity expressed as
a percent increase over resting control values from 2 to 58 minutes. (B) The distribution
of protease activity between the soluble (free) and particulate (bound) fractions expressed

as a percentage of total activity at each time point. * denotes a significant difference (p<0.05) for control and exercise groups.

Discussion.

These observations demonstrate that two modes of exercise, swimming or running, lead to marked differences in the activation of calpain. Calpain activity is decreased with swimming but increased in response to running. Running also prompted an increase in the particulate calpain activity which is taken to suggest a redistribution of the protease, possibly as a requirement for activation or as required for substrate targeting. When the distribution between soluble and particulate fractions are compared, it is evident that activation and distribution characteristics of skeletal muscle calpain-like activity are quite different in run as compared to swim exercise. The distribution of activation with running also shows differences when skeletal muscle is compared to cardiac muscle with the particulate fraction changing more rapidly in skeletal muscle than in cardiac muscle. In contrast, increases in the soluble fraction were only observed for cardiac muscle preparations, suggestive of an enhanced "total pool" of protease activity not resident in predominately fast-type skeletal muscle. In general, it may be concluded from this study that exercise promotes an early redistribution of activated calpain to the particulate (bound) fractions which appears to be linked to the increased demands of the exercise and that swim and run exercise models provide quite different responses of the calpain system.

The observation that resting total calpain-like activities vary within liver, cardiac and skeletal tissues agrees with a previous study reporting higher calpain activity in the liver compared to cardiac and skeletal muscle (158). Gopolakrishna and Barsky reported that 30 to 60% of the total tissue calpain activity was found associated with the particulate fraction in liver tissue (84), which agrees with our values for calpain-like activity in the particulate fraction (i.e. 38%). Whether these tissue-specific differences in calpain-like activity and distribution, measured under resting conditions, reflect differences in physiological function or importance remain speculative (202).

The finding that increased contractile activity promoted an early activation and redistribution of calpain-like activity for skeletal and cardiac muscles is a novel finding.

Exercise-induced increase in total calpain-like activity observed in rat skeletal muscle, measured after 1 hour of exercise, was comparable to changes previously reported for calpain isozymes although partitioning into soluble or bound fractions was not determined in the earlier study (17). Although the apparent time course of protease activation is slightly longer for skeletal muscle than cardiac muscle a shift in location of calpain towards the particulate fraction as a result of increased contractile activity was evident in both muscle types. The results presented here might be explained by a redistribution of a soluble (free) pool of calpain and/or activation of a previously bound inactive pool, however, it is apparent that both of these mechanisms are responsive to run exercise. For skeletal muscle, a reduction of calpain-like activity in the soluble fraction suggests a real redistribution, whereas the cardiac data is more readily explained activation of a previously inactive bound pool of calpain. This suggestion is partially supported in the literature, where activation and translocation of calpain between cytosolic and bound fractions (145,248), concurrent with increased intracellular Ca^{2+} levels, has been reported in cultured cells (214), red blood cells (127) and neurons (135). Because in vitro changes in localization of calpain have been associated with altered Ca^{2+} levels, it is important to emphasize that running exercise, as used in the study, has been shown to be accompanied by altered Ca^{2+} transport functions of the SR (18,178). Although the physiological regulation of calpain activity within the cell is not fully understood, clearly a number of factors, such as: i) intracellular Ca^{2+} concentrations; ii) state of autolysis of calpain and iii) localization and amounts of both calpain and calpastatin (82) contribute to its biological activity. Regulation of these factors is likely to be dependent on their spatial distribution within the cell. Therefore, the observation that protein damage of striated muscles following exercise is localized suggests also that the redistribution of calpain-like activity has a critical role in initiating protein degradation by regulation of the calpain-calpastatin system in vivo.

Despite the fact that the precise physiological functions of calpain are uncertain, these increases in calpain-like activity in muscle tissue(s) with running exercise and decrease with swimming exercise suggest that this non-lysosomal proteolytic system may be playing a specific role during prolonged exercise. Interestingly, prolonged running exercise is known to cause similar ultrastructural changes in both cardiac and skeletal

muscle myofibrillar protein compositions (22) which agrees with the calpain distribution changes measured following exercise in this study. There are a number of other conditions where calpain activity has previously been reported to be elevated i.e. following denervation (128), and muscular dystrophy (192,242), where SR and Z-line changes are common and suggestive of a shared mechanism underlying muscle injury. Whether redistribution of calpain activity occurs with these other models and contributes to the process of skeletal muscle injury waits to be determined. However, the data from this analysis lends credence to the hypothesis that calpain-mediated proteolysis occurs as a result of early onset subcellular redistribution for both skeletal and cardiac muscle with running exercise. With swimming however the decrease in calpain activity coupled with the lack of E64c's ability to alter the swim induced hypertrophy reported in the previous chapter argue that the swim is quite distinct and the calpain system is regulated in the opposite direction from that seen with running. As stated earlier, swim exercise may therefore provide a valuable model in which calpain activity can be reduced using a "physiological" mode of activity.

Conclusions.

The data presented here documents a number of features of the induction of calpain activity in run exercise, especially the early onset of increased activity which supports the view that calpain mediated processes contribute to exercise induced intracellular modifications including those leading to muscle cell damage or injury. In terms of the hypertrophy model of swim exercise compared to run exercise it is clear that the calpain system is being stimulated with run exercise and inhibited with swimming. The results allow me to reject the hypothesis that:

O The response of calpain to a single swim exercise session will be greater in the degree and/or pattern of activation than for a single run exercise session.

CHAPTER 5.

Calpain in isoproterenol induced hypertrophy.

As outlined in chapter 1 and more thoroughly in chapter 2, many of the myocardial responses to β-agonist stimulation (isoproterenol), in terms of ionic and ultrastructural modifications may be linked to the actions of calpain whose substrates include key proteins in the processes of ionic regulation and resulting contractile responses. There are however no studies available that have attempted to determine such a role for calpain in the process of cardiac hypertrophy. It seems that calpain does not underlie the alterations seen in response to the swim-induced model of hypertrophy and the associated physiological type of cardiac hypertrophy. However the activation of calpain in cardiac muscle during pathological states and the characteristic alterations present in the myocardium lead me to predict that calpain may be more important in pathological hypertrophy of the heart. Therefore the hypothesis to be tested in this study was.

O Isoproterenol induced cardiac hypertrophy is dependent upon the activation of
   calpain.

Introduction

In studying the process of cardiac hypertrophy the β-adrenergic system is commonly stimulated to induce cardiac growth (25,110,155). The action(s) of β-agonists are mediated by the cAMP/PKA system (98,277). The resulting phosphorylation of myocardial proteins such as phospholamban (PLB), inhibitory subunit of troponin (TnI) and cAMP response element binding protein (CREB), modulate myocardial function and gene expression (96,220,277). Although cell signaling via cAMP dependent activation of protein kinase-A is thought to be the major pathway during β-agonist stimulation, Hansen and Rupp have suggested that elevated intracellular Ca^{2+} levels which accompany β-agonist induced cardiac hypertrophy, may partially mediate the hypertrophic process (96). Indeed a common observation in various models of cardiac hypertrophy (i.e. isoproterenol-induced, or pressure overload) is the alteration in Ca^{2+} handling within the myocardium which leads to a pronounced elevation in Ca^{2+} influx (110). The consequences of this elevation in intracellular Ca^{2+} are likely to underlie

some of the functional and biochemical changes accompanying the hypertrophic process. These include re-organization of cytoskeletal proteins (273), increased Ca^{2+} sensitive protein degradation rates and modulation of selected ion channels (156,245). Ca^{2+} regulated processes are likely to play a role, either directly or indirectly in the development of cardiac hypertrophy by an unidentified mechanism. Because the biochemical and ultrastructural changes which accompany cardiac hypertrophy (see table 1 in chapter 1), are characteristic of the actions of calpain, I have speculated that this is one of the mechanisms by which Ca^{2+} may be involved in the alterations in response to β-agonist induced hypertrophy. Although the calpain-calpastatin system is known to be present in cardiac muscle (260), and its activity is increased under conditions of β-adrenergic stimulation and/or hypoxia (105), no direct evaluation of its role in cardiac hypertrophy is available in the literature. Therefore the aim of this study was to determine if indeed the calpain system could be responsible for the alterations seen in response to β-agonist-induced cardiac hypertrophy.

To test this hypothesis I employed a model of isoproterenol induced hypertrophy, together with the determination of Ca^{2+}-stimulated, calpastatin inhibited proteolytic calpain activity (calpain like activity) in individual hearts. The current assays of calpain activity in mammalian muscles required that a large amount of starting tissue be available to prepare a purified source of calpain (17). This makes the direct comparison of calpain activity and hypertrophy for individual muscles difficult to realize. To initially address the question of whether calpain activity and cardiac mass changes are linked, a muscle homogenate assay technique for proteolysis was applied which measures Ca^{2+} stimulated and calpastatin inhabitable protease activity. Furthermore, E64c was employed to help elucidate this possible role of the protease. This approach has been used by other investigators in studying the possible role of calpain in myocardial ischemia and stunning (142) and focal ischemic brain damage (15).

Therefore the purpose of this study was to determine if changes in calpain like activity for individual hearts were proportional to changes in cardiac mass, protein synthesis capacity, left ventricular function and β-adrenergic stimulation following isoproterenol-induced cardiac hypertrophy in the rat.

Methods.

Animal care and treatments.

The hypertrophic effects of isoproterenol are preceded by acute alterations such as damage and disruption to the myocardial cells. Single injection of 1mg/Kg body weight isoproterenol causes initial rapid appearance of necrotic myocytes followed by a progressive clearing or "healing process" which leaves the myocardium showing minimal evidence of necrosis after 48 hours and none after 72 hours (25). Therefore it was decided that a 72 hours post injection time point for the measurement of the variables was suitable. This time frame is also adequate for significant ventricular protein accumulation as opposed to edema formation as shown by Gordon et al who measured dried ventricular weights from isoproterenol injected animals and demonstrated an 18% increase three days after an injection of 5.25mg/Kg body weight of isoproterenol (85). Concern over edema also prompted measurement of myocardial water content which showed that with prolonged isoproterenol injection that cell water content of myocardial cells was increased only 0.7%.

Male Wistar rats (270-300g) were randomly assigned to one of three treatment groups. A control group which received a subcutaneous injection of 0.2ml saline (n=15). An isoproterenol group (iso) which received a subcutaneous injection of isoproterenol at a dosage of 1mg/Kg body weight (n=15). Finally, an E64c plus isoproterenol group, in which animals were administered a subcutaneous injection of the cysteine protease inhibitor E64c at a dosage of 1mg/Kg 1 hour prior to the isoproterenol injection (n=15). The animals were housed in a temperature controled room and were allowed standard rat chow and water. All experimental procedures were conducted in accordance with the guidelines of the Canada Council on Animal Care, and approved by the University of British Columbia ethics committee. All the measurements reflect the variables at three days post injection(s), except for the case of [cAMP], where both 24 and 72 hour post injection measures were made.

Cardiac growth, ventricular hypertrophy index: After administering 0.75 mg/g pentobarbitone sodium, i.p. Exsanguination and tissue extraction were completed quickly (within 4 minutes). Body weights were recorded prior to removal of hearts for cardiac function measurements and upon completion of the working heart procedure

where appropriate (see later in methods), the ventricles were trimmed free of any atrial material then blotted on tissue paper before being weighed. The ventricular weight (g) was divided by the body weight (Kg) for each animal and an increase in this ratio was taken to represent a ventricular hypertrophy (25).

Measurement of total RNA levels: The total cardiac RNA concentration was assayed for individual hearts and expressed as total RNA ug per mg of ventricular tissue. The RNA was measured using a guanidinium one step extraction protocol (48) in which RNA was measured by a 260-280 nm absorbance ratio.

Measurement of cardiac cAMP levels: The procedure used to determine cardiac cAMP content was identical to that reported previously in chapter three.

Total calpain like activity of cardiac muscle: Caesinolytic (calpain like activity) activity of the cardiac muscle was measured using a microplate assay procedure. Approximately 100mg of tissue is homogenized in buffer containing 100mM KCL, 20mM Tris (pH 7.5), 5mM EGTA & 5mM DTT. 200ul of sample is added to 2mg/ml caesin , 20mM DTT, and 50mM Tris (pH 7.5) and incubated for 30 minutes at $37^{o}C$. The assay is carried out with and without 0.8mM Ca^{2+} ($\pm$5mM EGTA). The amount of caesin degraded is measured with coomasie brilliant blue where 1 unit of calpain like activity = 0.1 ABS at OD 250.

Statistical analysis: All results presented are reported as mean values with the corresponding standard deviation. Ventricular weight to body weight ratios, total RNA and cAMP content and calpain like activities of cardiac muscle were compared across the three groups with a one way analysis of variance and a Tukeys post hoc test for individual group differences. The values for each of the three groups at each filling pressure in the functional measurements were compared using a one way analysis of variance with a Tukeys post hoc test to determine the group(s) responsible for any significant differences. An α level of P=0.05 was used for all statistical analyses.

Results.

Cardiac growth, ventricular hypertrophy index: Three days post isoproterenol injection, ventricular hypertrophy as indicated by an increase in ventricular weight to body weight ratio from 3.52 $\pm$ 0.29 in the control group to 4.26 $\pm$ 0.39 in the isoproterenol group

(p<0.05). The group injected with E64c 1 hour prior to the isoproterenol had a ventricular weight to body weight ratio of 3.95 ± 0.16 which is intermediate between that of the control and the isoproterenol group, table 4.

Group	LV wt / Body wt	RNA ug/g wet wt
Control	3.52±0.29 (n=6)	0.97±0.10 (n=3)
Isoproterenol	4.26±0.39* (n=9)	1.99±0.23* (n=3)
E64c + Isoproterenol	3.95±0.16* (n=8)	0.82±0.09 (n=3)

Table 5. Effects of isoproterenol injection (1mg/Kg subcutaneous) on ventricular weight (g) to body weight (Kg) ratio as compared to E64c (1mg/Kg) preinjected animals and controls. Total ventricular RNA content under the same three conditions are also shown. All measures were taken 72 hours post injections. * denotes significantly different from control values (p<0.05).

Total RNA concentrations. Isoproterenol injected animals showed increased total RNA concentration from 0.97 ± 0.10 (ug per mg wet wt) in the control animals to 1.99 ± 0.23 in the isoproterenol injected animals (p<0.05). Preinjection with E64c resulted in total RNA levels of 0.82 ± 0.09, this was not different from control levels (p>0.05), table 5.

Left ventricular function. Isoproterenol injection resulted in increased rates of development of left ventricular pressure (+dP/dt) as compared to control hearts. These increases were of 23% and 22% at the highest two filling pressures used (p<0.05). This increase was not present however in the hearts from animals pre-injected with E64c which showed +dP/dt values similar to controls at these higher left atrial filling pressures, figure 18.
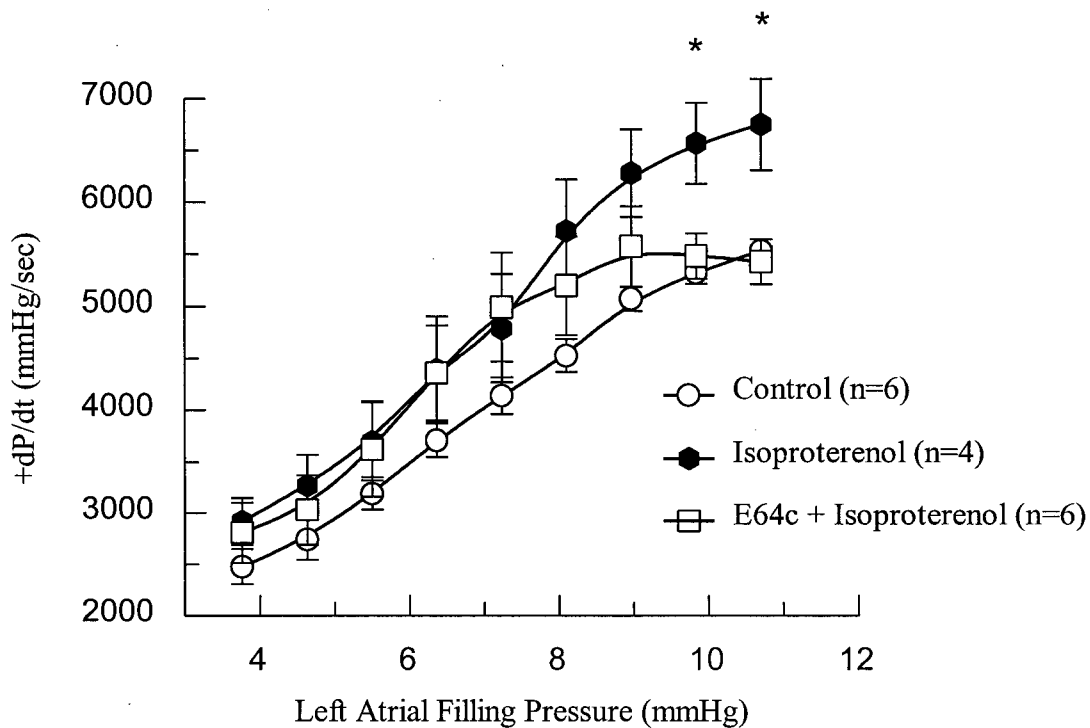
Figure 18. Effect of isoproterenol and E64c plus isoproterenol on the rate of rise of left ventricular pressure development (+dP/dt) over a range of left atrial filling pressures. Data was collected 72 hours after injections. Values shown are means ± standard deviation. * denotes significantly different from control (p<0.05).

In contrast, the isoproterenol group showed slightly greater rates of decline of left ventricular developed pressure (-dP/dt), than controls at the higher filling pressures (p>0.05), and the E64c treated group also showed no differences from the control curve (p>0.05), figure 19.
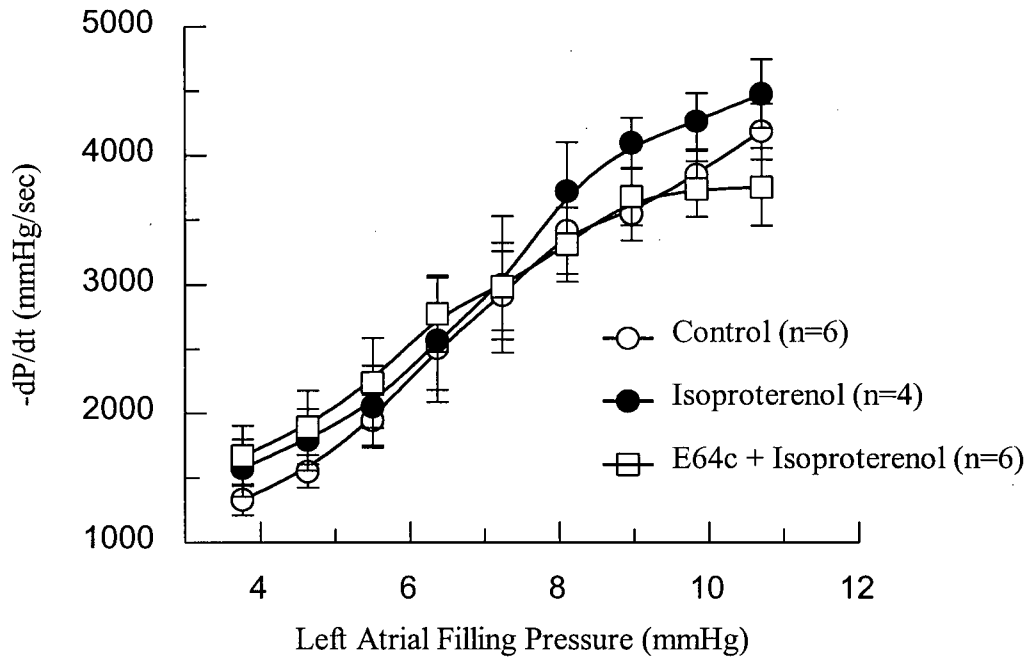
Figure 19. Effect of isoproterenol and E64c plus isoproterenol on the rate of decrease of left ventricular pressure (-dP/dt) over a range of left atrial filling pressures. Data was collected 72 hours after injections. Values shown are means ± standard deviation. * denotes significantly different from control (p<0.05).

Maximum developed left ventricular pressures from isoproterenol treated hearts were elevated compared to the control hearts. The three highest left atrial filling pressures tested showed maximum left ventricular pressures 18%, 21%, and 19% greater than control hearts (p<0.05). These increases in maximum developed pressure were prevented by preinjection of the E64c (p>0.05) figure 20.
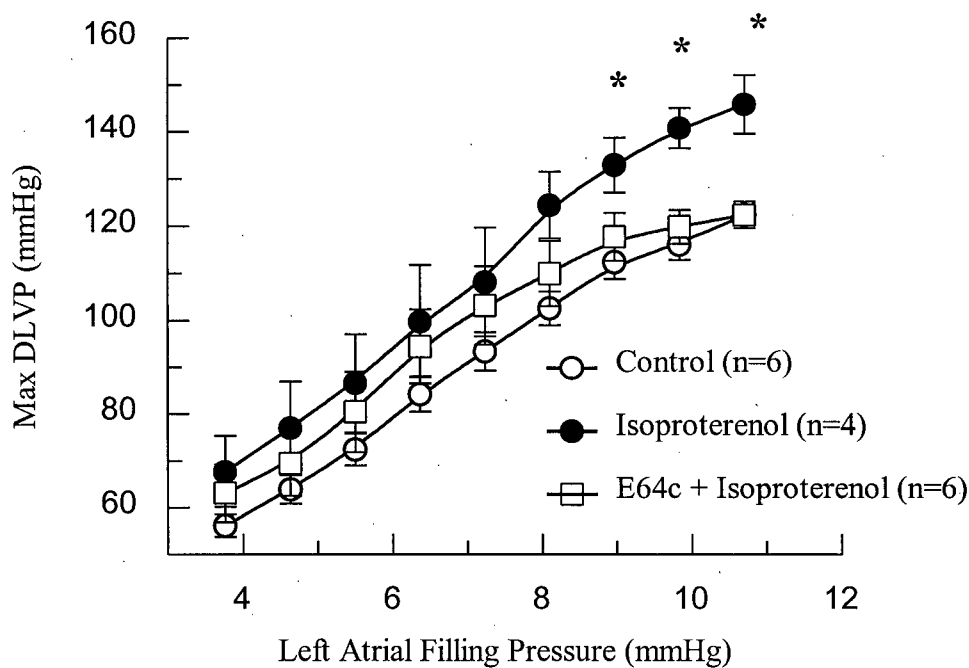
Figure 20. Effect of isoproterenol and E64c plus isoproterenol on the maximum developed left ventricular pressure (max DLVP) over a range of left atrial filling pressures. Data was collected 72 hours after injections. Values shown are means ± standard deviation. * denotes significantly different from control (p<0.05).

Preliminary experiments for this study showed that when E64c was administered 24 hours prior to the isoproterenol then there was no protective effect. When the order of isoproterenol and E64c injection was reversed, i.e. the isoproterenol given 24 hours prior to the E64c then there was also no inhibition of the isoproterenol induced increase in left ventricular function, these function curves are shown in appendix 3. In another pilot experiment, a series of working heart data was collected for a limited number of animals treated with only E64c injection. We found no significant alterations from control values in any of the functional variables mentioned when the animals received only E64c, see also appendix 3. Also shown in appendix 3 are working heart function curves generated from hearts of animals that were measured 24 hours after injections. Interestingly there are no real significant effects of E64c on the functional alterations seen in these 24 hour data sets.

cAMP content of cardiac muscle: Left ventricular cAMP content 24 hours after isoproterenol injection was increased from 606±124 to 937±205 pmol/g/wet/wt (p<0.05) and was 553±139 (p<0.05) with E64c preinjection, figure 21.
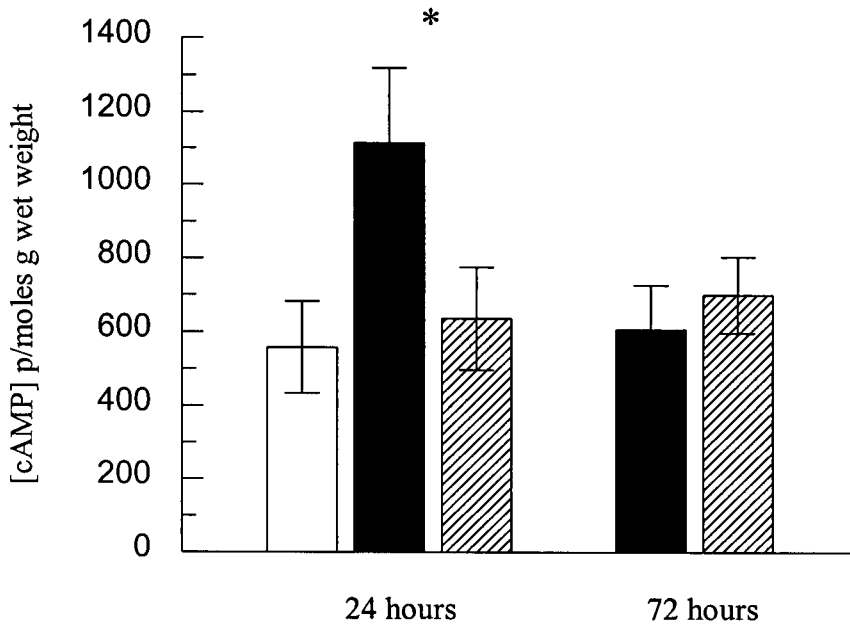
Figure 21. Effect of isoproterenol and E64c plus isoproterenol on left ventricular cAMP concentrations. Control (n=4) = open box, isoproterenol (n=6) = filled boxes, E64c plus isoproterenol = hatched box (n=4 for 24 hour group and n=5 for 72 hour group). Data represents ventricular tissue both 24 and 72 hours after injections. Values shown are means ± standard deviation. * denotes significantly different from control.

Calpain like activity of cardiac muscle: 72 hours after injection of isoproterenol the total myocardial calpain like activity was found to be 39.54 ± 4.96 Units/gram wet weight (n=5), this represents a 45% increase over control cardiac muscle values of 27.76 ± 4.64 (n=5) (p<0.05). This increase in calpain like activity was prevented by the preinjection of E64c and resulted in activity levels of 24.84 ± 1.41 (n=5) which was not significantly different from that of the control group, figure 22.
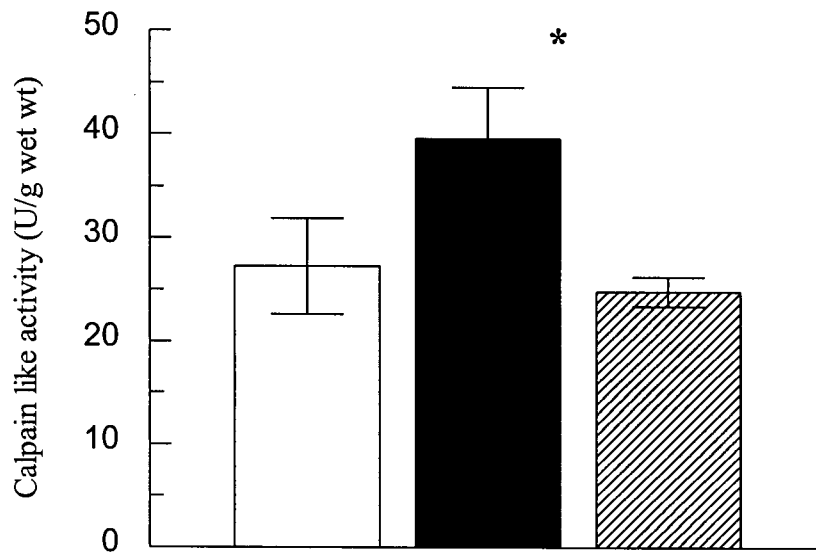
Figure 22. Effect of isoproterenol and E64c plus isoproterenol on left ventricular caseinolytic (calpain like) activity. Open box = control (n=5), filled box = isoproterenol (n=5), hatched box = E64c plus isoproterenol (n=5). Data represents cardiac tissue 72 hours after injections. Values shown are means ± standard deviation. * denotes significantly different from control.

Discussion.

The objective of this study was to determine if calpain plays any role in the biochemical or functional changes associated with β-adrenergic agonist induced cardiac hypertrophy. Biochemical and functional parameters that are known to change with isoproterenol induced hypertrophy were measured both with and without pre-treatment with the cysteine protease inhibitor E64c prior to the administration of isoproterenol. Acute isoproterenol injection resulted in significantly increased cardiac calpain activity concurrent with increases in cardiac mass, total cardiac RNA content, ventricular function, and cAMP levels. Pre-treatment with E64c prevented the increase in calpain like activity, reduced the cardiac mass gains seen with isoproterenol and prevented the isoproterenol induced increases seen in the other biochemical and functional variables measured. The results of this study demonstrate that calpain is likely to play a role in β-agonist induced hypertrophy.

Acute isoproterenol injection resulted in a significant hypertrophy after 72 hours. The degree of ventricular hypertrophy, measured as the ventricular weight (g) divided by

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body weight (Kg), was in accordance with previous studies using similar dosage and time course of isoproterenol (25,264). When E64c was given prior to the isoproterenol injection, the resulting ventricular weight to body weight ratio was intermediate between control and isoproterenol treated animals, see table 5. The measurement of left ventricular weights after working heart perfusion raised the question of whether the weights recorded may reflect increased uptake of water and edema during the perfusion process in the isoproterenol treated hearts. The values recorded for increases in left ventricular weight are similar in magnitude to those measured in previous studies using isoproterenol and measured with no prior perfusion. Hypertrophy data from pilot work with one and two days post injection animals also show significant hypertrophy when measured without undergoing perfusion (data not shown). It was also felt that the initial membrane damage to treated hearts, which may allow increased water uptake should have recovered by three days post injection (25) when the perfusion took place. The process of isoproterenol induced hypertrophy is accompanied by up regulation of a number of cardiac genes (34), this increased capacity for protein synthesis can be measured non-specifically as total RNA content of the myocardium. The elevated total RNA following hypertrophy inducing stimuli most likely represents an increase in ribosomal (rRNA) as a necessary precursory step that increases the cells capacity for protein synthesis (94). However very little is known concerning how this increase in "capacity" is coupled to actual increases in myocardial mass or protein synthesis. This increase in total RNA content seen with isoproterenol treatment is thought to be due to both increased rate of DNA transcription and increased RNA stability which are associated with accelerated protein synthesis and ribosome formation (34). Therefore the total RNA content , while reflecting the capacity for protein synthesis is not taken to represent or account for all of the increase in cardiac mass seen with hypertrophy. The data presented here demonstrates that with E64c present there is no such increase in total RNA content after isoproterenol injection. This suggests that E64c inhibits the process(es) responsible for the isoproterenol induced increase in RNA content i.e. a process dependent on a cysteine protease may be required to increase the capacity for protein synthesis.

Cardiac calpain like activity was increased after isoproterenol injection, this effect has been demonstrated previously by Iizuka et al (105). Calpain is absolutely dependent on increased Ca^{2+} levels for activation and may also require membrane phospholipids for activation (105). β-receptor coupled G_s proteins are thought to directly gate sarcolemmal Ca^{2+} channels (66) and therefore increase local Ca^{2+} levels. The sarcolemmal membrane in proximity to β-receptors could therefore provide an ideal location for calpain activation in response to isoproterenol. Indeed one of the theories proposed to explain how intracellular calpain activation occurs, states that calpain activation is a membrane associated event (248). By giving E64c injection one hour prior to isoproterenol injection we were able to prevent the increase in calpain activity seen in response to isoproterenol. In terms of other cysteine proteases in the heart, it is my contention that their role may be secondary to that of calpain in light of the structural and cytoskeletal alterations seen during the hypertrophy development. These ultrastructural changes are reminiscent of calpain action therefore suggesting that the calpain system is a likely regulator of the hypertrophy process rather than other more digestive lysosomal cysteine proteases.

Acute administration of isoproterenol is also known to result in a characteristic positive inotropic effect in response to altered intracellular Ca^{2+} and cAMP (66). We have demonstrated isoproterenol induced increased left ventricular function as determined by maximum developed left ventricular pressure, rate of rise of pressure development, but fail to show any lusitropic effect i.e. any increased rate of decline of left ventricular pressure. Acute isoproterenol injection typically increases the rate of both left ventricular contraction and relaxation (98). The primary reason for these alterations in contractile function include protein kinase A (PKA), dependent phosphorylations of regulatory proteins associated with membrane, SR and contractile proteins which are known to effect the performance of the myocardium (229). cAMP induced PKA dependent phosphorylation of voltage sensitive Ca^{2+} channels increase the influx of Ca^{2+} across the sarcolemma and PKA induced phosphorylation of phospholamban increases the rate and capacity of Ca^{2+} uptake by the SR Ca^{2+}-ATPase (98,277). These effects of cAMP result in a greater uptake and release of activating Ca^{2+}, which underlies the increased rate and force of contraction. The PKA dependent phosphorylation of

troponin-I decreases the affinity of troponin-C for Ca^{2+} and thereby allows faster dissociation of Ca^{2+} from the troponin complex. This is thought to contribute to the increased rates of relaxation following isoproterenol stimulation (278).

E64c pretreatment resulted in left ventricular function curves that were not significantly different from controls, (figures 20 and 22). Therefore it seems that the increased left ventricular function in response to isoproterenol is also in some way dependent on increased calpain activity. This could either be in response to the initial increased levels of cAMP, which can also be prevented by E64c or calpain may directly regulate some protein(s) associated with the regulation of contractile function. If the latter is true, then examination of the proteins thought to regulate functional changes in the myocardial cell may reveal possible targets for calpain. Proteolysis of these could affect contractile function directly or indirectly as they adapt to an acute isoproterenol treatment. Such possible targets may include the SR mechanisms of Ca^{2+} release and uptake, troponin-C, troponin-I, troponin-T and C-protein, all of which are thought to be involved in the functional changes and regulated by PKA phosphorylation (66).

The mechanism(s) that allow the heart to hypertrophy and alter its function in response to many hormonal or physical factors are numerous and likely to involve increased cAMP through a direct response of the G-protein, adenylate cyclase signal transduction pathway (66). Therefore the increase in cardiac cAMP content measured in our isoproterenol injected group 24 hours after injection was as expected. If indeed the isoproterenol induced biochemical and functional changes measured in this study are dependent on, or directed by cAMP regulated processes, then it is of interest that the increase in cAMP in response to β-adrenergic stimulation could be prevented by injection of E64c. This suggests that some cysteine protease regulated process (es) under elevated intracellular Ca^{2+} concentrations may be required for the transduction of the various signals, including cAMP, that direct the hypertrophy process. The precise location and nature of calpain involvement during the hypertrophy process is unclear. Speculation from the in vitro substrates and the fact that the 24 hour cAMP levels are returned to normal with E64c suggests that a likely area of involvement is upstream from cAMP production. This matches the data reported in the acute swim exercise model where the increase in cAMP can be prevented by E64c administration. This effect may indicate an

involvement of calpain in the signal transduction process at, or near the sarcolemmal membrane however the scope of such an involvement is likely to be wide as there are a range of proteins and membrane associated processes contributing to the increase in cAMP production. Further experiments are obviously warranted in order to determine exactly what role calpain may play within the signaling process in hypertrophy and at what level within the cell is this involvement occurring.

Conclusions.

The data presented in this chapter supports the hypothesis that.

○ Isoproterenol induced cardiac hypertrophy is dependent upon the activation of calpain.

Significant in this study is the fact that as expected, isoproterenol induced increased calpain activity and pretreatment with E64c was able to prevent this. Thus further scrutiny of possible calpain mediated processes with this model is possible.

CHAPTER 6.

Time course of isoproterenol induced adaptations.

It was demonstrated in the previous chapter that the overall activity of the calpain proteolytic system is increased following isoproterenol injection. The ability of E64c to prevent these increases in calpain activity and to reduce certain biochemical and functional variables suggests that calpain may be involved in these processes. To date there have been no published reports to characterize any such calpain mediated effects in cardiac muscle during hypertrophy with respect to these functional alterations. Therefore the aims of this study were to further characterize the response of the calpain system by measuring the activity of calpain-1, calpain-2, and calpastatin.

Functional changes with β-agonist induced hypertrophy likely reflect alterations in either the regulation of intracellular Ca^{2+} or alterations in the elements of the thin filaments that regulate the process of contraction in response to increased Ca^{2+} (229,258). Data from Tang and Taylor has shown increased Ca^{2+} transients leading to functional increases yet no real change in the Ca^{2+} sensitivities of the proteins of the myofibril at or around regular sarcomere lengths. However, differences in the Ca^{2+} sensitivity of certain myofibrillar proteins were noticed at higher sarcomere length (258). Both the structural and regulatory proteins of the myofibrillar apparatus (22) and also elements of the SR system (80) are known to be substrates for calpain. For example the regulatory troponin complex is known to be susceptible to calpain mediated degradation with exercise, diabetes (19), and ischemia (260). Likewise, the SR release channel is a substrate for calpain and its proteolysis alters its regulation and Ca^{2+} release from the SR (80). Therefore the modifications of either the myofibrillar proteins responsible for mediating contractile regulation or the Ca^{2+} handling characteristics of the SR were measured in this study in order to test the following hypothesis.

O Isoproterenol induced hypertrophy is dependent on contractile and SR proteins that are mediated by the effects of calpain-calpastatin activation.

Functional differences in substrate recognition and actions between calpain-1 calpain-2 remain unclear. Our lab has recently shown that in response to exercise both calpain 1 and 2 activities are increased in skeletal muscle (10) while the endogenous and specific inhibitor calpastatin was increased more slowly and over a longer time frame. Therefore secondary hypotheses for this study were:

○ The activities of calpain-1 and calpain-2 will be increased to a similar degree following isoproterenol administration.

○ Calpastatin activity will be increased following the administration of the exogenous inhibitor E64c.

Introduction.

The non specific β-adrenergic agonist isoproterenol acutely influences the myocardium by two main routes. As outlined in the previous chapter, the main route is via stimulation of β-adrenergic receptors to produce G-protein mediated signals which activates adenylate cyclase to produce cAMP. The resulting activation of PKA by cAMP results in phosphorylation of many of the key regulatory proteins of such as components of the sarcolemmal membrane (282), SR (98,220), and myofibrillar apparatus (229,244). More directly, isoproterenol is thought to promote opening of sarcolemmal Ca^{2+} channels resulting in elevated intracellular Ca^{2+} (285). The resulting functional alterations of the heart are manifest as increased rates of ventricular pressure generation, relaxation, and maximal pressure production by the heart. Data from the previous chapter (5), showed that indices of left ventricular function and the capacity for protein synthesis are increased following an acute dose of isoproterenol concurrent with increased activity of calpain. These changes are significantly reduced when the cysteine protease inhibitor E64c is given prior to isoproterenol. It was concluded that calpain may play a role in determining the myocardium's response to isoproterenol. Of particular interest was the ability of E64c to prevent the functional alterations seen in response to isoproterenol, which were still apparent 72 hours after administration. This suggested that the adaptive response of the contractile system consists of more than just a transient ionic or metabolic

effect as isoproterenol is relatively short lived and these variables are known to return to baseline fairly shortly after the initial response to isoproterenol (93). The kinetics of elimination of isoproterenol from plasma measured from the vena cava of rats shows a half time of approximately fifty minutes (93), and metabolism by the liver is totally complete within about 6 hours. The early drop in systolic blood pressure (SBP), and elevated heart rate following acute isoproterenol administration are also returned to normal quickly. Indicators of myofibrilar damage that develop rapidly after isoproterenol injection are essentially absent three days after the acute dose of isoproterenol (197).

To investigate the possible mechanism(s) responsible for these functional changes it was necessary to study the effects of isoproterenol on certain proteins of the myofibril and the Ca^{2+} handling characteristics of isolated SR fractions. It was decided to follow selected myofibrillar proteins of the myocardium that may be playing a role in underlying the altered function evident at 72 hours post isoproterenol injection. These functionally important yet sensitive myofilament proteins were followed via SDS-PAGE in order to detect any altered distribution or levels of degradation in the initial period after isoproterenol injection where significant ultrastructural alterations are known to occur. In order to determine a calpain dependence of any changes, studies both with and without pretreatment with E64c were carried out.

There are long lasting effects in response to both acute and chronic isoproterenol exposure and the functional and structural alterations seen. Therefore it was felt that a time course of the myocardial variables measured up to 72 hours should be followed in order to characterize the temporal pattern of these responses. In order to achieve these objectives the following methods were applied.

Methods.

Animal care and treatments: Male Wistar rats (270-300g) were randomly assigned to one of four treatment groups. 1) Control = this group received a subcutaneous injection of 0.2ml phosphate buffered saline (PBS) (n=30). 2) Isoproterenol = this group received a subcutaneous injection of isoproterenol in PBS at a dosage of 1mg/Kg body weight (n=60). 3) E64c + Isoproterenol = treated as above plus a subcutaneous injection of E64c in PBS at a dosage of 1mg/Kg 1 hour preceding isoproterenol injection (n= 60).

4) E64c control = this group were given the same E64c dose as the group above but then received only 0.2ml of PBS one hour later (n= 30).

All animals were housed in a temperature controled room and allowed standard rat chow and water. Experimental procedures were conducted in accordance with the guidelines of the Canada Council on Animal Care and approved by the University of British Columbia ethics committee.

Determination of tissue concentrations of adenine nucleotides: A perchloric acid extract of frozen muscle samples (approximately 70mg) was prepared by grinding / crushing muscle in a mortar and pestle under liquid nitrogen. The powder was reconstituted with 0.5ml of 0.5M perchloric acid and 0.15ml of 2M Tris, pH 6.75. The extract was centrifuged at 22,000 x g for 10 minutes. The supernatant was kept on ice and assayed for ATP, ADP and AMP concentrations using reverse phase HPLC (55). Briefly, aliquots of the samples (20ul) were made equal volume with $0.1M$ KH_2PO_4, pH 6.50. The adenosine phosphates were eluted in 30 minutes with a combination of buffer A ($0.1M$ KH_2PO_4, pH 6.0) and Buffer B ($0.1M$ KH_2PO_4, pH 6.0 10% (v/v) of CH_3OH) at a flow rate of 1.3 ml/min from a Bio-Sil c18 HL 90-5s column. The retention times and peaks were assessed at 254nm and compared against standards of ATP, ADP and AMP prepared in Buffer A at concentrations ranging from 0.625 to 5.0 mM.

Isolation and quantification of Calpain-1, 2 and Calpastatin: Pooled cardiac muscle (3.5g) was homogenized in 5 volumes of ice-cold homogenization buffer. The homogenate was then centrifuged at 13,000 g for 30 minutes and the supernatant decanted through glass wool. The supernatant (20 ml for calpain isoform isolation) was mixed with 40 ul of 1 mM-leupeptin and 4 ml of Phenyl-Sepharose beads which had been previously washed with Buffer A (20mM Tris/HCl, 0.1 mM $CaCl_2$, 10 mM DTT and 20 uM leupeptin) + 0.25M NaCl. To this mixture, 1.2 ml of 5M NaCl was added. The solution was then shaken for 5 minutes (while immersed in ice), 0.1 M $CaCl_2$ was then added and shaken again for a further 10 minutes. This solution was then poured into a 10 ml Econo-pac chromatography column. Elutions were then carried out with 8 ml of: (1) buffer A with 0.2 M NaCl, (2) buffer A without NaCl, and (3) buffer A without leupeptin (this series of washes acts to rid the column of abstract muscle proteins). Calpain II was

eluted with 16 ml of buffer B (20mM Tris/HCl, 1mM EGTA, and 10 mM DTT) with 0.1M NaCl. Calpain I was eluted with 8 ml of buffer B alone (i.e. no NaCl).

To isolate calpastatin, 1 ml of the supernatant was boiled for 5 minutes, followed by a 10 minute centrifugation at 3000 rpm. The calpastatin supernatant was then collected and stored at -80°C until analyzed.

Calpain assay: Ca^{2+}-dependent proteolytic activity was determined by measuring the release of trichloroacetic acid-soluble peptides from Hammersten casein, absorbing at 280 nm (286). 3 ml samples of eluted solution were combined with 1.5 ml of Ca^{2+} assay medium and incubated at 25°C for 30 minutes. Caseinolysis was stopped by adding 0.25 mls of ice-cold 50% trichloroacetic acid. The solution was then centrifuged at 3000 rpm for 10 minutes and the supernatant (i.e. soluble digestion products) measured spectrophotmetrically at 280 nm. All activities were measured in triplicate.

Calpastatin assay: Calpastatin activity was assayed based on its ability to inhibit calpain-2 activity. Inhibitory activity was measured by adding 150 ul of boiled fractions of isolated calpastatin to 0.2 enzyme units of calpain II. This solution was then pre-incubated at 25°C for 10 minutes. Following the pre-incubation, the remaining protease activity was determined using the protein assay described above (i.e. calpain assay). 1 unit of calpastatin activity was defined as inhibiting 1 unit of calpain-2 completely.

Sarcoplasmic reticulum Ca^{2+} uptake: Hearts were crushed to a fine powder under liquid nitrogen with mortar and pestle and suspended in a 1M KCl and 10mM Imadizole at pH 7. A cocktail of protease inhibitors were present (2um leupeptin, 1M DTT, 1mM PMSF, 10mM antipain, 28uM TPCK, 28uM TLCK, 10uM pepstatin, 1mM ATP-TRIS, and 1mM EGTA). This suspension was further homogenized with a polytron homogenizer prior to a 20 minute centrifugation at 10000g. The supernatant was discarded and the pellet resuspended to the original volume with buffer and spun again at 6000g. The resulting pellet was discarded and the supernatant was spun at 24000g for 25 minutes and the small pellet discarded. The SR component of the remaining sample was isolated by centrifugation of the solution through a sucrose density gradient of 3mls of 35% sucrose in 1M KCL with 10mM Imidazole with 5mls of 25% sucrose solution in the same buffer. 10mls of supernatant were layered onto these and spun at 41000g for 2 hours. The white fuzzy layer was harvested and diluted 1:1 with 1M KCl, 10mM

imadazole. A further spin was carried out at 41000g for 1 hour. The pellet was then resuspended in 30% sucrose, 20mM Tris pH 7, containing 2uM leupeptin and 1mM DTT. Small aliquots were then stored in liquid nitrogen until further analysis.

The following solutions were used for the measurement of Ca^{45} uptake of the isolated SR. A general buffer containing 300mM Sucrose and 25-50mM Dik PIPES pH 7. A rinse solution which contained 50uM ruthenium red, 5mM $MgCl_2$, 5mM K oxalate, 1mM EGTA in sucrose pipes. A reaction buffer containing SR fraction, 100uM EGTA, 5mM sodium azide, 5mM K oxalate in sucrose PIPES. The hot stock solution contained 5mM Mg-ATP, 200uM Ca containing 10000 counts/100ul. In eppendorf tubes, the reaction mixture was added and allowed to equilibriate at 37 degrees C. 45 micron HAWP Millipore filters were wetted with rinse buffer and the reaction was started with the addition of hot stock. At various time points, 100ul samples were removed from the reaction mixture and filtered through the wetted filters. These filter membranes were washed twice with 400-500ul of rinse buffer and then placed in scintilation vials. Membranes were allowed to sit overnight in scintillation fluid before counting.

Myofibril purification: Preparation of myofibrils involved homogenization of left ventricular tissue in 20 volumes per weight of homogenization buffer. The samples were then centrifuged at 2900 rpm for 12 minutes, the supernatant was retained and marked "cytosolic-1", while the pellet was resuspended with buffer II. The new solution was then centrifuged again, as above. Following this centrifugation, the supernatant was retained (marked "cytosolic-2") and the pellet resuspended in wash buffer and centrifuged again (as above). The supernatant was then discarded and the above step repeated. Following the last wash, the myofibrils were resuspended in suspension medium. A quantitative protein assay was used to quantify the yield of protein for each phase (i.e. homogenate, cytosolic-1, cytosolic-2 and myofibril). This protein assay was adapted from the procedure of Lowry et al (134). If these robust myofibrils were stored, centrifugation was repeated and they were resuspended in a low salt buffer and stored at -80°C.

Poly-acrylamide Gel Electrophoresis (PAGE): Myofibrillar proteins were separated by electrophoresis under denaturing and reducing conditions with one dimensional 5-15% gradient gels using a discontinuous buffer system. Concentrations of protein in the homogenate were adjusted to 50 mg/ 80 uL well. Of the 80 microliters final

volume, the SDS-PAGE sample buffer always composed a minimum of 5 uL (62.5 mM Tris-HCL, pH=6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.5% (w/v) bromophenol blue (in water)). Stock solutions were used to synthesize 1.5 mm thick gradient gels. The 5% gel solution (approx. 23 mL) was made of 3.75 mL 30% acrylamide, 5.63 mL Tris-OH (pH=8.8), 225 uL 10% SDS, 12.83 mL distilled water, 67.5 uL of 10% ammonium persulphate, and 7.5 uL TEMED. The 15% gel solution (approx. 23 mL) was composed of 11.25 mL 30% acrylamide, 5.63 mL Tris-OH (pH=8.8), 225 uL 10% SDS, 5.39 mL glycerol, 13.5 uL 10% ammonium persulphate, and 7.5 uL TEMED. A 4% stacking gel (2.6 mL 30% acrylamide, 5.0 mL Tris (pH=6.8), 200 uL SDS, 12.2 mL distilled water, 100 uL 10% ammonium persulphate, and 200 uL TEMED) was used. Ectrophoresis was carried out at constant amperage of 40 mA for 16 hours (i.e. for 4 concurrently running gels: therefore 10 mA per gel). They were stained with 0.025% Coomassie Blue (R-250) stain (45% methanol, 45% distilled water, 10% glacial acetic acid and 2.75g/1100 mL R-250) for 45 minutes (Morrisey, 1981). Following staining, the gels were destained in 2 washes of destain I (45% methanol, 45% distilled water, 10% glacial acetic acid) overnight. Finally, the gel was destained in destain II (20% methanol, 5% acetic acid, and 75% distilled water) for approximately 3 hours. The completed gel was then dried in 70-100% methanol for 5 minutes, mounted between two sheets of cellophane paper (Bio-Rad, CA, U.S.A.) and clamped between 2 perspex frames. The relative protein composition of each SDS-PAGE preparation (i.e. band intensity) was determined by a MacIntosh II PC utilizing a Hewlett Packard Scanner and IP Lab Gel Image Analysis software version 1.5 (Signal Analytics Corporation). The program acts to provide a specific quantitative value for each gel band (i.e. TnI, TnT, TM) which has been corrected for the background intensity. The intensity value for each band was also quantified relative to a preselected band (i.e. actin band at ~ 40 kDa) to account for potential loading error during sample injection. Actin was the internal standard as it is easily identifiable, significant in density, resistant to proteolysis and close in proximity to the bands of interest (17,261,275). A representative scan of a gel is shown in figure A14.

Results.

Cardiac growth and left ventricular hypertrophy: A significant hypertrophy was evident from as early as 12 hours after injection of isoproterenol. The ratio of ventricular weight to body weight continued to increase and reached a plateau at 24-48 hours, remaining significantly elevated at 3.12 ± 0.12 compared to 2.68 ± 0.10 for the control group (p>0.05), figure 25. The groups given E64c prior to isoproterenol showed a small initial rise but by 24 hours it is clear that the hypertrophy response was blunted and at 72 hours the ratio for this group was only 3.8% above control (p>0.05). E64c control animals also had ratio values not different from controls (p>0.05) figure 23.
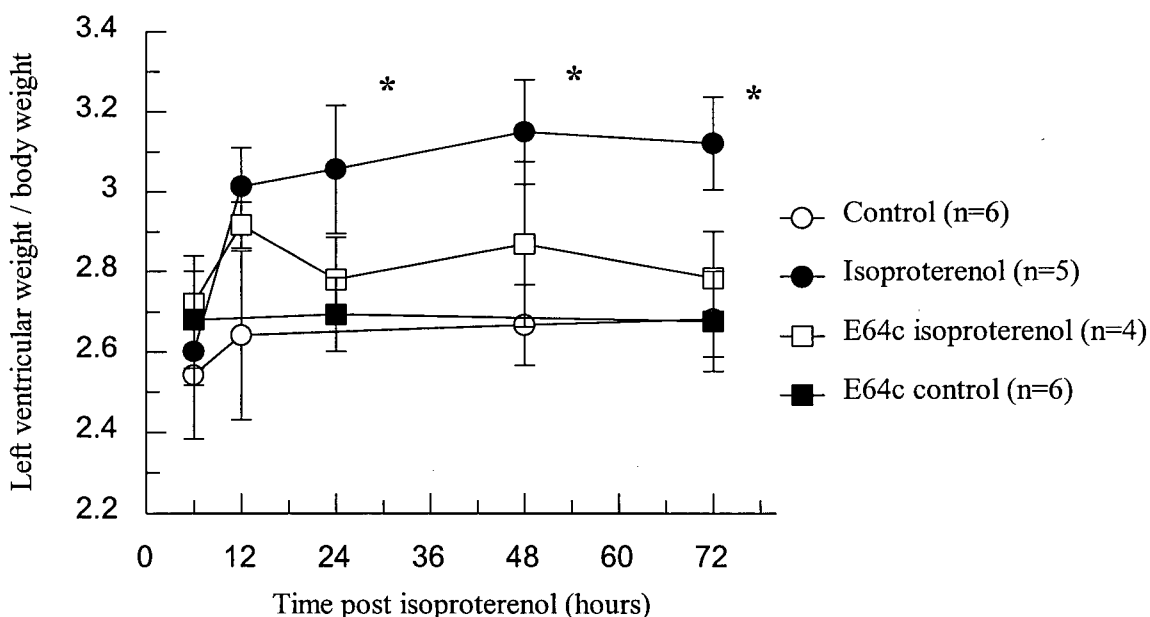
Figure 23. Effect of isoproterenol and E64c plus isoproterenol on left ventricular weight (g) to body weight (Kg) ratio over 72 hours following injections. Values shown are means $\pm$ standard deviation. * denotes significantly different from control (p<0.05).

It should be noted that the left ventricular weights in the isoproterenol group did not increase significantly until 24 hours and the increased ratio at the early time point reflected the drop in body weight seen at 12 hours post injection. Indeed the left ventricular weights of the groups all dip down a little at 12 hours before increasing over the next 12 hours, figure 24.
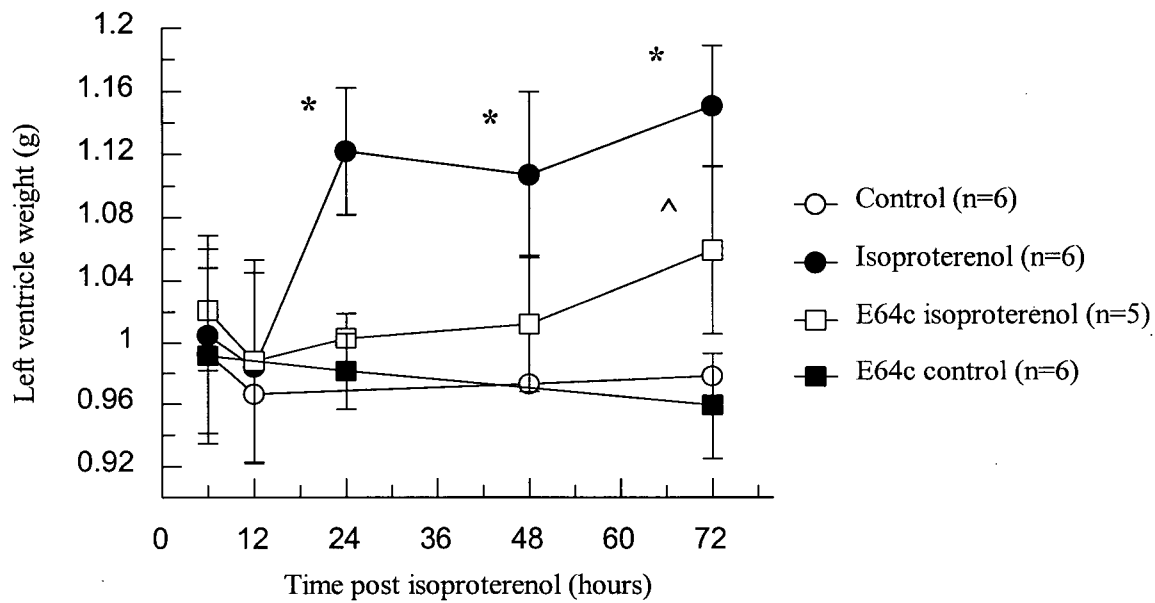
Figure 24. Effect of isoproterenol and E64c plus isoproterenol on left ventricular weight (g) over 72 hours following injections. Values shown are means ± standard deviation. * denotes significantly different from control (p<0.05). ^ denotes significantly different from isoproterenol group (p<0.05).

When the body weights were studied a transient drop in body weight following the injections was evident. The hypertrophy as shown by the left ventricular weights and the ventricle to body weight ratio were therefore only detected following 24 hours. This agrees with data on isoproterenol infusion by Brand et al who showed a significantly increased ratio 24 hours into a constant infusion protocol which then peaked at a 40% increase at 72 hours (34).
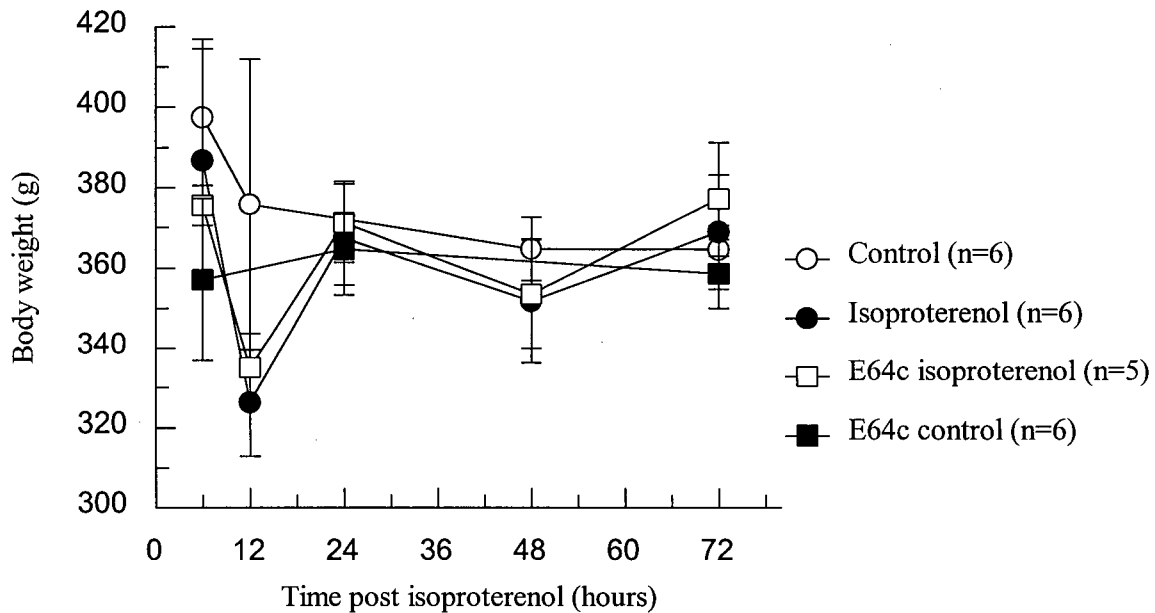
Figure 25. Effect of isoproterenol and E64c plus isoproterenol on body weight (g) over 72 hours following injections. Values shown are means ± standard deviation. * denotes significantly different from control (p<0.05).

The results demonstrate that E64c can reduce isoproterenol stimulated hypertrophy of the left ventricle.

Compilation of hypertrophy data from both isoproterenol studies: The data presented above showed a significant decrease in hypertrophy with E64c administration. The corresponding data in chapter 5 did not reach significance. This led me to combine the data from these two independent studies to compare the effects of E64c over a larger overall number of samples. The results are presented below as percentage changes from control because the different ages of the animals available meant the ratio values were not comparable between groups due to different starting body weights. When the changes are presented as a percentage of control, the combined results again show that E64c significantly prevents the hypertrophy in response to isoproterenol, figure 26. Graphs showing the left ventricular weight data and body weight data were also compared and are shown in appendix 3, figures A12 and A13 respectively. The mean left ventricular weights were significantly greater than control (p<0.05) and E64c plus isoproterenol left ventricular weights were significantly lower than the isoproterenol treated hearts (p<0.05). Body weights were not found to change significantly as a result of isoproterenol injection or E64c combined with isoproterenol (p>0.05).
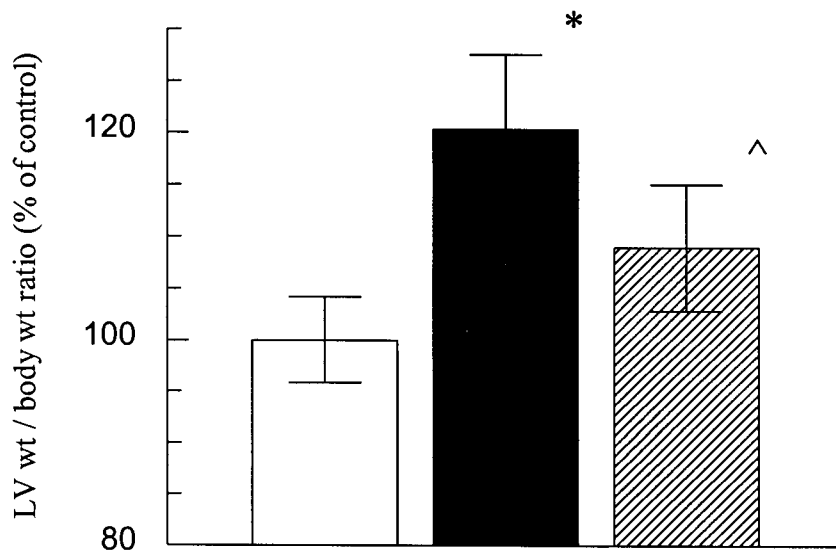
Figure 26. Left ventricular weight to body weight ratios comparing controls (open columns, n=12), with 72 hours after isoproterenol (filled column, n= 16) and 72 hours after E64c and isoproterenol (hatched column, n=13). Data shown is mean percentage of control and standard deviation, * denotes significantly different from control (p<0.05), ^ denotes significantly different from isoproterenol (p<0.05).

Calpain Activity: At the 6 hour time point after isoproterenol injection the activities of both calpain-1 and calpain-2 were significantly elevated (p<0.05). Peak levels in activity were recorded at the 12 hour time point with 0.693 ± 0.036 U/g and 0.703 ± 0.047 U/g for calpain-1 and calpain-2 respectively, (p<0.05). Activities then declined over time but remained elevated at the 72 hour time point, figures 27 & 28.
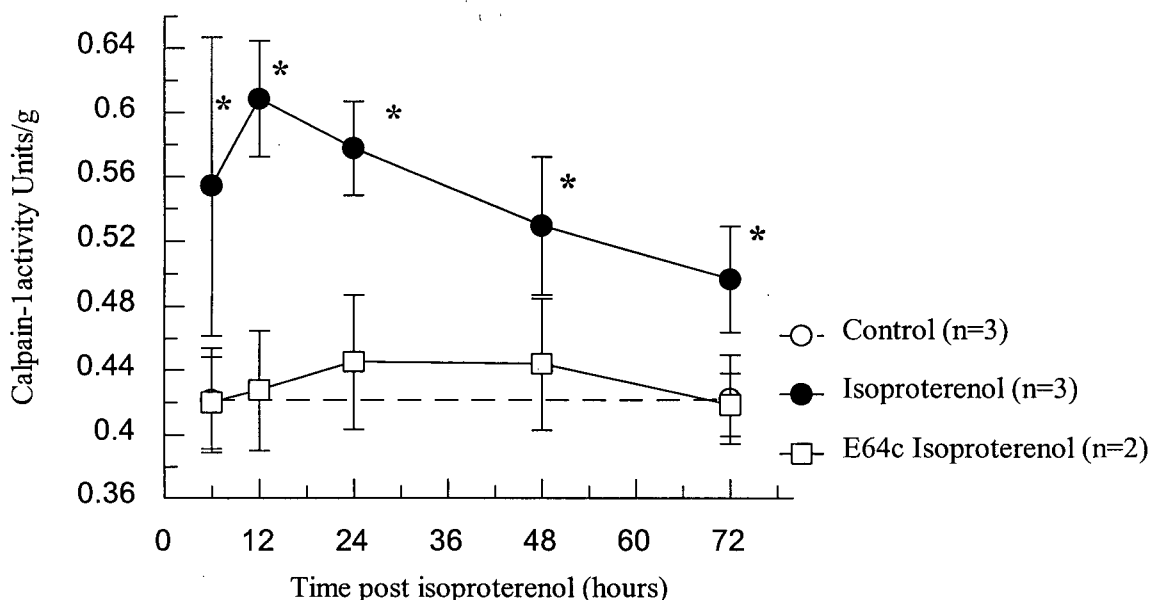
Figure 27. Effect of isoproterenol and E64c plus isoproterenol on calpain-1, activity over 72 hours following injections. Values shown are means $\pm$ standard deviation, control data only available at 6 and 72 hour time points. * denotes significantly different from control (p<0.05).
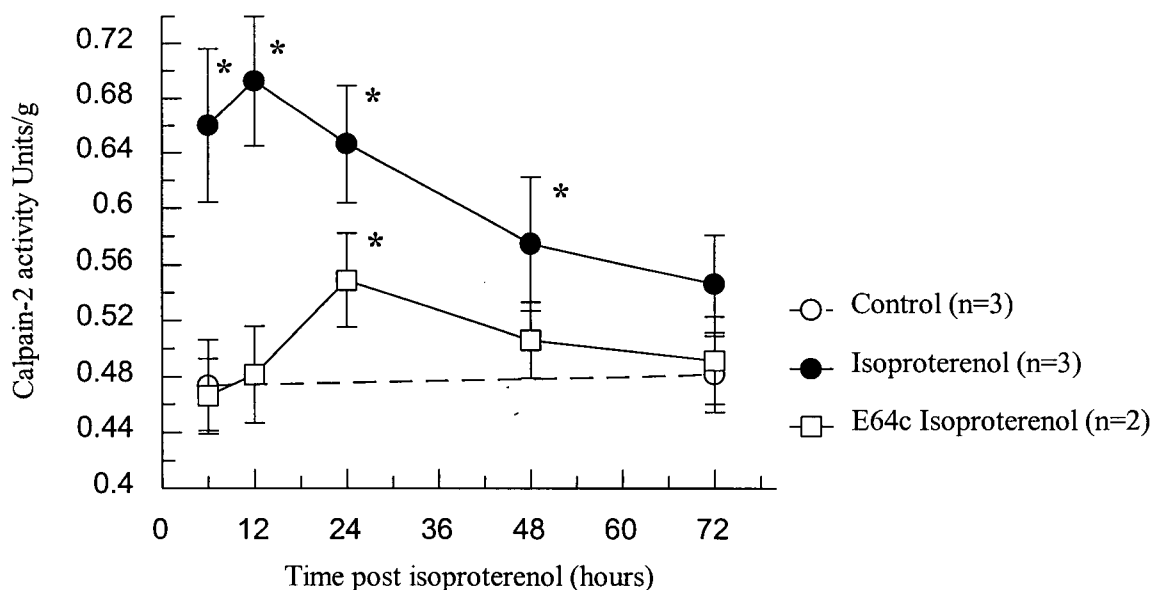
Figure 28. Effect of isoproterenol and E64c plus isoproterenol on calpain-2 activity (higher Ca^{2+} isoform), over 72 hours following injections. Values shown are means $\pm$ standard deviation, control data only available at 6 and 72 hour time points. * denotes significantly different from control (p<0.05).

It can also be seen in figures 27 and 28 that E64c prevented the increase in calpain activity associated with isoproterenol injection. Although at 24 hours calpain-2 activity

is 0.549±0.033 U/g which is significantly greater than control (p<0.05), this value is significantly less than the isoproterenol group (p<0.05). E64c values were not significantly different from controls (see data in appendix 2). It can be clearly seen that activities of both isoforms increase over the first 12 hours and then return towards control levels. E64c given one hour prior to the isoproterenol can prevent these increases in calpain activity, confirming the earlier report on whole homogenate calpain activity (5).

Calpastatin activity following isoproterenol was increased and showed a slower response than the calpains, reaching a maximum at the 24 hour time point at 0.696±0.063 U/g and then returning to control levels at 72 hours (p>0.05) figure 29. A different pattern was found in the group given E64c prior to isoproterenol. Calpastatin activity was elevated to higher levels than with isoproterenol alone and remained elevated even at 72 hours. Its activity of 0.761±0.053 U/g was still significantly above both the control and isoproterenol groups at 0.608±0.047 U/g and 0.634±0.041 U/g respectively (p>0.05) figure 29.
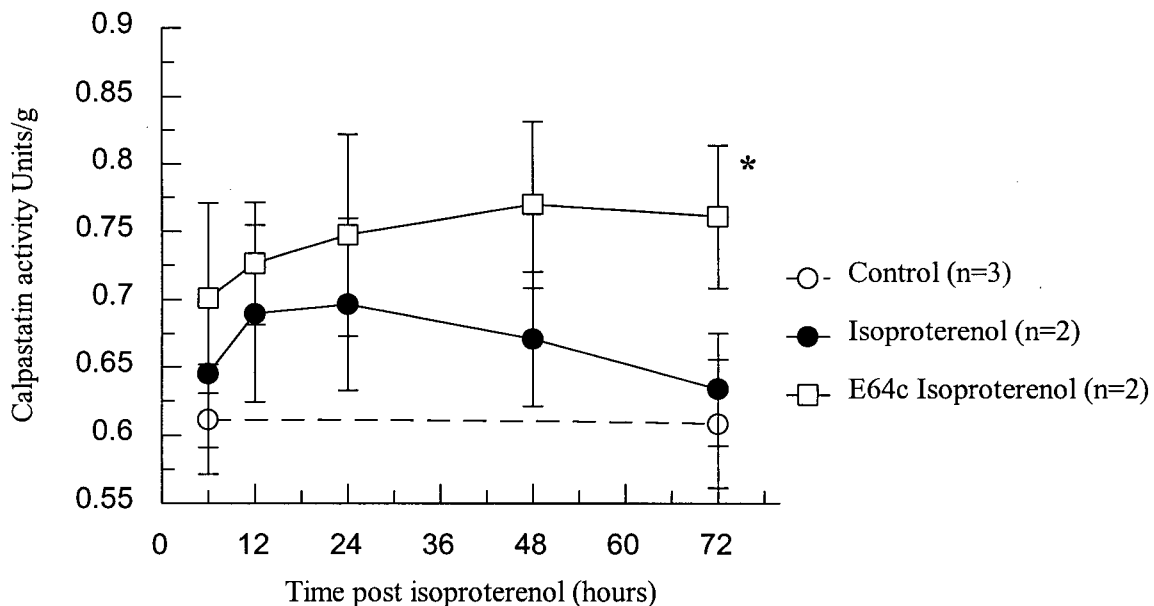
Figure 29. Effect of isoproterenol and E64c plus isoproterenol on calpastatin activity over 72 hours following injections. Values shown are means ± standard deviation for control data with only two samples available at each time point for the isoproterenol conditions.

Calpain to calpastatin ratio: The ratio of calpain to calpastatin is shown below in figures 30 & 31. This ratio can be taken as an indicator of Ca^{2+} activated cellular

proteolytic potential and a ratio greater than one may reflect a situation where the inhibitor is unable to completely regulate calpain activity and therefore allow calpain-mediated effects. The ratios for both isoforms of calpain are initially increased significantly following isoproterenol injection. These ratio's remain elevated although they decrease towards control levels at the 72 hour time point. The ratio of protease to inhibitor is reduced for both isoforms when E64c is given prior to isoproterenol.
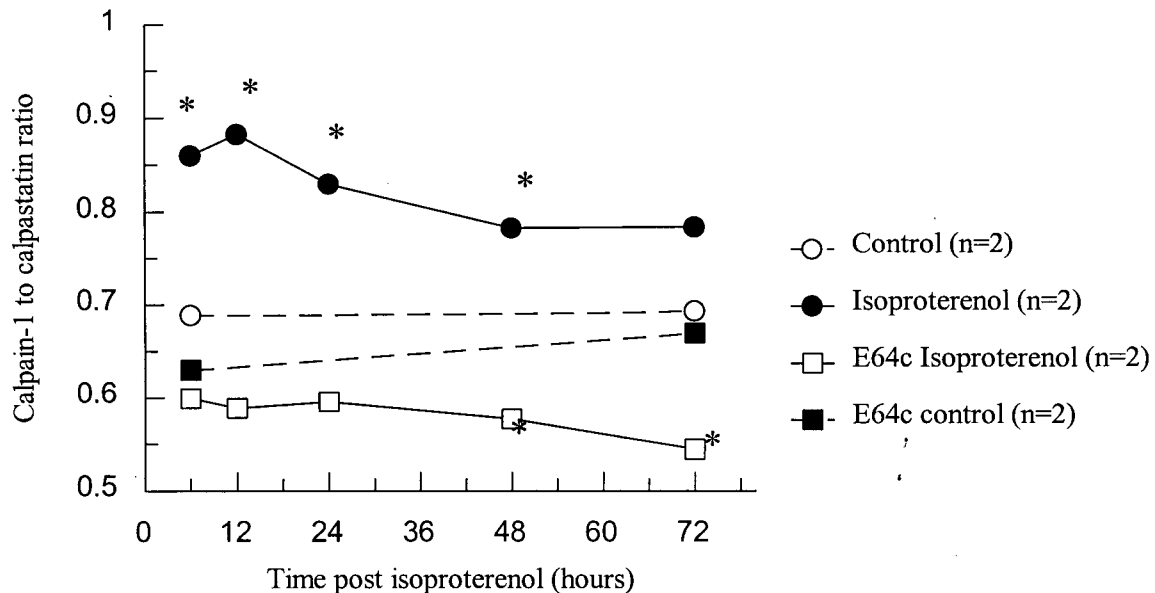
Figure 30. Effect of isoproterenol and E64c plus isoproterenol on calpain-1 to calpastatin ratio activity over 72 hours following injections. Values shown are in ratio form with control data only available at the 6 and 72 hour time points.

120
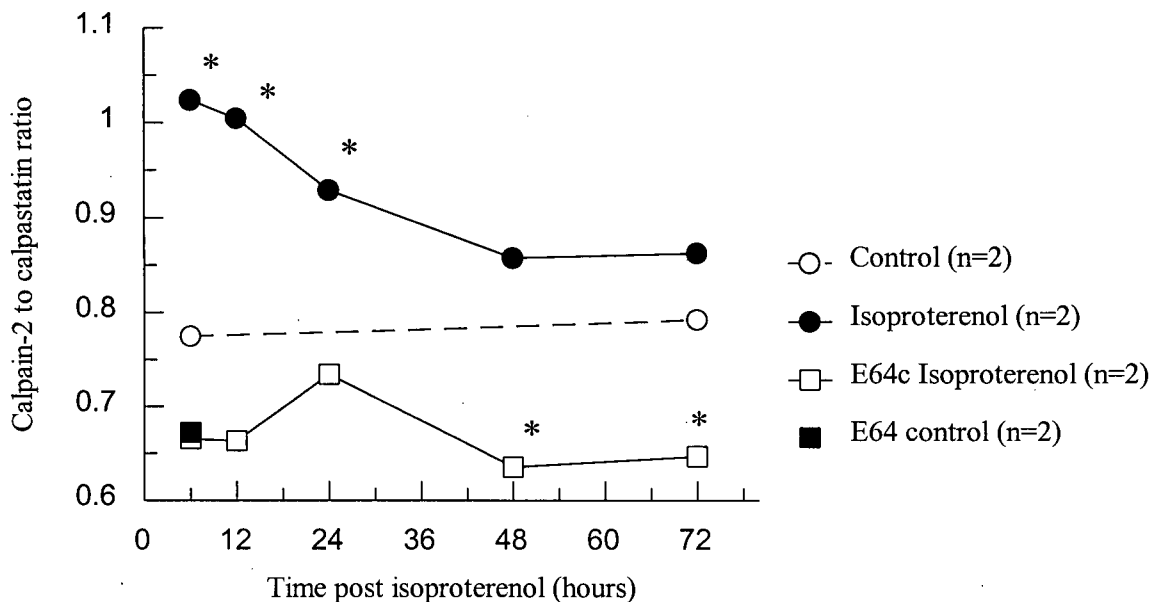
Figure 31. Effect of isoproterenol and E64c plus isoproterenol on calpain-2 to calpastatin ratio over 72 hours following injections. Values shown are as a ratio with the control data only available at the 6 and 72 hour time points.

Adenine nucleotides: As expected in this model, ATP concentration was lowered from control levels of 4.17 ± 0.26mM and at 24 hours post isoproterenol was decreased significantly by 30.7% to 2.89 ± 0.37mM ($p<0.05$), figure 32. Interestingly, E64c reduced this drop to only 3.65 ± 0.37 mM which is 17.5% lower than control values at the 24 hour time point. The same trend was seen with the total adenylate values although E64c is not as protective here against the isoproterenol induced decreases, figure 33. With [ADP] and [AMP] the variability was larger and the trend was for lower values following isoproterenol injection but not dramatically, however it seems that the E64c groups are reduced less. E64c controls showed no significant differences compared to controls (see data in appendix 2).
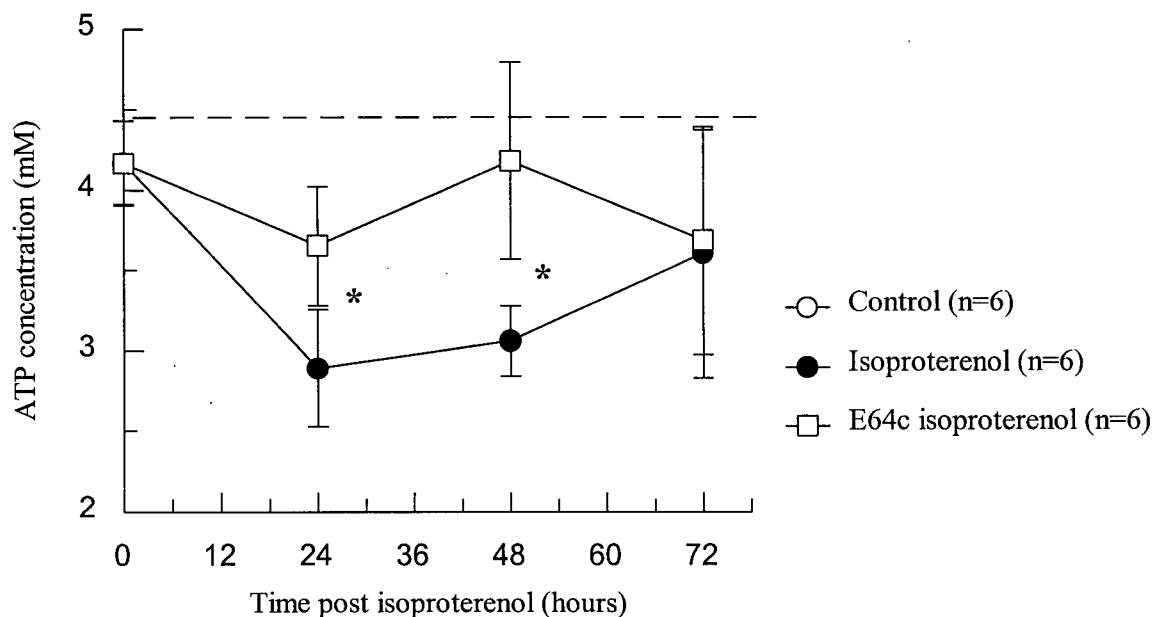
Figure 32. Effect of isoproterenol and E64c plus isoproterenol on ATP concentration of the left ventricle over 72 hours following injections. Values shown are means $\pm$ standard deviation. Control data was only available at a zero time point therefore typical control levels are represented by the dashed line. * denotes significantly different from control

(p<0.05).
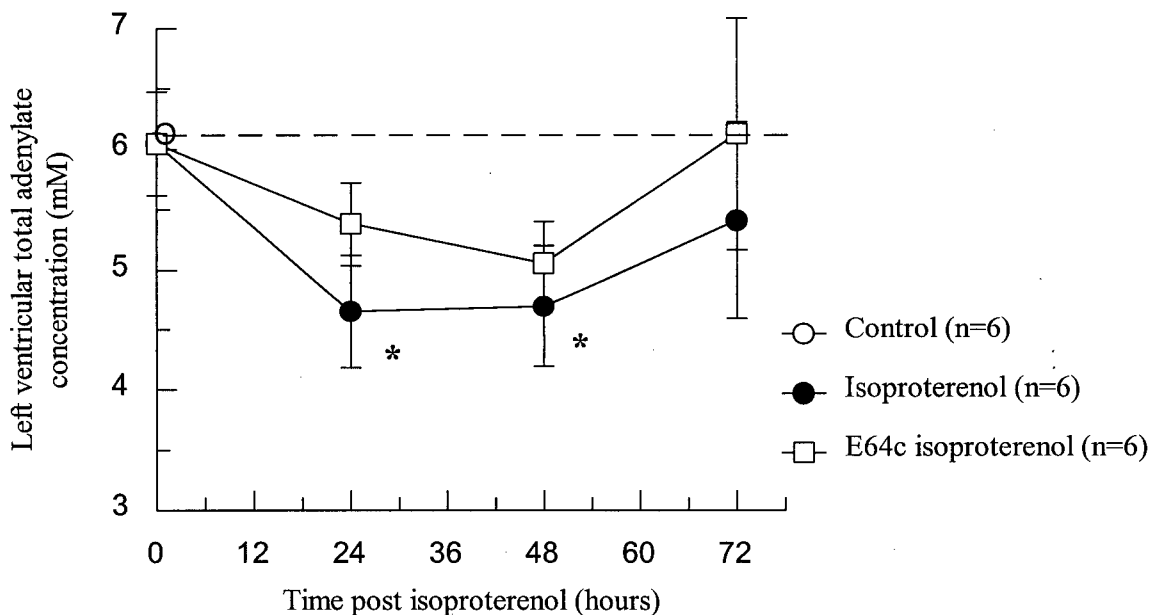
Figure 33. Effect of isoproterenol and E64c plus isoproterenol on total adenylate concentration of the left ventricle over 72 hours following injections. Values shown are means ± standard deviation. Control data was only available at a zero time point therefore typical control levels are represented by the dashed line. * denotes significantly different from control (p<0.05).

Sarcoplasmic reticulum Ca^{2+} uptake: The SR isolated from isoproterenol treated animals showed increased Ca^{2+} uptake rates, which were variable between individuals. The 24 and 72 hours post isoproterenol data showed 137.3% and 227% increases in SR Ca^{2+} uptake respectively, figure 34.
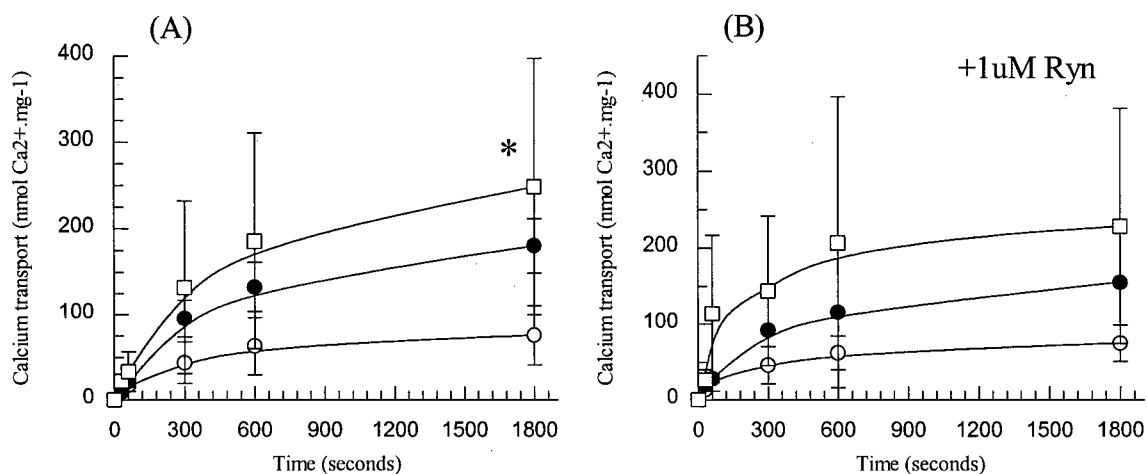
Figure 34. Effect of isoproterenol on SR Ca^{2+} uptake measured up to 30 minutes (all groups n=4). (A) Control = o--o, 24 hours post isoproterenol = •-•, 72 hours post

isoproterenol = []-[]. (B) Same legend with SR Ca^{2+} uptake measured in the presence of 1uM ryanodine. * denotes significantly different from control (p<0.05).
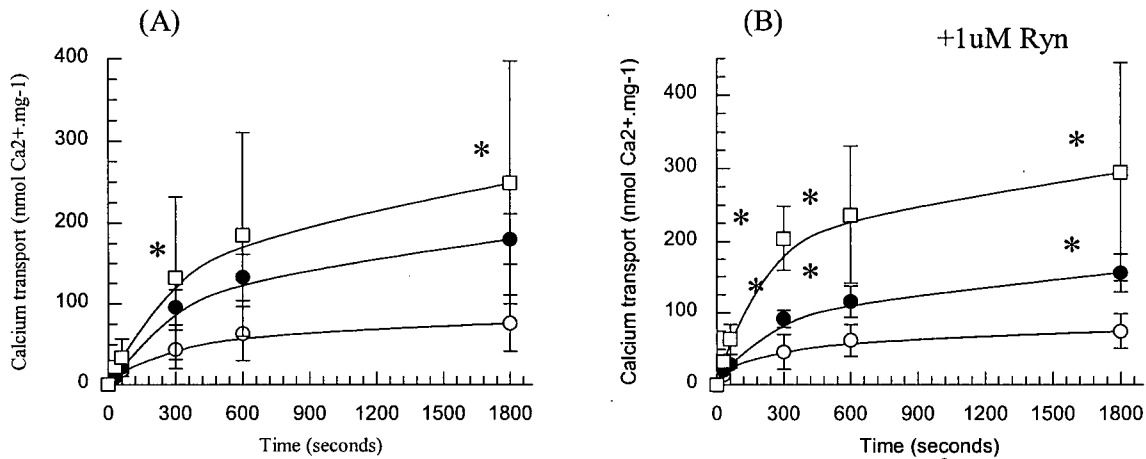
Figure 35. Effect of isoproterenol and E64c plus isoproterenol on SR Ca^{2+} uptake 24 hours after injections (all groups n=4). (A) Control = o--o, Isoproterenol = •-•, E64c + Isoproterenol = []-[]. (B) Same legend with SR Ca^{2+} uptake measured in the presence of 1uM ryanodine. * denotes significantly different from control (p<0.05).
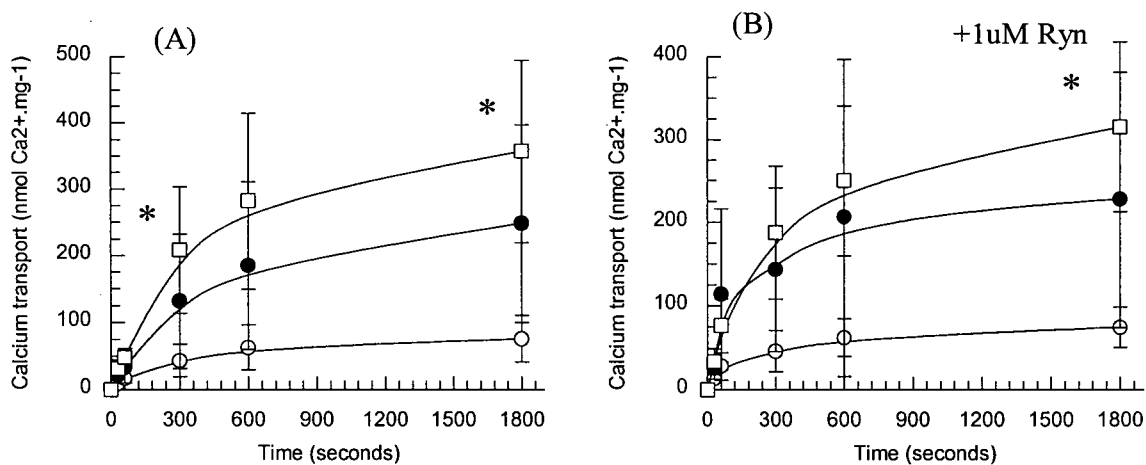
Figure 36. Effect of isoproterenol and E64c plus isoproterenol on SR Ca^{2+} uptake 72 hours after injections (all groups n=4). (A) Control = o--o, Isoproterenol = •-•, E64c + Isoproterenol = []-[]. (B) Same legend with SR Ca^{2+} uptake measured in the presence of 1uM ryanodine. * denotes significantly different from control (p<0.05).

The corresponding data from assays containing 1uM of the Ca^{2+} release channel blocker ryanodine (Ryn) present showed essentially the same pattern with a slight reduction in Ca^{2+} transport values at the 24 and 72 hour time points and even larger variation in these groups, figures 35-36. In terms of the response of the SR from the E64c plus isoproterenol group the results demonstrate a greater increase in Ca^{2+} transport, figures 34-36. It is apparent that this effect is significant with considerable increases over those

from isoproterenol only groups. At 24 hours this stimulation or increase with E64c is 404% of control which is greater than the 293% of control transport values found at 72 hours. This pattern is essentially the same in the samples measured in the presence of 1uM Ryn with only a very slight decrease in Ca^{2+} transport evident.

Levels of selected myofibrilar proteins: Densitometric analysis of the SDS-PAGE demonstrated the relative densities of proteins associated with a fibril fraction, a cytosolic fraction and a whole homogenate fraction.

Alpha-actinin: The fibril associated alpha-actinin showed an early and consistent decrease in density after 6 hours exposure to isoproterenol, the density values being 25.3 % less than control ($p < 0.05$). Fibril associated alpha-actinin levels remained lower up to 72 hours however this decrease was not seen in the corresponding E64c pretreated group ($p > 0.05$). The probable relocation from the fibril to the cytosolic fraction was demonstrated as an increase in content of alpha-actinin in the cytosolic fraction at most of the time points measured. This significant increase in the density at the alpha actinin band showed a maximum increase of 45.8% above control levels at the 48 hour time point ($p < 0.05$). The E64c plus isoproterenol group showed an 8.3% decrease at the same time point ($p > 0.05$). The density of this protein band from isoproterenol treated animals in the homogenate fraction of myofibrils tended to be lower than controls over the 72 hours although the decreases were not statistically significant. It is noticeable however that the E64c plus isoproterenol values were consistently lower at all time points, figure 37.

Desmin: Density of the desmin bands from the fibril fraction showed a significant ($p < 0.05$) and early drop of 29.7% with respect to the control. Again, this is not the case with the E64c plus isoproterenol group where there was a small and non-significant decrease of 5.6%. The densities of the desmin bands had returned towards control levels at 72 hours after isoprterenol treatment. Desmin content of the cytosolic fraction showed an increase in the isoproterenol treated groups which peaks at 34.7% higher than control at 48 hours ($p < 0.05$). These increases were not present with the E64c plus isoproterenol group. By comparison, this group at 48 hours showed only a 1.3 % increase which was not significantly different from control ($p > 0.05$). The density of the

desmin bands from the homogenate fractions from myofibrils showed no striking changes across the groups studied or across time, figure 38.

Troponin-I: The density of troponin-I bands showed a low point at 12 hours of 34.4 % below control and these lower levels recovered over the remaining time points. The corresponding E64c plus isoproterenol group showed only a 10.4 % decrease at the same time point. In the cytosolic fraction there was a significant increase at the 12 hour time point of 28.2 % above control ($p<0.05$) and with E64c pretreatment this increase was 11.8% greater than control. At the 48 and 72-hour time points the differences seen between the groups were small. Troponin-I density from the homogenate fractions showed consistent and sizable decrease especially at the later time points where at 48 hours the values are 24.3% lower than controls and the E64c plus isoproterenol groups show variable responses but in general are lower than those of the isoproterenol group, figure 39.
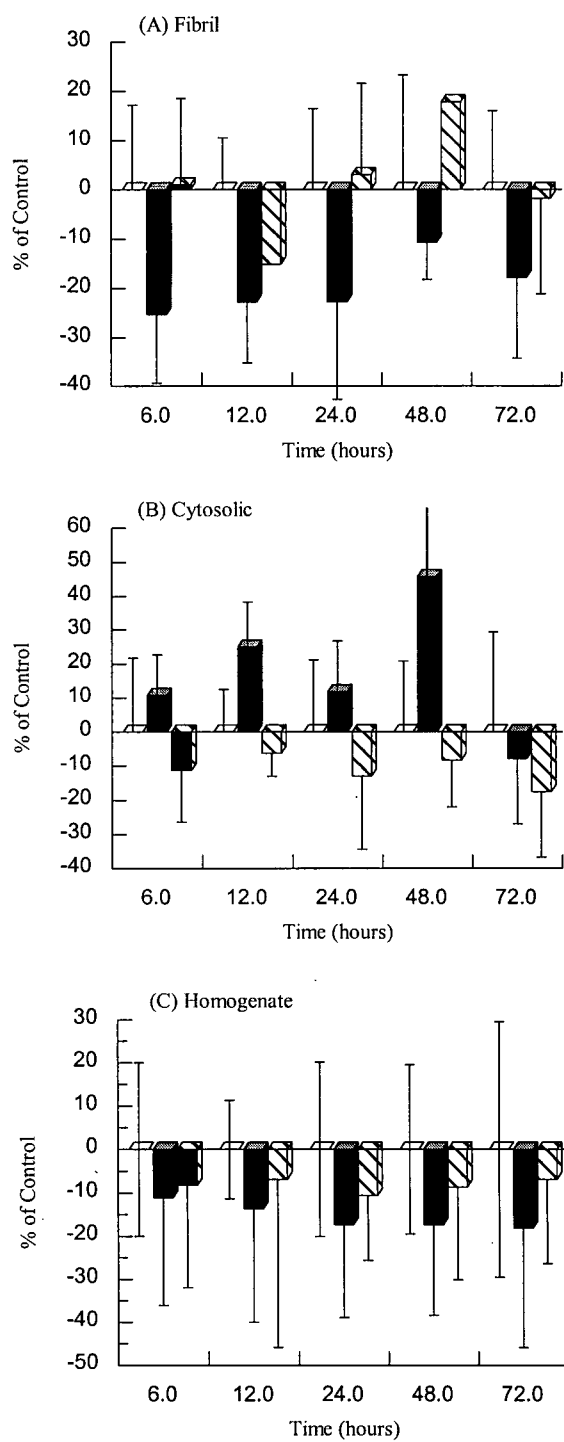
Figure 37. Alpha-actinin protein band density from SDS-PAGE analysis. Control = 0%,
isoproterenol = solid bars, E64c plus isoproterenol = hatched bars. (A) Fibril fraction.
(B) Cytosolic fraction. (C) Homogenate fraction.
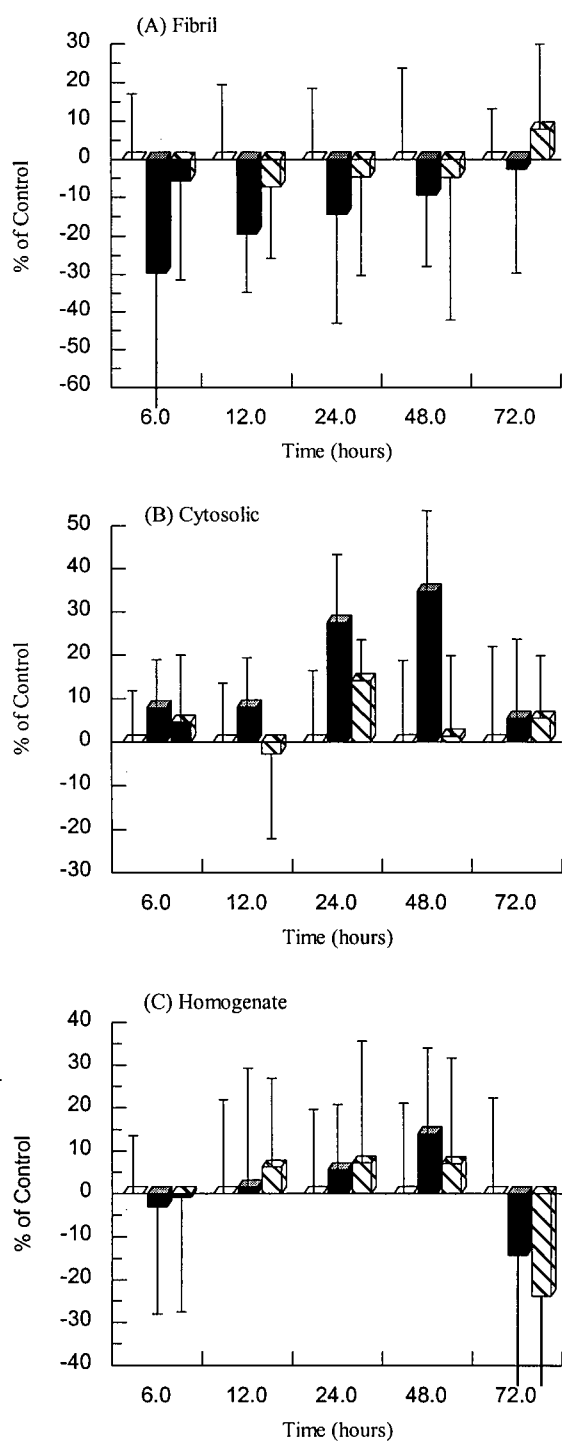
Figure 38. Desmin protein band density from SDS-PAGE analysis. Control = 0%, isoproterenol = solid bars, E64c plus isoproterenol = hatched bars. (A) Fibril fraction. (B) Cytosolic fraction. (C) Homogenate fraction.
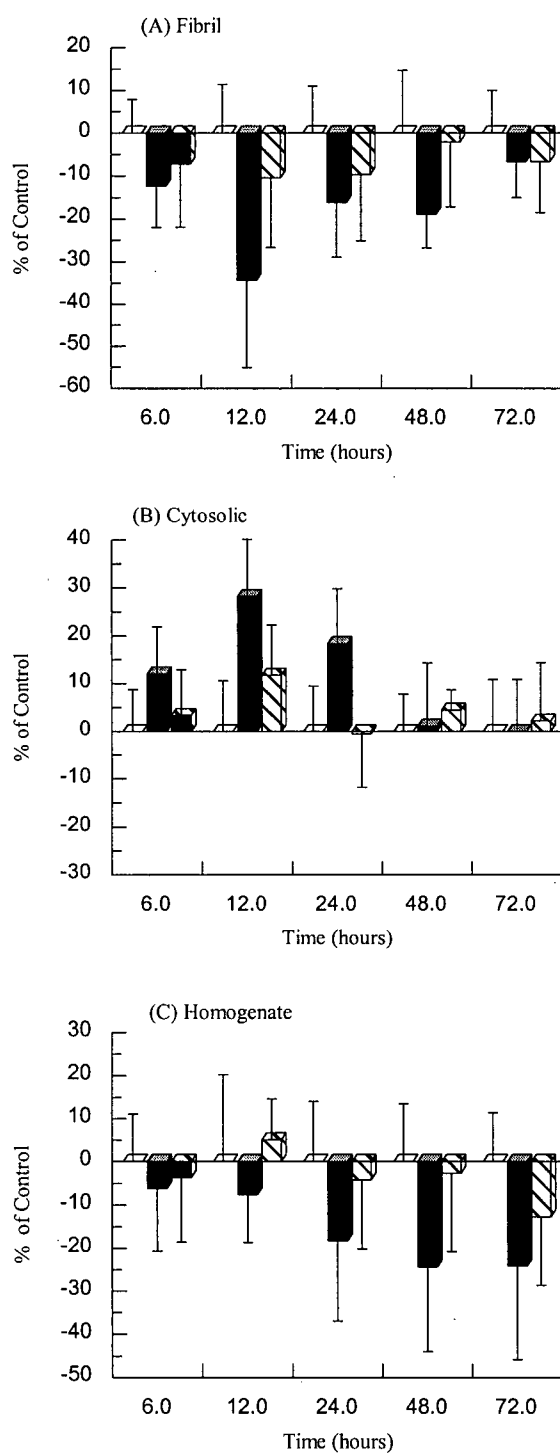
Figure 39. Troponin-I protein band density from SDS-PAGE analysis. Control = 0%, isoproterenol = solid bars, E64c plus isoproterenol = hatched bars. (A) Fibril fraction. (B) Cytosolic fraction. (C) Homogenate fraction.

Discussion.

This study followed selected aspects of the myocardium following isoproterenol injection both with and without prior administration of E64c. Aspects of cellular Ca^{2+} handling and selected myofibrilar proteins both with and without this inhibitor were measured so a clearer view of the role of calpain in determining the functional changes in response to isoproterenol would be indicated. Anthropometric data, activities of calpain and calpastatin, metabolic status, SR Ca^{2+} uptake and myofibrillar protein levels were measured at time points from 6 to 72 hours following isoproterenol injection. It was found that E64c treatment prior to isoproterenol could prevent increases in both calpain-1 and 2 and also accentuated increases of the endogenous calpain inhibitor calpastatin. E64c reduced the degree of hypertrophy recorded and was protective towards selected myofibrillar proteins while increasing the Ca^{2+} transport response of the SR. Therefore these acute changes seen in response to isoproterenol may reflect alterations due in some part to calpain mediated events.

The degree of hypertrophy demonstrated here is consistent with those in studies that have used β-adrenergic agonists to induce hypertrophy (34,197,289). It should be noted however that the left ventricular weights showed a slight decrease immediately after injection of the animals. This was accompanied by a decrease in body weight. The resulting left ventricular weight to body weight ratio therefore shows an increase, despite an actual small and transient drop in left ventricular weight. It is likely that this initial drop in body weight reflects a possible impact on the rats food intake as expected from the significant physiological effects. These include haemodynamic effects and disturbances such as the rapid shallow breathing and lack of activity seen for the first 30 minutes to one hour following isoproterenol injection. I am confident however that at time points beyond 24 hours the increased ratios seen do in fact reflect an increase in ventricular mass. Other groups have demonstrated that the increased ventricular weight to body weight ratios following isoproterenol, even after only 24 hours, is a reflection of increased protein synthesis and increased dry mass of the heart and not due to edema (25,258). The hypertrophy later in the time course is similar to those found previously (25,289) and the results demonstrate that E64c pretreatment can prevent this increase in ventricular mass. Combining the data from chapter 5 to summarize the percentage

changes in left ventricular weight to body weight ratios also reveals a significant overall reduction in hypertrophy when E64c is present. This confirms the ability of calpain inhibition to prevent increased left ventricular mass following isoproterenol injection. In order to conclude any role for calpain it was necessary to show that the E64c did in fact prevent increased calpain activity. Our measures of both calpain-1 and calpain-2 isoforms demonstrate that E64c does indeed do this, confirming the results from the cell homogenate total calpain assay used previously in chapter 5 (5). The ability of E64c to inhibit the activity of calpain and calpain-dependent processes has been demonstrated previously although this inhibitor is not thought to be as cell permeable as other agents used for in vivo applications. It does however have the ability to prevent the activation of calpain in this whole animal model as well as in exercise studies of skeletal muscle calpain (10). This ability of E64c to prevent the increase in both calpain isoforms was coupled with increased activity of the endogenous inhibitor calpastatin. It is known that the interaction of calpain and calpastatin is likely to involve degradation of the calpastatin molecule by active calpain (231). In the case where E64c is present and calpain activity is maintained at basal levels, increased levels of calpastatin could reflect reduced levels of calpastatin proteolysis by calpain. Calpastatin levels were increased over a more prolonged time frame than calpain which has been noted previously for calpastatin in skeletal muscle following acute running exercise where calpastatin activity lags behind that of calpain and is increased to greater levels when E64c is given prior to the exercise bout (10).

The regulation and targeting of calpain substrates may well involve various co-factors and protein targeting modifications etc (19). Indeed it seems that calpain regulation is probably tissue and isoform specific in nature (236). While this makes the protease all the more intriguing it makes comparisons across tissues and or models difficult to interpret until recognition of tissue specific isoforms is assured and physiological substrates are defined. The values we have measured for the two isoforms generally agree with those previously reported although comparisons remain difficult due to methodological differences. The ratio of the calpain isoforms to the endogenous inhibitor calpastatin increases significantly following isoproterenol administration. This ratio could indicate an increased in calpastatin proteolysis. The administration of E64c

prior to isoproterenol reduced this ratio below control levels, i.e. reduced the Ca^{2+} dependent proteolytic potential. Therefore the E64c model employed here achieved its goal of preventing increased calpain activation despite β-agonist stimulation. In this respect the administration of E64c achieved its goal and while it may have affected other cysteine proteases in the heart, the quantitative significance of these is not thought to be so great (287).

Given that the E64c was able to reduce calpain changes, then how could this reduce the hypertrophy response? In terms of hypertrophy induction, it is known that isoproterenol induces both increased activation of AC via G-protein mediated stimulation and subsequently PKA mediated phosphorylation of various intracellular proteins including the CREB which in turn increase the expression of muscle proteins (34,266). However isoproterenol also acts to open L-type Ca^{2+} channels and increase Ca^{2+} influx. It is possible that this increase in Ca^{2+} can stimulate a Ca^{2+} dependent pathway leading to increased protein expression and hypertrophy (96). The fact that E64c can prevent or reduce the extent of the hypertrophy suggests that calpain at some level in one of these pathways could be playing a role in the signal transduction or even in the expression of the signal after it reaches the nucleus. Typical of calpain studies is the fact that many proteins in vitro are substrates but actual intracellular modifications mediated by calpain have been difficult to demonstrate. However there is evidence that limited proteolysis by calpain can modify the immediate early genes c-fos and c-jun which are expressed following adrenergic stimulation of the heart (91). Calpain has been assigned a possible role in the regulation of meiosis as it rapidly cleaves pp39mos, the protein product of the proto-oncogene c-mos which is responsible for arresting the cell in the M-phase of meiosis (272). Roles of the intermediate filaments are thought to extend beyond purely structural ones because protein subunits derived from these filaments are thought to act like transcription factors and participate in the activation of nucleosomes (263). These intermediate filament proteins often contain alpha-helical dimerization interfaces and a basic DNA binding region (173). All non epithelial intermediate filament proteins tested (including vimentin and desmin) have been found to be nucleic acid binding proteins (263). Indeed the activation of transcriptional ability of these intermediate filaments might be achieved by limited truncation by calpain (158). It was hypothesized that

calpain may regulate the release of partially degraded subunit proteins from the insoluble intermediate filaments, which as soluble DNA binding proteins could enter the nucleus and regulate gene expression (158). It has also been suggested that the AP-1 transcriptional complex activity may be regulated partly by calpain mediated proteolysis of selected proto-oncogenes (40). Therefore a mediatory role for calpain in the complex processes of protein transcription and translation is not unreasonable and may explain why E64c reduced the hypertrophy effect and prevented the increase in total cell RNA content. Elucidation of the precise role of calpain here will require considerable study to target precisely where such a role might be.

The increased energy cost of cross bridge cycling following isoproterenol administration, increased ion handling and metabolic processes were reflected by the decreases in total adenylate content and specifically the drop in $[ATP]_i$ (91). It is interesting, that giving Forskolin, which increases cAMP directly, does not reduce $[ATP]_i$, (281) and protein synthesis rates are elevated. This suggests that the actions of isoproterenol on the L-type Ca^{2+} channels which increases Ca^{2+} entry can set off a cascade of energy dependent processes that will reduce $[ATP]_i$. E64c seems able to provide some protection against this in that $[ATP]_i$ does not drop as low as with isoproterenol alone. Calpain was initially known as a "kinase activating factor" due to its ability to increase the activity of certain enzymes of metabolism therefore it is probable that some of the increased metabolic activity may be calpain assisted or triggered (103). This could explain why E64c was able to reduce the drop in $[ATP]_i$. Ultrastructural alterations visualized by electron microscopy following isoproterenol administration were also lessened by E64c administration although this was only studied in one heart from each group and therefore were not presented here. With certain cytoskeletal and membrane-associated proteins being regarded as likely substrates for calpain then E64c may act to protect against proteolysis of such membrane associated proteins and as a result, maintain better integrity of the cell membrane and reduce ionic disturbances. This could suggest a role for calpain early in the response to isoproterenol due to the Ca^{2+} influx.

The functional responses to isoproterenol recorded with the working heart perfusion presented in the previous chapter (5) showed increases in max DLVP and

+dP/dt at the higher filling pressures studied. It has been shown that twitch force is elevated at longer diastolic sarcomere lengths in skinned trabeculae following a chronic model of isoproterenol induced hypertrophy (258). This agrees with the functional data where changes in max DLVP and +dP/dt are evident at the higher filling pressures studied i.e. where presumably sarcomeres are at their longest. E64c could prevent these alterations measured 72 hours after injections (figures 18 & 20) but not those in hearts measured 24 hours post injections (figures A1-A3, appendix 3). This may reflect two different underlying mechanisms for the alterations in function. The 24 hour effects may be independent of calpain activity however the 72 hour differences in function seem to be preventable with E64c administration suggesting a calpain link. The 24 hour changes may reflect a more short term cAMP dependent alteration while the changes at 72 hours may reflect a more permanent change such as with protein conformation or expression. The ability of E64c to prevent increase cAMP at the 24 hour time point is intersting and the reasons or possible sites of calpain influence are many but remain speculative. These include, receptor proteins, sub membrane cytoskeletal proteins etc. Changes in contractile function are likely to reflect either altered rates or amounts of Ca^{2+} being delivered to the contractile mechanisms, or altered responses of the contractile or regulatory proteins to a given level of activating Ca^{2+}. The fact that SR Ca^{2+} handling increases in our model at both 24 and 72 hours suggests that this may play a role. The phosphorylation of phospholamban increases the rate of SR Ca^{2+} uptake via the SERCA2a protein complex and therefore the increased cAMP levels at 24 hours may still allow increased Ca^{2+} uptake as shown in this data. However the even greater rates of Ca^{2+} uptake at 72 hours after isoproterenol requires a different explanation. Also intriguing is the fact that the E64c plus isoproterenol group showed even greater levels of SR Ca^{2+} uptake than the isoproterenol group. In terms of the data from the isolated SR, it could be that isoproterenol related effects increase Ca^{2+} uptake despite a calpain sensitive aspect of channel structure and / or function and E64c administration may act to protect the channel and allow the isoproterenol directed increase to show up even more. Previous work with calpain substrates has demonstrated that in some cases, limited truncation or proteolysis stimulates the activity and or function of the substrate. However when proteolysis is continued or is more severe then activity or function of the protein is

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lost (4). Whether this occurs with some aspect of the Ca^{2+} handling machinery is speculative but the Ca^{2+} release channel is known to be a substrate for calpain mediated proteolysis in skeletal muscle at least (80,222). Proteolysis does not significantly alter Ca^{2+} handling although it can increase the percentage of time the Ca^{2+} release channel is open. Previously it has been shown that SR Ca^{2+} uptake is increased in the early stages of compensated hypertrophy in response to pressure overload and was decreased in response to volume overload hypertrophy (100). In general it seems that Ca^{2+} uptake by the SR may be reduced in various models of hypertrophy and heart failure and contribute to impaired relaxation. The precise causes of reduced Ca^{2+} uptake are unclear and may be due to reduced density of SERCA2a pumping sites (222). Kiss et al showed in a pressure overload model of hypertrophy that SERCA2a levels were depressed, as was Ca^{2+} uptake ability (122). Thus there seem to be divergent mechanisms depending on the model studied and its severity. It is not unreasonable that in the case of an acute response to isoproterenol that initial alterations induce increased SR Ca^{2+} uptake which becomes evident as increased Ca^{2+} uptake values in both the 24 hour and 72 hour isoproterenol models. Increased rates of Ca^{2+} uptake at the 72 hour time point may reflect that there is increased expression of the SERCA2a proteins as has previously been demonstrated in myocardium recovering from stunning which is thought to allow the injured myocardium to handle the increased levels of Ca^{2+} (222).

The evidence for calpain proteolysis of both cytoskeletal and myofibrillar proteins is extensive with most specifically the proteins desmin (19,263), vimentin (263), and alpha-actinin (190). Indeed the proteolysis or removal of myofibrillar proteins by calpain has been reported using SDS-PAGE scanned relative to actin as an internal standard because actin is typically resistant to proteolysis and remains unaltered even after 60 minutes of complete global ischemia (275) and is therefore an ideal internal standard (19,261,275). Desmin is known to be a calpain substrate and is degraded rapidly from control and diabetic myofibrils by exogenous calpain (19). The reduced content of desmin in our fibril fraction is evident at the first time point measured and demonstrates a rapid degradation or loss, the removal or loss of desmin from this fraction matches the increased densities of the desmin bands associated with the cytosolic fraction. If the only process occurring was a removal of these proteins from their intracellular locations then

the homogenate data would be essentially unchanged, i.e. the same as control as it reflects a total whole sample. However the calpain mediated effects could well be severe enough to result in significant amounts of proteolytic digestion products (protein fragments) that are smaller than native desmin and therefore do not contribute to the density of the desmin band. This would result in lower values for the homogenate data as seen for the alpha-actinin and troponin-I proteins. The Desmin data from the homogenate samples however show some increases, although these are within the standard deviations measured. Alpha-actinin is also a good substrate for calpain and is removed from cardiac myofibrils by exogenous calpain (19), although it is removed a little slower than desmin it is also lowered significantly by the six hour time point in this study. It was observed in electron micrographs of myocardium from each of the groups studied that distinct z-line disruptions and areas of unaligned filaments were evident in the isoproterenol heart but not in the E64c/isoproterenol heart. Loss of alpha-actinin, which anchors the myosin filament proteins at the z-line in the muscle, is characteristic also of cardiac muscle three days into a coronary ligation model (115).

The thin filament regulatory proteins are known to be regulators of the Ca^{2+} signal via TnC through the TnT, TnI, and tropomyosin proteins. Therefore any alteration of these proteins will likely effect changes in the force production in response to the activating Ca^{2+} signal (193). TnI, or fragments of it, are released during or following acute cardiac events (268) and the diagnostic and clinical importance of these processes are reflected in the current use of these proteins as markers for cardiac infarcts (139). The results of experiments employing ischemia or coronary ligation (261), and stunning (142), have shown that calpain may well be responsible for alterations of these regulatory proteins. Specifically, the loss of TnI is a common feature as shown by densitometric scans of 15-30% SDS (261). The size and spatial organization of these proteins may also influence their susceptibility with TnI being the outermost positioned of the regulatory filaments and therefore thought to be more susceptible to alteration. Light chains 1 and 2 are known to be relatively well preserved after coronary occlusion while TnI is lost. TnI is known to be degraded to a limited extent producing large fragments which are also characteristic of calpain mediated proteolysis.

In myofibrillar samples from isoproterenol injected animals a significant decrease in the fibril associated fraction was accompanied by an increased density of the TnI bands from the cytosolic fraction within the first 12 hours. As with the other proteins, the E64c treated group showed levels close to those of controls suggesting that these changes are indeed calpain mediated. TnI degradation has been seen within 6 hours coupled with increased Ca^{2+} sensitivity (pCa_{50}) and co-operativity in complete global ischemia despite the concurrent thin filament protein degradation (261). This is in agreement with the data presented here in that we see significant alterations in protein densities yet cardiac function is obviously not limited as seen from the increased max DLVP and +dP/dt recorded at higher filling pressures. It is recognized however that the homogenization process is likely to exaggerate any weakened intracellular links or binding of these proteins and therefore the physiological significance may be much more subtle in vivo and especially in longer term adaptations to chronic stimuli leading to hypertrophy and ultimately heart failure. This data does however provide valuable clues as to which elements may be limiting or susceptible during the hypertrophy process in response to calpain.

The initial alterations seen during cardiac pathologies such as hypertrophy argue against a role for lysosomal proteases due to the early modifications of the proteins and systems mentioned previously. The lysosomotropic inhibitors methylamine and chloroquine (287) do not prevent the early changes seen in protein levels. These authors also showed that Cathepsin B had no effect on alpha-actinin or tropomyosin at $37^{o}C$ for 17 hours at pH 3.9-6.8. TnC was also shown to be resistant to proteolysis. While definitive evidence of a role for calpain is not available in striated muscle due to its complex nature (39,167), it seems likely that calpain regulation is important in cardiac pathology and involves more than just intracellular Ca^{2+} alone. For instance with β-adrenergic agonist stimulation there are alterations in cellular homeostasis (66,277) which are likely to impact substrate susceptibility and or targeting by intracellular proteases.

Conclusions.

This study addressed the hypothesis that:

○ Isoproterenol induced hypertrophy is dependent on contractile and SR proteins that are mediated by the effects of calpain-calpastatin activation.

In light of the results obtained it can be concluded that any calpain mediated effect due to isoproterenol injection were more pronounced in the case of myofibrilar protein alterations than the changes recorded in SR Ca^{2+} transport. The activities of calpain-1 and calpain-2 were increased to a similar degree in response to isoproterenol injection. However calpastatin activity was not reduced following the administration of isoproterenol, rather it increased transiently following isoproterenol. The effects of E64c were, as predicted, to decrease calpain activation and promote calpastatin activity. The fact that calpain activities are increased in response to isoproterenol and stabilized by E64c coupled with the corresponding changes seen in heart function and biochemical parameters demonstrate that calpain may play a multifactorial role within the myocardium. More specific experiments that target precisely the location and nature of these roles are required.

CHAPTER 7:

The hypotheses addressed in this study and the conclusions drawn.

<u>Main hypothesis.</u>

O Calpain-calpastatin is the mechanism through which Ca^{2+} mediates biochemical and contractile responses in cardiac hypertrophy.

This hypothesis was not accepted because calpain - calpastatin did not underlie all of the biochemical and functional variables studied in the models of hypertrophy used here. The specificty of calpain activation and regulation is reflected in the fact that it does seem to influence certain aspects of the hypertrophy process in one of the models studied here. This is reflected in the following sub hypothesis and the conclusions to them.

<u>Exercise (swim) induced cardiac hypertrophy.</u>

O Calpain activity will increase following acute swim exercise.

This was not the case, calpain activity decreased following a single swim session..

O Increased calpain activity is necessary to promote cardiac functional and biochemical adaptations with repeated exercise (i.e. chronic swim training).

This was not true as calpain activity decreases with a single swim and functional adaptations are unaffected by E64c.

O The response of calpain to a run exercise session will be greater in the degree and/or pattern of activation than for a single swim exercise session.

The degree of activation was opposite between these two modes of exercise with run exercise increasing calpain activity and swim exercise decreasing it. The calpain activity changes were also unequal between the different exercises and tissues assayed.

<u>Isoproterenol induced cardiac hypertrophy.</u>

O Isoproterenol induced cardiac hypertrophy is linked (promoted by) to the activation of calpain.

This hypothesis was supported by the data collected.

◯ Isoproterenol induced cardiac hypertrophy is dependent on contractile and sarcoplasmic reticulum proteins/processes that are mediated by the effects of calpain-calpastatin activation.

The calpain -calpastatin system influences both these SR Ca^{2+} handling and myofibrillar proteins.

◯ The activities of calpain-1 and calpain-2 will be increased to a similar degree in response to isoproterenol injection.

This was found to be true as both isoforms responded with the same pattern and magnitude.

◯ Calpastatin activity will be reduced following the administration of isoproterenol.

This was not the case as calpastatin activity transiently increased following isoproterenol.

◯ Administration of an exogenous inhibitor (E64c) will decrease calpain activation and promote calpastatin activity.

This was the case as calpain increases were prevented and calpastatin levels were increased.

CHAPTER 8.

Final discussion.

Role of calpain and calpastatin: The combination of complex activation requirements, tissue specific isoforms and an endogenous specific inhibitor protein conspire to make an all encompassing model of calpain function a formidable task. Currently much of the interest directed towards calpain reflects that it has the potential to cause a number of pathological conditions. Calpain proteolysis is limited in degree and is specific to only certain substrates. As such, its roles are likely to be regulatory in nature rather than purely degradative. This is also reflected in the fact that calpain activation can be rapid and although the nature of its activation and substrate preferences are still being revealed, it is quite likely that calpain acts specifically and locally in response to increased Ca^{2+}. Spatial regulation is likely to be a key feature of calpains activation, as the high Ca^{2+} concentration required in vitro are unlikely to be found in vivo unless localized to sites such as Ca^{2+} release or entry channels. Despite the many questions regarding calpain regulation there have been significant recent advances and defined roles for calpain are emerging. These calpain functions are thought to include potentiation of memory and mediation of certain neurological conditions such as Alzheimer's disease and focal ischemic damage. Calpain has also received significant attention as a possible mediator of acute alterations in striated muscle associated with the initiation of myofibrillar turnover and remodeling. In striated muscle the calpain system is in part likely to be responsible for the myofibrillar alterations seen with and or underlying exercise induced muscle damage and may direct the adaptive process. Calpain is also thought to direct the hypertrohy process in skeletal muscle in response to growth promoting agents in agricultural animals. It is generally accepted that alterations in proteolytic activity can alter the rates of protein degradation or synthesis and affect meat quality. In cardiac muscle it may be responsible for pathological alterations seen with ischemic episodes associated with myocardial infarctions and stunning. Studying the available literature including data from within our lab regarding exercise and calpain, it seemed likely that this protease system was likely to underlie some of the changes seen in the myocardium of hypertrophying hearts. No published studies had yet documented a role for the calpains in cardiac hypertrophy, despite considerable evidence suggesting that this

protease would be well suited to underlie many of the cellular changes associated with hypertrophy development. Such a role for calpain in cardiac muscle was an attractive model as protease seems ideal to mediate adaptive responses within the myocardial cell. Therefore in this series of studies my goals were to determine any role for calpain in the development of physiological and pathological hypertrophy.

The use of the cysteine protease inhibitor E64c had proved effective in reducing the degree of activation of calpain. Therefore my initial aim in these studies was to determine if the use of this inhibitor would also reduce the magnitude of myocardial effects resulting from treatments that are known to induce myocardial hypertrophy. Ultimately it was my goal to define which systems or substrates were underlying the biochemical and functional alterations characteristic to the development of cardiac hypertrophy using a physiological (swim) and a pathological (isoproterenol) model of hypertrophy.

Is calpain involved in swim exercise induced hypertrophy? Cardiac hypertrophy, measured as an increase in left ventricular weight to body weight ratio and reflecting an increased left ventricular mass became apparent after a swim training protocol. E64c administration did not prevent development of this hypertrophy, although there was a trend towards lowered ventricular weight to body weight ratios. This was not pursued any further as the effect of a single session of swim exercise was to actually decrease left ventricular cell homogenate calpain activity and not increase it as predicted. E64c administration did not significantly effect the swim induced changes in calpain activity, also confirming the lack of calpain dependent effects in any of the variables measured. The exception to this was the ability of E64c to prevent the increase in cAMP typically seen post exercise. The mechanism underlying this is unclear but E64c was also found to prevent isoproterenol induced increases in cAMP. The hallmark of physiological hypertrophy is the increased or maintained function that occurs as opposed to the functional decrements that accompany pathological hypertrophy. I was interested in determining if calpain actions might underlie some of the alterations in function seen with swim induced hypertrophy. Functional indices measured soon after acute swim exercise are known to be depressed. This decrease in contractile function is likely to reflect alterations such as acute endocardial ischemia and fatigue including depressed

Ca^{2+} uptake of the SR. The functional alterations in this study following single swim bouts were unaffected by E64c except in the case of +dP/dt and -dP/dt where the reduced function was slightly accentuated. The fact that cardiac calpain activity was reduced following a single swim does match the fact that function on the whole was decreased, but obviously no real conclusions can be made to link these effects. Functional measures recorded on swim trained hearts were performed 24 hours after the last swim session therefore acute fatigue effects were unlikely to have caused the reduced functional indices observed. Interestingly, decreased indices of function were found. The majority of studies following swim induced hypertrophy demonstrate increases in function or no changes. However the majority of studies showing increased function do so using contractile responses of trabeculae and isolated ventricular strips. I can only conclude that the protocol used and the nature of the working heart data reflect an accurate assessment of the function of the left ventricles of the animals studied here as the results recorded were reproducible and consistent. The evidence taken as a whole regarding the functional alterations showed that calpain was unlikely to be playing a role in response to swimming exercise.

Is calpain involved in isoproterenol induced cardiac hypertrophy? Contrary to the swimming model, the initial analysis of whole cell homogenate calpain activity of left ventricular muscle following administration of isoproterenol revealed a significant increase in calpain activity. The ability of E64c pre-treatment to prevent this increase was obviously of great interest as it allowed the study of the responses to isoproterenol both with and without calpain activation. When the ventricular weight to body weight ratio was studied, E64c also showed a slight ability to prevent but not totally eliminate these increases. This trend was sufficient to warrant further investigation since the total RNA content, which can be taken to represent protein synthesis capacity of the cells, was increased with isoproterenol and no such increase occurred when E64c was given prior to isoproterenol. The fact that calpain inhibition reduced the extent of hypertrophy (Chapter 5) was confirmed upon further study of the effects of isoproterenol (Chapter 6). The combination of the data from the two studies using injection of isoproterenol showed that at 72 hours post injection, the difference between E64c plus isoproterenol and isoproterenol treated groups was statistically significant. This ability of E64c to prevent

the hypertrophy likely stems from some role of the protease in the process of gene regulation, protein transcription and or protein synthesis. To date the regulation of muscle hypertrophy has been studied with specific focus on meat production and meat quality and the consensus is in favor of a role for calpain although the conclusions are usually limited to general rates of cellular protein breakdown versus synthesis rates. Calpain may influence hypertrophy by more specific actions. As mentioned previously, (chapter 6), some calpain substrates are known to be intimately involved in the process of protein synthesis for example calpain is suggested to regulate meiosis by cleaving pp39mos, the protein product of the proto-oncogene, c-mos, that is responsible for arresting the cell in the M-phase of meiosis. In addition the specific cleavage of a number of intermediate filaments produce partially degraded subunit proteins which may enter the nucleus and regulate gene expression. Such proteins have been isolated from the myocardium of spontaneously hypertensive rats and shown to stimulate myocyte growth. Also among calpain substrates are the immediate early response genes, protein oncogenes and members of the protein co-factors thought necessary for the functions of the AP-1 transcriptional complex. The use of different models and methods of calpain measurement however deters any clear overall description from being made. What I can conclude here is that calpain may underlie some of the increased capacity for protein synthesis and increased mass of the left ventricle that occurs in response to isoproterenol.

The clinical importance of hypertrophy development is significant as the alterations that occur lead to failure of the heart. The regulatory and limited nature of calpain proteolysis is suggestive of a controlled and specific ability to alter certain substrates such as kinases, cytoskeletal, myofibrillar, and ion handling proteins. Energy dependent processes likely to be increased after isoproterenol injection include increased cross bridge cycling, increased ionic transport, possible stimulation of energy dependent kinases, and significant ultrastructural adaptations and subsequent repair. The decreases in ATP and total adenylate content of the myocardial cells were therefore to be expected. Recovery of normal cellular adenylate levels showed a time dependent rise towards control values at 72 hours. This suggests a sizable disruption in energy balance over the three days following isoproterenol administration. The ability of E64c to reduce the magnitude of these changes could reflect the reduced potential for proteolysis by calpain

and therefore lesser modifications of possible calpain substrates. These substrates include kinases such as phosphorylase kinase-b, which shows increased activity upon calpain activation and a key regulator of glycolysis. Creatine kinase, the key energy shuttling kinase within muscle cells is also a substrate that shows activation after initial proteolysis. Indeed the regulation of metabolic enzymes is thought to depend on the maintenance of strict localization within or upon internal scaffolds of intermediate filaments and cytoskeletal elements. These are ideal calpain substrates, and can be disrupted during various conditions where calpain activity is elevated. Both ion transporting proteins and key ultrastructural proteins are calpain substrates and are likely to increase energy costs within the cell when subject to proteolysis. These proteins are typically disrupted during conditions where calpain activity is increased, suggesting a general uncoupling of metabolism and contractile responses which could lead to reduced efficiency of both metabolism and of force transmission through the muscle and therefore higher energy demands for a given functional output.

Increased or altered calpain activity in the cell would be an ideal candidate to underlie the modifications in functional characteristics associated with hypertrophy development. As such it was felt that any possible role of calpain and calpastatin in the functional changes during the hypertrophy process should be determined. The functional data recorded here demonstrated the response of the left ventricle to dynamic pressure changes and the rates of development and relaxation of the isolated perfused working heart. This allows an insight into the nature of adaptations across a range of functional demands.

With the isoproterenol model however, there were consistent and significant alterations in function seen at the higher filling pressures, which were not present in hearts of animals treated with E64c. These alterations in function at the higher filling pressures were apparent 72 hours after isoproterenol injection. The nature of these alterations are intriguing in that the functional indices shown by a sub group of hearts measured 24 hours after isoproterenol showed increased function over a greater range of filling pressures however these increases were not prevented by E64c. This suggests a time dependent effect where E64c inhibited effects are not manifest until a later time point and are also only evident at the higher filling pressures. Also interesting is why

there were no increases seen at 72 hours for the -dP/dt data. The answers to these questions likely reflect changes in either cellular Ca^{2+} handling characteristics and/or the responsiveness of the contractile proteins to the activating Ca^{2+} signal.

Key to the processes of Ca^{2+} regulation are the SR and its associated Ca^{2+} handling proteins. Despite significant alterations to the processes described above the left ventricle demonstrates increased function at 24 hours and at higher workloads at 72 hours. As stated previously there are a number of processes that are likely to be influenced by an active calpain - calpastatin system. With calpain being present and active following isoproterenol administration then the two main areas that are likely to modulate the functional alterations that seem sensitive to E64c treatment are Ca^{2+} handling by the SR and certain myofilament proteins that respond to the activating Ca^{2+} signal. In order to determine if calpain was affecting the level of Ca^{2+} handling by the SR. I measured the rate of Ca^{2+} uptake by isolated SR fractions from each of the groups studied. The rate of Ca^{2+} uptake into SR fractions was elevated at 24 and 72 hours after isoproterenol indicating an intrinsic modification of the SERCA2a, the protein complex responsible for the sequestration of Ca^{2+} into the SR. The increases in Ca^{2+} uptake 72 hours post isoproterenol were greater than those from the 24 hour group. In terms of a role for calpain in this effect the results of E64c treatment were to actually increase Ca^{2+} uptake. An explanation for this effect could be that certain substrates for calpain do indeed show biphasic responses where initial moderate proteolysis increases activity but if the degree of proteolysis is increased then function is lost. This occurs for kinases and other substrates and could help explain why E64c treatment in combination with isoproterenol might show even greater rates of Ca^{2+} uptake than isoproterenol alone. This is speculative and would require accompanying structural data such as electrophoresis of the SERCA2a complex to determine the extent of any proteolysis that was occurring. It should also be recognized that the group which received E64c prior to isoproterenol showed increased Ca^{2+} uptake into the SR yet did not show the increased function seen at the higher filling pressures.

Myocardial function also reflects the ability of the myofilament proteins to transduce the Ca^{2+} signal into force generation and transmission. Therefore I decided to assess any modifications of proteins involved in these processes of force generation and

transmission. Certain proteins that play these roles are known to be susceptible to calpain proteolysis as shown by previous research showing increased calpain activity resulting in their modification or loss from muscle. Previous work in the literature also suggests that calpain is likely to underlie the loss of selected proteins such as the troponin complex proteins and other key structural proteins. Obviously these changes would have significant functional consequences, therefore I attempted to follow such key proteins following isoproterenol administration. While it is recognized that the process of homogenization is severe and likely to exagerate any modifications in protein interactions in the intact myocardium, the analysis of the fractional content of the proteins by SDS-PAGE allowed general conclusions to be made. The pattern found for all three proteins was one of release or loss from the myofibrillar fraction and appearance in the soluble associated fraction. In the case of TnI and alpha-actinin the proteolysis may have been extensive enough to produce protein fragments that did not migrate associated with the whole protein. Thus the band representing that protein from the homogenate fraction would show values less than those of controls. A valuable addition to these kinds of data would be specific antibodies to the protein products of calpain mediated cleavage which would allow more accurate quantification of the proteolytic products. The degrees of protein loss or modifications seen were reduced significantly with E64c pre treatment. Thus inhibiting the activation of calpain does prevent the alterations seen in the proteins studied here. Therefore calpain is playing a role here but the nature of the alterations require closer study as numerous factors are likely to affect the interaction of calpain with these substrates. The location of these proteins for example could determine the degree to which they are affected and the functional significance of their alterations.

To assess wether the different responses seen in the models used were ultimately determined by differences in calpain activity, the degree and direction of any alterations to calpain and calpastatin were measured. If the changes associated with hypertrophy were to be linked to calpain mediated effects then alterations in calpain activity would have to be in the appropriate direction and the effects of E64c treatment should reflect situations where calpain activity was not increased despite hypertrophy inducing stimuli. In response to acute swim exercise it was apparent that whole cell homogenate calpain activity was reduced and this was the opposite of run exercise which was studied using an

assay that allowed determination of soluble and bound fractions of calpain activity. Previous data had shown increased calpain activity at the termination of an exhaustive run session lasting approximately one hour. The data presented from the run model used here shows that there is an early and significant increase in calpain activity. This is a novel finding and supports the fact that calpain may, in skeletal muscle underlie ongoing processes during exercise and not just direct or regulate post exercise modifications. The precise nature of such roles await more specific studies but calpain would be well suited to regulate processes such as metabolic responses, fatigue, training adaptations and in more severe cases the inflammatory response associated with the delayed onset of muscle soreness. The exercise data also supports the concept of alterations in calpain localization occurring during activation and regulation. Increased activity associated with contractile activity in skeletal muscle shows that there is a shift towards a particulate associated localization. Cardiac muscle from run exercised animals also showed increased total activity, reflecting an increase in both particulate and soluble calpain while liver activities remained unchanged. Therefore we can see tissue specific responses in calpain distribution and activation. Swim exercise influences calpain activation characteristics quite differently to run exercise in that the total skeletal muscle activity is reduced, reflecting less activity in the soluble fraction of the cell. Total cardiac muscle activity is also decreased after swim exercise and unfortunately I do not have data for the distribution between the soluble and particulate fractions. However it is clear that the activation characteristics and distribution patterns of calpain show distinct differences between swimming and running in the rat. Calpain responses are therefore both tissue specific and as far as exercise is concerned are also model specific. This should be of interest to the applied exercise physiologist or biochemist with an interest in manipulating calpain levels with whole body models. With the model of isoproterenol injection the intracellular alterations such as increases in free intracellular Ca^{2+} were proposed to stimulate increased calpain activity. This was indeed the case as shown by the initial measures of total cell calpain activity and then further characterized by measuring the activity of both the isoforms and of the inhibitor protein calpastatin.

The increases in calpain activity followed essentially the same pattern for both isoforms which does not give us any clues as to which of the these isoforms may be more

important or whether there is any substrate and or role specificity between the two. The physiological significance of both calpain isoforms targeting essentially the same substrates while requiring different Ca^{2+} levels for activation remains a major question for the study of calpain. Activity of both the calpain isoforms were increased within the first 6 hours following isoproterenol injection which is not surprising as cellular responses to isoproterenol are known to be rapid. Activity of both the isoforms remained elevated for a considerable time following isoproterenol administration.

Concurrent with these increases was an increased calpastatin activity in a similar pattern. The administration of E64c significantly reduced the magnitude of the calpain activity increase. When calpastatin levels were studied it became apparent that their levels were increased further and remained elevated longer. Calpain inhibition by E64c is achieved by a non-competitive irreversible formation of a thioether bond with the active thiol of the protease. Therefore the increased calpastatin levels seen with E64c treatment prior to isoproterenol could reflect reduced proteolysis of native calpastatin which is known to be a substrate for calpain. Thus an inhibition of calpain could increase calpastatin levels and promote further calpain inhibition. The nature of increased calpain and calpastatin levels in terms of Ca^{2+} dependent proteolytic potential can be gauged by the ratio of calpain to calpastatin. The response to isoproterenol shows a significant and early rise in proteolytic potential following isoproterenol. E64c acts to reduce this net proteolytic potential to levels below those found in control myocardium, again showing the effectiveness of this agent in this model.

As mentioned before, the significance of the release of myofibrillar proteins or their fragments is considerable as a clinical tool for assessing the nature and severity of cardiac events such as myocardial infarction. That these proteins are actually released by calpain mediated proteolysis is shown by the protective effect of calpain inhibitors, which reduce TnI release from stunned myocardium. The precise nature of calpains effect is unknown and requires closer determination to indicate where the proteolysis occurs within the TnI protein and how this would impact on the interaction of the TnI with the other proteins of the troponin-tropomyosin complex. A concurrent measurement of plasma levels of TnI or fragments produced from TnI proteolysis was initially an aim of the study in chapter 6 however technical limitations and other difficulties prevented this.

It was expected that the fragments detected would demonstrate likely calpain mediated modifications in terms of their size and location on the proteins. Valuable information would have been gained in terms of a cause and effect relationship if E64c prevented increased TnI levels in the plasma.

Currently much of the interest focusing on calpain reflects its potential to contribute to, or cause, a number of pathological conditions. With regards to the ongoing research of the calpain system in cardiac muscle the results presented here from cardiac hypertrophy models are significant in that they indicate a role for this protease in the development of alterations characteristic to a condition that ultimately leads to heart failure. As such the actual characteristics of calpain activation within cardiac muscle and their regulation is of great importance and the continued study of aspects of these processes is warranted. These processes are still not clear despite considerable study within other tissues and systems. In terms of calpain activation and regulation, the distribution and localization of the protease and its endogenous inhibitor calpastatin are likely to be very important in the process of activation due to the need for high intracellular Ca^{2+} concentrations. These can occur in specific areas of the cell and the ability of phospholipids to lower the Ca^{2+} concentration required suggests that spatial responses and their regulation are very important. There is considerable evidence focusing on in vitro substrates for calpain. However, more work is required to determine precisely the response of calpain upon activation in vivo, the goal of such experiments should be to indicate where calpain activation occurs and which protein substrates are modified. This could be achieved by visualization of calpain autolysis and localization of calpain produced fragments via antibodies generated towards likely calpain substrates and visualized through such techniques as labeling with fluorescent probes etc.

In focusing on the hypertrophy response that is influenced by calpain activity it would be valuable to understand why isoproterenol induced increases in total protein synthesis capacity, as reflected by increased RNA content are prevented by calpain inhibition. The fact that majority of cellular RNA is ribosomal RNA and that approximately 90% of ribosomes are fully active normally suggests that during the hypertrophy following isoproterenol that there must be a significant increase in ribosome content. A study focusing on the content of ribosomes and their distribution after

isoproterenol stimulation both with and without calpain inhibition would be valuable especially as the localization of ribosomes are known to include specifically the intermediate filaments such as desmin which is modified under conditions of increased calpain activity. The importance of the intermediate filaments such as desmin and vimentin have already been hinted at as they are ideal substrates for calpain and the products of calpain mediated proteolysis contain what appear to be DNA binding properties and may influence the process of protein synthesis. Thus any further characterization of these effects would obviously help to determine how calpain inhibition can prevent the increase in total RNA content. The effects of isoproterenol administration on SR Ca^{2+} uptake were interesting in that they demonstrated a significant increase in Ca^{2+} transport yet the increase was even greater with E64c pretreatment. An explanation for this that would fit with a calpain mediated effect would be that limited proteolysis of the SERCA2a complex stimulates its Ca^{2+} transport abilities and that in this case the E64c can provide limited protection to the complex and therefore allow greater increases than seen with isoproterenol alone. Such a pattern is actually seen with creatine kinase where limited proteolysis increases its activity but as proteolysis becomes more severe then function is reduced. Visualization of the SERCA2a proteins and determining if there is any significant proteolysis and whether it is in fact greater with isoproterenol alone rather than with E64c plus isoproterenol would help explain these SR Ca^{2+} handling results.

As a whole, therefore there is a probable role for the calpain - calpastatin proteolytic system acting at a number of sites, and as such, directing aspects of the hypertrophy process. It is hoped that the use of cell permeable inhibitors specific to either calpains catalytic or Ca^{2+} binding domains, which are now available, will allow a more detailed investigation and description of calpains role during cardiac hypertrophy. The continued development and study of these inhibitors will advance our understanding of the calpain proteolytic system and ultimately help to manage or prevent the deleterious effects associated with pathological cardiac hypertrophy.

Fuure directions for this area of research should focus on determining sites where calpain substrates are known to play roles in the hypertrophy process. These are numerous and some will be more appealing than others for study. The membrane associated proteins

involved in beta agonist responses such as the G-protein complexes and receptor molecules are positioned ideally for a membrane associated calpain response. However the interatcions at this point on the signalling pathway are very diverse and will require some very well controlled and designed studies to determine any calpain role beyond those hinted at from in vitro work. The substrates such as calcineurin and various transcription factors suggest a more direct calpain role and deserve further study, as does the ability of calpain inhibition to reduce the increase in total RNA levels. The localisation of ribosomes to the calpain substrate desmin and desmin alterations in hypertrophy suggest a link between the cytoskeletal elements and hypertrophy. The abiltity of cell stretch or stress to induce hypertrophy may reflect the importance of this possible relationship.

The achievement of such future research such goals does however require that more cell permeable and specific inhibitors of calpain be available. This coupled to the demonstration of calpain activation at the Ca^{2+} levels found in vivo would allow demonstration of calpain activation, substrate hydrolysis and a physiological or pathological effect. The ability to intervene and regulate these events will ultimately allow better strategies and therapies for preventing and treating pathologies of the myocardium.

APPENDIX 1.

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APPENDIX 2.

Results and Data tables.

Condition	Bdy (Kg)	LV Wt(g)	Ratio(g/Kg)	Condition	Bdy (Kg)	LV Wt(g)	Ratio
Control	0.539	1.403	2.6	Swim	0.521	1.315	2.52
Control	0.430	1.141	2.65	Swim	0.506	1.523	3.01
Control	0.492	1.226	2.49	Swim	0.45	1.387	3.08
Control	0.489	1.209	2.47	Swim	0.489	1.59	3.25
Control	0.509	1.143	2.25	Swim	0.481	1.441	3
Control	0.508	1.172	2.31	Swim	0.452	1.38	3.05
Control	0.461	1.07	2.32	Swim	0.465	1.36	2.92
Control	0.492	1.138	2.31	Swim	0.491	1.601	3.26
Control	0.486	1.23	2.53	Swim	0.5	1.3	2.6
Control	0.481	1.28	2.66	Swim	0.482	1.52	3.15
Control	0.430	1.15	2.67	Swim	0.466	1.361	2.92
Control	0.538	1.25	2.32	Swim	0.469	1.375	2.93
Control	0.461	1.18	2.56	Swim	0.484	1.33	2.75
Control	0.504	1.168	2.32	Swim	0.502	1.3	2.59
Control	0.499	1.05	2.1	Swim	0.449	1.171	2.61
Control	0.448	1.205	2.69	Swim	0.447	1.214	2.72
Control	0.502	1.03	2.05				
Control	0.411	0.923	2.25				
Control	0.451	0.967	2.14				
Control	0.444	1.14	2.57				
Mean	0.479	1.154	2.413	Mean	0.478	1.386	2.898
Std Dev	0.035	0.110	0.201	Std Dev	0.023	0.123	0.240
				E64c Swim	0.486	1.321	2.72
				E64c Swim	0.449	1.213	2.7
				E64c Swim	0.476	1.185	2.49
E64c Con	0.499	1.205	2.41	E64c Swim	0.495	1.32	2.67
E64c Con	0.483	1.106	2.29	E64c Swim	0.508	1.421	2.8
E64c Con	0.51	1.178	2.31	E64c Swim	0.424	1.072	2.53
E64c Con	0.507	1.321	2.61	E64c Swim	0.458	1.283	2.8
E64c Con	0.457	1.034	2.26	E64c Swim	0.46	1.225	2.66
E64c Con	0.524	1.134	2.16	E64c Swim	0.465	1.319	2.84
Mean	0.497	1.163	2.34	Mean	0.469	1.262	2.69
Std Dev	0.024	0.098	0.155	Std Dev	0.025	0.101	0.120

TableA1. Weight data from swim trained groups.

Condition	Bdy Wt(Kg)	LV Wt(g)	Ratio(g/Kg)
Control 24hr	0.299	1.049	3.610
Control 24hr	0.258	0.853	3.610
Control 24hr	0.266	0.906	3.450
Control 24hr	0.260	0.896	3.450
Control 24hr	0.276	0.975	3.530
Control 24hr	0.275	1.046	3.800
Control 24hr	0.248	0.915	3.680
Control 24hr	0.307	1.124	3.660
Control 24hr	0.299	0.978	3.320
Control 24hr	0.277	1.045	3.770
Mean	0.277	0.979	3.588
Std Dev	0.020	0.086	0.151
Iso 24hr	0.268	1.272	4.750
Iso 24hr	0.257	1.067	4.160
Iso 24hr	0.237	1.020	4.310
Iso 24hr	0.253	1.186	4.690
Iso 24hr	0.275	1.235	4.490
Iso 24hr	0.274	1.215	4.430
Iso 24hr	0.283	1.185	4.190
Mean	0.264	1.169	4.431
Std Dev	0.016	0.091	0.230
E64c/I 24hr	0.252	1.064	4.230
E64c/I 24hr	0.252	1.005	3.990
E64c/I 24hr	0.249	1.078	4.330
E64c/I 24hr	0.259	1.083	4.190
Mean	0.253	1.058	4.185
Std Dev	0.004	0.036	0.143

Table A2. Weight data from animals 24 hours post injections.

Condition	Bdy Wt(Kg)	LV Wt(g)	Ratio(g/Kg)
Con 72hr	0.272	1.019	3.750
Con 72hr	0.280	0.923	3.300
Con 72hr	0.300	1.138	3.790
Con 72hr	0.315	1.128	3.580
Con 72hr	0.269	0.819	3.050
Con 72hr	0.268	0.980	3.660
Mean	0.284	1.001	3.522
Std Dev	0.019	0.122	0.289
Iso 72hr	0.259	1.226	4.730
Iso 72hr	0.271	1.238	4.570
Iso 72hr	0.237	1.020	4.310
Iso 72hr	0.253	1.186	4.690
Iso 72hr	0.303	1.304	4.300
Iso 72hr	0.295	1.200	4.070
Iso 72hr	0.258	1.076	4.170
Iso 72hr	0.284	1.150	4.050
Iso 72hr	0.281	0.981	3.490
Mean	0.271	1.153	4.264
Std Dev	0.021	0.107	0.385
Iso/E 72hr	0.252	1.064	4.230
Iso/E 72hr	0.252	1.005	3.990
Iso/E 72hr	0.281	1.190	4.050
Iso/E 72hr	0.247	0.968	3.920
Iso/E 72hr	0.244	0.976	4.000
Iso/E 72hr	0.261	1.016	3.890
Iso/E 72hr	0.284	1.045	3.670
Iso/E 72hr	0.281	1.090	3.880
Mean	0.263	1.044	3.954
Std Dev	0.017	0.072	0.160

Table A3. Weight data from animals 72 hours post injections.

Condition	Wt(Kg)	LV Wt(g)	Ratio(g/Kg)	Condition	Wt(Kg)	LV Wt(g)	Ratio(g/Kg)
Con 6hr	0.401	1.099	2.686	E64c 6hr	0.377	1.067	2.830
Con 6hr	0.390	1.080	2.767	E64c 6hr	0.332	0.978	2.776
Con 6hr	0.320	1.031	2.387	E64c 6hr	0.345	1.011	2.637
Con 6hr	0.383	0.912	2.382	E64c 6hr	0.341	0.901	2.644
Con 6hr	0.384	0.954	2.488	E64c 6hr	0.380	1.024	2.701
Con 6hr	0.387	0.987	2.551	E64c 6hr	0.369	0.968	2.490
Mean	0.377	0.993	2.515	Mean	0.357	0.992	2.680
Std Dev	0.029	0.073	0.157	Std Dev	0.020	0.057	0.120
Con 12hr	0.341	1.019	2.347	E64c 24hr	0.357	1.010	2.829
Con 12hr	0.388	0.970	2.501	E64c 24hr	0.370	0.991	2.667
Con 12hr	0.344	0.816	2.588	E64c 24hr	0.352	0.945	2.686
Con 12hr	0.331	0.991	2.994	E64c 24hr	0.357	1.001	2.736
Con 12hr	0.365	0.981	2.691	E64c 24hr	0.376	0.959	2.551
Con 12hr	0.389	1.021	2.623	E64c 24hr	0.367	0.982	2.677
Mean	0.360	0.966	2.624	Mean	0.363	0.981	2.691
Std Dev	0.025	0.076	0.217	Std Dev	0.009	0.025	0.091
Con 48hr	0.378	1.010	2.674				
Con 48hr	0.360	0.974	2.704				
Con 48hr	0.361	0.915	2.533				
Con 48hr	0.366	1.017	2.776				
Con 48hr	0.359	0.968	2.696				
Con 48hr	0.361	1.004	2.785				
Con 48hr	0.376	0.986	2.625				
Con 48hr	0.375	0.908	2.542				
Mean	0.367	0.973	2.667				
Std Dev	0.008	0.041	0.095				
Con 72hr	0.366	1.001	2.737	E/C 72#1	0.354	0.919	2.598
Con 72hr	0.355	0.966	2.718	E/C 72#2	0.353	0.956	2.710
Con 72hr	0.367	0.990	2.701	E/C 72#3	0.362	0.964	2.663
Con 72hr	0.361	1.011	2.800	E/C 72#4	0.370	0.984	2.657
Con 72hr	0.367	0.949	2.585	E/C 72#5	0.365	0.924	2.533
Con 72hr	0.372	0.953	2.550	E/C 72#6	0.348	1.007	2.896
Mean	0.365	0.978	2.682	Mean	0.359	0.959	2.676
Std Dev	0.006	0.026	0.095	Std Dev	0.009	0.034	0.124

Table A4. Weight data from isoproterenol time course groups (part 1).

Condition	Wt(Kg)	LV Wt(g)	Ratio(g/Kg)	Condition	Wt(Kg)	LV Wt(g)	Ratio(g/Kg)
Iso 6hr	0.350	0.911	2.604	E/I 6hr	0.369	1.014	2.752
Iso 6hr	0.412	1.099	2.668	E/I 6hr	0.380	0.977	2.573
Iso 6hr	0.386	1.010	2.618	E/I 6hr	0.378	1.022	2.702
Iso 6hr	0.408	1.015	2.487	E/I 6hr	0.376	1.072	2.858
Iso 6hr	0.409	1.031	2.520	E/I 6hr			
Iso 6hr	0.356	0.964	2.706	E/I 6hr			
Mean	0.387	1.005	2.601	Mean	0.376	1.021	2.721
Std Dev	0.028	0.063	0.084	Std Dev	0.005	0.039	0.118
Iso 12hr	0.337	1.072	3.184	E/I 12hr	0.335	0.977	2.913
Iso 12hr	0.308	0.908	2.951	E/I 12hr	0.341	0.986	2.893
Iso 12hr	0.324	0.969	2.994	E/I 12hr	0.335	0.977	2.977
Iso 12hr	0.341	1.011	2.958	E/I 12hr	0.321	0.909	2.834
Iso 12hr	0.322	0.958	2.976	E/I 12hr	0.344	1.091	2.966
Mean	0.326	0.984	3.013	Mean	0.335	0.988	2.917
Std Dev	0.013	0.062	0.097	Std Dev	0.009	0.065	0.058
Iso 24hr	0.357	1.192	3.341	E/I 24hr	0.372	1.012	2.720
Iso 24hr	0.344	1.080	3.138	E/I 24hr	0.388	0.995	2.567
Iso 24hr	0.381	1.118	2.938	E/I 24hr	0.365	1.013	2.778
Iso 24hr	0.375	1.140	3.041	E/I 24hr	0.368	1.021	2.775
Iso 24hr	0.377	1.110	2.944	E/I 24hr	0.375	1.000	2.666
Iso 24hr	0.371	1.091	2.939	E/I 24hr	0.360	0.977	2.874
Mean	0.367	1.122	3.057	Mean	0.371	1.003	2.730
Std Dev	0.014	0.040	0.160	Std Dev	0.010	0.016	0.106
Iso 48hr	0.363	1.085	2.989	E/I 48hr	0.367	0.965	2.633
Iso 48hr	0.352	1.103	3.131	E/I 48hr	0.338	0.992	2.933
Iso 48hr	0.354	1.183	3.341	E/I 48hr	0.342	1.081	3.162
Iso 48hr	0.364	1.124	3.089	E/I 48hr	0.353	1.022	2.898
Iso 48hr	0.326	1.040	3.193	E/I 48hr	0.367	0.999	2.719
Mean	0.352	1.107	3.149	Mean	0.353	1.012	2.869
Std Dev	0.015	0.053	0.131	Std Dev	0.013	0.044	0.206
Iso 72hr	0.388	1.184	3.050	E/I 72hr	0.379	1.093	2.756
Iso 72hr	0.386	1.162	3.013	E/I 72hr	0.358	1.054	2.944
Iso 72hr	0.370	1.135	3.066	E/I 72hr	0.383	1.014	2.650
Iso 72hr	0.368	1.151	3.125	E/I 72hr	0.396	1.130	2.855
Iso 72hr	0.367	1.181	3.220	E/I 72hr	0.371	1.004	2.709
Iso 72hr	0.354	1.073	3.035				
Iso 72hr	0.351	1.168	3.330				
Mean	0.369	1.151	3.120	Mean	0.377	1.059	2.783
Std Dev	0.014	0.038	0.116	Std Dev	0.014	0.053	0.117

Table A5. Weight data from isoproterenol time course groups (part 2).

Condition	[RNA] ug/g
Control	0.987
Control	0.858
Control	1.056
Mean	0.967
Std Dev	0.101
Iso	1.769
Iso	2.221
Iso	1.975
Mean	1.988
Std Dev	0.226
E64c Iso	0.919
E64c Iso	0.806
E64c Iso	0.738
Mean	0.821
Std Dev	0.091

Table A6. Total left ventricular [RNA] 72 hours post injections.

Single Swim	Calpain U/g	Repeated Swim	Calpain U/g
Control	26.83	Control	23.80
Control	33.35	Control	20.90
Control	21.60	Control	22.70
Control	24.38	Control	23.89
Control	30.16	Control	18.44
		Control	27.13
Mean	27.26	Mean	22.81
Std Dev	4.64	Std Dev	2.95
		Swim	22.21
		Swim	14.74
		Swim	16.41
Swim	19.46	Swim	20.64
Swim	14.41	Swim	17.12
Swim	16.69	Swim	33.66
Swim	13.28	Swim	18.73
Mean	15.96	Mean	20.50
Std Dev	2.73	Std Dev	6.33
E64c Swim	14.08		
E64c Swim	22.37		
E64c Swim	16.39	E64C Swim	17.09
E64c Swim	18.52	E64C Swim	22.29
E64c Swim	14.24	E64C Swim	30.22
E64c Swim	14.34	E64C Swim	15.96
Mean	16.66	Mean	21.39
Std Dev	3.29	Std Dev	6.50
		E64C Control	13.33
		E64C Control	18.48
		E64C Control	17.81
		E64C Control	17.27
		Mean	16.72
		Std Dev	2.31

Table A7. Calpain activity of single swim and swim trained groups.

Time	Plantaris-total	Cardiac-total	Plantaris-S	Plantaris-B	Cardiac-S	Cardiac-B
0 min	100.00	100.00	47.00	53.00	30.00	70.00
			63.00	37.00	37.00	63.00
			55.00	45.00	40.00	60.00
			52.00	48.00	44.00	56.00
			58.00	42.00	34.00	66.00
Mean	100.00	100.00	55.00	45.00	37.00	63.00
Std Dev			6.04	6.04	5.39	5.39
2 min	23.00	3.00	21.00	79.00	44.00	56.00
	29.00	3.00	39.00	61.00	48.00	52.00
	26.00	2.00	34.00	66.00	62.00	38.00
	28.00	4.00	26.00	74.00	58.00	42.00
	24.00	3.00	30.00	70.00	53.00	47.00
Mean	26.00	3.00	30.00	70.00	53.00	47.00
Std Dev	2.55	0.71	6.96	6.96	7.28	7.28
5 min	4.00	19.00	44.00	56.00	56.00	44.00
	5.00	23.00	38.00	62.00	65.00	35.00
	4.00	16.00	41.00	59.00	60.00	40.00
	6.00	26.00	35.00	65.00	53.00	47.00
	5.00	21.00	32.00	68.00	62.00	38.00
Mean	4.80	21.00	38.00	62.00	59.20	40.80
Std Dev	0.84	3.81	4.74	4.74	4.76	4.76
15 min	18.00	11.00	38.00	62.00	56.00	44.00
	22.00	13.00	29.00	71.00	53.00	47.00
	14.00	14.00	43.00	57.00	61.00	39.00
	21.00	17.00	33.00	67.00	59.00	41.00
	15.00	15.00	47.00	53.00	51.00	49.00
Mean	18.00	14.00	38.00	62.00	56.00	44.00
Std Dev	3.54	2.24	7.28	7.28	4.12	4.12
30 min	28.00	37.00	35.00	65.00	45.00	55.00
	25.00	34.00	46.00	54.00	52.00	48.00
	17.00	25.00	35.00	65.00	60.00	40.00
	23.00	31.00	35.00	65.00	44.00	56.00
	21.00	28.00	24.00	76.00	59.00	41.00
Mean	22.80	31.00	35.00	65.00	52.00	48.00
Std Dev	4.15	4.74	7.78	7.78	7.52	7.52
60 min	27.00	67.00	33.00	67.00	46.00	54.00
	31.00	59.00	24.00	76.00	44.00	56.00
	35.00	72.00	28.00	72.00	39.00	61.00
	32.00	75.00	19.00	81.00	49.00	51.00
	30.00	62.00	27.00	63.00	42.00	58.00
Mean	31.00	67.00	26.20	71.80	44.00	56.00

| Std Dev | | 2.92 | 6.67 | 5.17 | 7.12 | 3.81 | 3.81 |

Table A8. Time course of calpain activity changes in skeletal and cardiac muscle with running exercise.

Condition	Calpain U/g
Control	26.83
Control	33.35
Control	21.60
Control	24.38
Control	30.16
Mean	27.26
Std Dev	4.64
72Hr Iso	41.87
72Hr Iso	37.30
72Hr Iso	31.89
72Hr Iso	42.58
72Hr Iso	44.04
Mean	39.54
Std Dev	4.96
72Hr E64c Iso	23.84
72Hr E64c Iso	25.84
72Hr E64c Iso	25.19
72Hr E64c Iso	26.35
72Hr E64c Iso	22.96
Mean	24.84
Std Dev	1.41

Table A9. Calpain activity 72 hours post isoproterenol injection.

Condition	Calpn-1	Calpn-2	Condition	Calpn-1	Calpn-2	Condition	Calpn-1	Calpn-2
Control	0.427	0.461	Con 72hr	0.402	0.480			
Control	0.399	0.514	Con 72hr	0.462	0.457			
Control	0.416	0.502	Con 72hr	0.473	0.508			
Control	0.475	0.502						
Control	0.432	0.465						
Control	0.380	0.399						
Mean	0.421	0.474	Mean	0.446	0.482			
Std Dev	0.033	0.043	Std Dev	0.038	0.026			
			E64c/Iso 6hr	0.398	0.428			
Iso 6hr	0.459	0.610	E64c/Iso 6hr	0.420	0.492			
Iso 6hr	0.561	0.720	E64c/Iso 6hr	0.460	0.469	E64c 6hr	0.375	0.442
Iso 6hr	0.644	0.651	E64c/Iso 6hr	0.402	0.475	E64c 6hr	0.412	0.399
Mean	0.554	0.660	Mean	0.420	0.466	Mean	0.394	0.420
Std Dev	0.093	0.056	Std Dev	0.029	0.027	Std Dev	0.026	0.030
Iso 12hr	0.634	0.726	E64c/Iso 12hr	0.401	0.506			
Iso 12hr	0.583	0.659	E64c/Iso 12hr	0.454	0.457			
Mean	0.609	0.693	Mean	0.428	0.482			
Std Dev	0.036	0.047	Std Dev	0.037	0.035			
Iso 24hr	0.550	0.687	E64c/Iso 24hr	0.456	0.585	E64c 24hr		
Iso 24hr	0.575	0.602	E64c/Iso 24hr	0.480	0.518	E64c 24hr		
Iso 24hr	0.608	0.652	E64c/Iso 24hr	0.399	0.544	E64c 24hr		
Mean	0.578	0.647	Mean	0.445	0.549	Mean		
Std Dev	0.029	0.043	Std Dev	0.042	0.033	Std Dev		
Iso 48hr	0.577	0.575						
Iso 48hr	0.517	0.623	E64c/I 48hr	0.415	0.487			
Iso 48hr	0.495	0.527	E64c/I 48hr	0.473	0.525			
Mean	0.529	0.575	Mean	0.444	0.506			
Std Dev	0.043	0.048	Std Dev	0.041	0.027			
Iso 72hr	0.463	0.584	E/I 72hr	0.438	0.463	E 72hr	0.438	
Iso 72hr	0.528	0.515	E/I 72hr	0.400	0.525	E 72hr	0.423	
Iso 72hr	0.498	0.539	E/I 72hr	0.418	0.486	E 72hr	0.400	
Mean	0.497	0.546	Mean	0.418	0.492	Mean	0.420	
Std Dev	0.033	0.035	Std Dev	0.019	0.031	Std Dev	0.029	

Table A10. Time course of calpain isoform activities following injections.

Condition	Calpastatin U/g	Condition	Calpastatin U/g	Condition	Calpastatin U/g
Control	0.654	Con 72hr	0.559	E64c 6hr	0.662
Control	0.606	Con 72hr	0.614	E64c 6hr	0.588
Control	0.574	Con 72hr	0.652	Mean	0.625
Mean	0.612	Mean	0.608	Std Dev	0.053
Std Dev	0.040	Std Dev	0.047		
Iso 6hr	0.607	E64c/Iso 6hr	0.730	E64c 72hr	0.652
Iso 6hr	0.683	E64c/Iso 6hr	0.672	E64c 72hr	0.604
Mean	0.645	Mean	0.701	Mean	0.628
Std Dev	0.054	Std Dev	0.041	Std Dev	0.034
Iso 12hr	0.736	E64c/Iso 12hr	0.695		
Iso 12hr	0.643	E64c/Iso 12hr	0.758		
Mean	0.690	Mean	0.726		
Std Dev	0.065	Std Dev	0.045		
Iso 24hr	0.652	E64c/Iso 24hr	0.695		
Iso 24hr	0.741	E64c/Iso 24hr	0.800		
Mean	0.696	Mean	0.747		
Std Dev	0.063	Std Dev	0.075		
Iso 48hr	0.706	E64c/Iso 48hr	0.813		
Iso 48hr	0.636	E64c/Iso 48hr	0.726		
Mean	0.671	Mean	0.770		
Std Dev	0.049	Std Dev	0.062		
Iso 72hr	0.663	E64c/Iso 72hr	0.724		
Iso 72hr	0.604	E64c/Iso 72hr	0.798		
Mean	0.634	Mean	0.761		
Std Dev	0.041	Std Dev	0.053		

Table A11. Time course of calpastatin activity following injections.

LAFP mmHg	Control 1	Control 2		Mean	Std Dev
3.76	86.24	85.09	(mmHg)	85.67	0.81
4.63	97.37	97.73		97.55	0.25
5.50	109.00	114.30		111.65	3.75
6.37	123.30	132.20		127.75	6.29
7.23	136.80	146.10		141.45	6.58
8.10	155.00	151.90		153.45	2.19
8.97	171.30	143.70		157.50	19.52
9.84	176.10	138.60		157.35	26.52
10.70	169.90	136.80		153.35	23.41
LAFP mmHg	E64c1	E64c2	E64c3	Mean	Std Dev
3.76	82.51	94.81	78.57	85.30	8.47
4.63	92.01	109.60	90.11	97.24	10.75
5.50	106.00	127.90	100.00	111.30	14.69
6.37	116.00	143.50	114.30	124.60	16.39
7.23	126.90	156.90	126.40	136.73	17.47
8.10	136.10	183.70	140.60	153.47	26.28
8.97	143.40	165.50	153.40	154.10	11.07
9.84	147.40	166.40	144.60	152.80	11.86
10.70	152.10	160.60	140.20	150.97	10.25

Table A12. Left ventricular max DLVP from control and E64c control groups 72 hours post injections.

LAFP mmHg	Control 1	Control 2		Mean	Std Dev
3.76	2812.00	2702.00	mmHg/sec	2757.00	77.78
4.63	3313.00	3106.00		3209.50	146.37
5.50	3922.00	3663.00		3792.50	183.14
6.37	4668.00	4431.00		4549.50	167.58
7.23	5413.00	5256.00		5334.50	111.02
8.10	6103.00	5566.00		5834.50	379.72
8.97	6478.00	4992.00		5735.00	1050.76
9.84	7226.00	4768.00		5997.00	1738.07
10.70	7433.00	4654.00		6043.50	1965.05
LAFP mmHg	E64c1	E64c2	E64c3	Mean	Std Dev
3.76	2682.00	3348.00	2534.00	2854.67	433.60
4.63	2801.00	4188.00	3187.00	3392.00	715.86
5.50	3242.00	5329.00	3721.00	4097.33	1093.21
6.37	3630.00	6235.00	4590.00	4818.33	1317.42
7.23	4009.00	7000.00	5328.00	5445.67	1498.97
8.10	4342.00	7408.00	5822.00	5857.33	1533.31
8.97	4120.00	7245.00	6132.00	5832.33	1583.91
9.84	4393.00	6713.00	5719.00	5608.33	1163.95
10.70	4608.00	6256.00	5685.00	5516.33	836.85

Table A13. Left ventricular +dP/dt from control and E64c control groups 72 hours post injections.

LAFP mmHg	Control 1	Control 2		Mean	Std Dev
3.76	1453.00	1521.00	mmHg/sec	1487.00	48.08
4.63	1770.00	1853.00		1811.50	58.69
5.50	2111.00	2418.00		2264.50	217.08
6.37	2521.00	2921.00		2721.00	282.84
7.23	3362.00	3608.00		3485.00	173.95
8.10	4044.00	3809.00		3926.50	166.17
8.97	5172.00	3306.00		4239.00	1319.46
9.84	5744.00	3171.00		4457.50	1819.39
10.70	4892.00	3111.00		4001.50	1259.36
LAFP mmHg	E64c1	E64c2	E64c3	Mean	Std Dev
3.76	1448.00	1664.00	1238.00	1451.00	301.23
4.63	1706.00	2133.00	1547.00	1840.00	414.36
5.50	2168.00	3116.00	1827.00	2471.50	911.46
6.37	2606.00	3584.00	2498.00	3041.00	767.92
7.23	3166.00	3878.00	3260.00	3569.00	436.99
8.10	3645.00	4809.00	4190.00	4499.50	437.70
8.97	3402.00	4946.00	4818.00	4882.00	90.51
9.84	3511.00	4682.00	3850.00	4266.00	588.31
10.70	3530.00	4468.00	3595.00	4031.50	617.30

Table A14. Left ventricular -dP/dt from control and E64c control groups 72 hours post injections.

LAFPmmHg	Control	Control	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	2244	2558	2251	1941	2808	3065	2177	2434.86	394.14
4.63	2493	2728	2682	2060	2983	3499	2473	2702.57	451.58
5.5	2810	3062	3182	2972	3199	3926	2803	3136.29	382.91
6.37	3189	3589	3808	3566	3713	4393	3384	3663.14	381.63
7.23	3591	4178	4284	4308	3708	4767	3931	4109.57	401.82
8.1	4149	4648	4657	4243	4276	5199	4695	4552.43	363.63
8.97	4688	5058	5187	5380	4793	5340	5285	5104.43	271.85
9.84	5124	5403	5360		5058	5689	5873	5417.83	316.26
10.7	5537	5603	5412	5515	5426	5683	6034	5601.43	212.94

FPmmHg	Swim	Swim	Swim	Swim	Swim			Mean	Std Dev
3.76	2208	1981	1976	1922	1274			1872.20	352.03
4.63	2352	2172	2243	2064	1380			2042.20	384.75
5.5	2453	2335	2528	2307	1547			2234.00	394.30
6.37	2601	2648	2789	2597	1873			2501.60	359.91
7.23	2562	2958	3110	3015	2238			2776.60	366.41
8.1	2462	3303	3405	3665	2976			3162.20	462.75
8.97	2669	3553	3796	4140	3565			3544.60	544.26
9.84	2122	4062	4112	4829	4498			3924.60	1054.76
10.7	2434	4106	4369	5185	4870			4192.80	1069.46

FPmmHg	E64c Swim	E64c Swim	E64c Swim	E64c Swim	Mean	Std Dev
3.76	1509	2012	1677	1480	1669.50	244.29
4.63	1671	2134	1859	1595	1814.75	240.02
5.5	1906	2403	2003	1804	2029.00	262.24
6.37	2170	2802	2097	2046	2278.75	352.53
7.23	2496	3159	2339	2251	2561.25	411.18
8.1	2873	3586	2879	2413	2937.75	484.16
8.97	3191	3856	3402	2729	3294.50	468.10
9.84	3492	4302	3698	3128	3655.00	491.51
10.7	3809	4502	4238	3352	3975.25	504.18

Table A15. Left ventricular +dP/dt from single swim groups.

LAFP	Control	Control	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	1589	1770	1349	962	1135	1233	1537	1367.79	281.99
4.63	1806	1879	1557	1252	1188	1494	1927	1586.14	297.15
5.5	1852	1995	2237	1573	1364	1799	2699	1931.29	439.86
6.37	2102	2469	2717	1938	1656	2316	3939	2448.14	743.91
7.23	2592	2470	3208	2286	2072	3095	4366	2869.86	776.53
8.1	3146	2944	3563	2961	2475	3776	4762	3375.29	746.05
8.97	3742	3582	3586	3476	2893	4196		3579.17	421.55
9.84	4198	4042	3733	3822	3222	4569	3725	3901.57	424.42
10.7	4989	4556	3722	3910	3518	4590	4817	4300.29	575.81
LAFP	Swim	Swim	Swim	Swim				Mean	Std Dev
3.76	1304	1288	1398	965				1238.80	188.74
4.63	1470	1495	1647	1119				1432.75	223.31
5.5	1667	1733	1985	1280				1666.25	291.69
6.37	2019	1934	2363	1618				1983.50	306.22
7.23	2392	2239	2863	1981				2368.75	370.59
8.1	2789	2483	3472	2684				2857.00	429.21
8.97	3109	2749	3856	3311				3256.25	462.49
9.84	3602	2959	4368	3380				3577.25	590.77
10.7	3608	3170	4682	4217				3919.25	665.51
LAFP	E64c Swim	E64c Swim	E64c Swim	E64c Swim				Mean	Std Dev
3.76	1051	1172	1124	800				1036.65	165.68
4.63	1248	1307	1196	1033				1196.00	117.75
5.5	1477	1592	1349	1159				1394.25	185.6
6.37	1664	1998	1585	1365				1653.00	262.5
7.23	1925	2328	1860	1513				1906.50	334.17
8.1	2162	2765	2347	1719				2248.25	433.72
8.97	2409	3118	2773	1963				2565.75	495.25
9.84	2618	3470	3288	2163				2884.75	604.76
10.7	2894	3615	3744	2436				3172.25	617.09

Table A16. Left ventricular -dP/dt from single swim groups.

LAFP	Control	Control	Control	Control	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	85.82	81.31	72.07	61.60	63.18	51.41	47.86	55.04	57.85	64.02	13.1549
4.63	92.66	90.89	75.74	66.11	73.39	59.47	51.35	66.53	66.69	71.43	13.5686
5.5	99.28	101.00	80.32	70.74	83.22	67.47	59.77	76.66	77.05	79.50	13.6602
6.37	110.40	109.10	85.82	82.20	96.40	77.97	70.55	89.47	88.56	90.05	13.3491
7.23	119.00	117.40	91.20	84.29	103.90	87.26	81.57	100.10	103.20	98.66	13.6895
8.1	132.70	125.50	97.59	96.45	116.50	99.43	91.95	108.00	103.10	107.91	14.0772
8.97	141.50	132.60	103.30	105.90	121.60	109.60	100.00	115.50	121.80	116.87	13.895
9.84	151.20	139.70	108.00	113.70	129.00	118.00	106.70	118.80	110.10	121.69	15.3333
10.7	158.50	135.50	114.90	123.30	127.50	125.50	113.00	119.10	125.30	126.96	13.6399

LAFP	Swim	Swim	Swim	Swim	Mean	Std Dev
3.76	83.55	85.86	76.59	61.91	76.98	10.7901
4.63	89.50	93.84	82.85	66.37	83.14	12.059
5.5	95.88	103.20	91.78	71.29	90.54	13.6735
6.37	106.00	111.60	101.10	80.92	99.91	13.3639
7.23	115.60	121.30	112.30	91.02	110.06	13.2233
8.1	126.20	130.10	124.20	105.40	121.48	10.9931
8.97	134.40	139.50	133.10	118.50	131.38	9.01679
9.84	150.40	148.80	143.90	127.20	142.58	10.6165
10.7	151.10	155.70	150.20	143.40	150.10	5.07477

LAFP	E64c Swim	E64c Swim	E64c Swim	E64c Swim	Mean	Std Dev
3.76	74.05	82.07	68.87	67.40	73.10	6.62687
4.63	80.66	86.72	73.40	74.40	78.80	6.18347
5.5	89.55	95.55	79.56	81.75	86.60	7.34595
6.37	97.59	108.40	86.21	87.90	95.03	10.2297
7.23	108.80	118.00	94.71	94.11	103.91	11.592
8.1	120.70	130.70	105.80	101.10	114.58	13.615
8.97	129.80	139.80	116.30	109.30	123.80	13.6443
9.84	138.70	152.60	125.00	115.90	133.05	16.0525
10.7	149.30	158.30	135.70	125.10	142.10	14.6542

Table A17. Left ventricular max DLVP from single swim groups.

P mmHg	Control	Control	Control	Control		Mean	St Dev
3.76	2599	2866	2490	1932		2471.75	392.98
4.63	3022	3095	2677	2459		2813.25	298.34
5.50	3271	3478	2975	2731		3113.75	328.20
6.37	3660	4031	3283	3206		3545.00	379.91
7.23	4576	5043	3586	3845		4262.50	668.19
8.10	5254	5650	4146	4309		4839.75	728.26
8.97	5532	6411	4723	5008		5418.50	741.66
9.84	6134	7156	5394	5686		6092.50	771.56
10.70	6761	6480	5819	6469		6382.25	399.08

LAFP mmHg	Swim	Swim	Swim	Swim	Swim	Mean	St Dev
3.76	2301	1786	2147	2093	2122	2089.80	187.90
4.63	2673	1947	2432	2352	2289	2338.60	262.93
5.50	3138	2055	2648	2563	2461	2573.00	389.17
6.37	3551	2290	3062	2764	2828	2899.00	459.84
7.23	4248	2357	3441	3201	3154	3280.20	677.68
8.10	4736	2690	4043	3570	3892	3786.20	746.27
8.97	5454	3098	4602	3945	4499	4319.60	870.55
9.84	6544	3984	5292	4506	5632	5191.60	995.16
10.70	7177	4664	5845	4049	6705	5688.00	1323.91

LAFP mmHg	E64c Swim	E64c Swim	E64c Swim	E64c Swim		Mean	St Dev
3.76	2306	2135	1601	1755		1949.25	327.00
4.63	2702	2386	1799	2039		2231.50	395.54
5.50	2899	2653	1999	2202		2438.25	411.16
6.37	3332	3031	2253	2435		2762.75	504.40
7.23	3641	3613	2571	2545		3092.50	617.38
8.10	4178	4336	3235	2912		3665.25	698.88
8.97	4843	5228	3372	3576		4254.75	918.91
9.84	5464	5818	4016	4329		4906.75	869.51
10.70	6122	6599	5227	5003		5737.75	750.58

mmHg	E64c Control	E64c Control				Mean	St Dev
3.76	2668	2888				2778	155.56
4.63	3016	3035				3026	13.44
5.50	3344	3302				3323	29.70
6.37	3530	3766				3648	166.88
7.23	4049	4142				4096	65.76
8.10	4502	4717				4610	152.03
8.97	5026	5656				5341	445.48
9.84	5918	6565				6242	457.50
10.70	7050	7508				7279	323.85

Table A18. Left ventricular +dP/dt from swim trained groups.

LAFP mmHg	Control	Control	Control	Control		Mean	St Dev
3.764	1322	1796	1032	1626		1444.00	337.47
4.631	1630	2126	1121	2108		1746.25	475.93
5.499	2018	2438	1530	2482		2117.00	443.71
6.366	2333	2758	1753	2815		2414.75	490.79
7.234	3667	3378	2049	3294		3097.00	716.70
8.1	3471	3843	2231	3654		3299.75	728.51
8.968	4065	4412	2509	4325		3827.75	891.44
9.836	4908	5187	2725	4697		4379.25	1120.94
10.703	5067	4675	2911	5265		4479.50	1074.02

LAFP mmHg	Swim	Swim	Swim	Swim	Swim	Mean	St Dev
3.764	1523	862	1213	1104	998	1140.04	250.30
4.631	1854	932	1498	1414	1109	1361.40	357.83
5.499	2324	1117	1824	1791	1375	1686.20	463.03
6.366	2681	1408	2377	1992	1749	2041.40	502.70
7.234	3203	1525	2689	2186	1952	2311.00	652.08
8.1	3673	1784	3152	2432	2398	2687.80	733.55
8.968	4299	2105	3715	2661	2740	3104.00	884.32
9.836	4923	2670	4104	3349	3464	3702.00	851.28
10.703	5386	3025	4913	3830	4238	4278.40	922.39

LAFP mmHg	E64c Swim	E64c Swim	E64c Swim	E64c Swim		Mean	St Dev
3.764	1267	938	1527	1175		1226.73	243.50
4.631	1584	1279	1884	1488		1558.75	251.46
5.499	1909	1594	2097	1765		1841.25	213.65
6.366	2252	2078	2396	2111		2209.25	145.58
7.234	2446	2375	2784	2460		2516.25	182.34
8.1	2859	2787	3259	2788		2923.25	226.36
8.968	3318	3203	3775	3357		3413.25	249.87
9.836	3780	3707	4283	4068		3959.50	266.08
10.703	4354	4235	4912	4555		4514.00	296.38

LAFP mmHg	E64c Control	E64c Control				Mean	St Dev
3.76	1452	1527				1489.5	53.033
4.63	1844	1777				1810.5	47.3762
5.50	2258	2188				2223	49.4975
6.37	2730	2731				2730.5	0.70711
7.23	3324	3015				3169.5	218.496
8.10	3826	3346				3586	339.411
8.97	4292	4038				4165	179.605
9.84	4942	4779				4860.5	115.258
10.70	5296	5429				5362.5	94.0452

Table A19. Left ventricular -dP/dt from swim trained groups.

LAFP mmHg	Control	Control	Control	Control		Mean	St Dev
3.764	66.46	66.98	50.68	64.75		62.22	7.75
4.631	76.06	74.33	57.29	74.54		70.56	8.88
5.499	83.71	85.82	67.97	82.11		79.90	8.10
6.366	93.47	96.78	75.77	92.16		89.55	9.39
7.234	104.80	114.30	85.40	105.30		102.45	12.18
8.1	120.30	124.20	95.82	115.00		113.83	12.58
8.968	125.80	135.20	105.00	128.10		123.53	12.98
9.836	136.60	144.70	115.30	139.20		133.95	12.88
10.703	144.60	136.80	121.80	153.60		139.20	13.48

LAFP mmHg	Swim	Swim	Swim	Swim	Swim	Mean	St Dev
3.764	60.46	45.02	58.37	48.26	52.59	52.94	6.53
4.631	68.74	49.67	64.92	57.05	58.04	59.68	7.41
5.499	81.03	54.46	72.06	66.64	64.35	67.71	9.80
6.366	91.49	62.92	83.93	74.03	74.32	77.34	10.86
7.234	105.00	66.48	93.00	84.10	81.66	86.05	14.26
8.1	114.30	73.88	105.70	92.96	95.93	96.55	15.22
8.968	126.20	83.91	115.40	101.00	104.30	106.16	15.91
9.836	142.30	99.91	125.90	115.20	120.00	120.66	15.46
10.703	151.60	112.20	135.90	103.90	134.70	127.66	19.33

LAFP mmHg	E64c Swim	E64c Swim	E64c Swim	E64c Swim		Mean	St Dev
3.764	54.34	48.17	64.00	52.02		54.63	6.74
4.631	62.98	56.83	71.80	57.25		62.22	6.98
5.499	69.45	61.75	79.05	63.29		68.39	7.85
6.366	79.88	70.49	87.97	70.61		77.24	8.40
7.234	86.39	76.52	99.20	78.43		85.14	10.30
8.1	97.11	86.56	113.00	93.37		97.51	11.21
8.968	109.20	102.00	126.70	98.30		109.05	12.61
9.836	120.30	113.60	136.30	112		120.55	11.10
10.703	133.50	124.30	146.60	130.9		133.83	9.36

LAFP mmHg	E64c Control	E64c Control				Mean	St Dev
3.76	60.71	64.96				62.835	3.01
4.63	70.41	68.96				69.685	1.03
5.50	79.49	76.34				77.915	2.23
6.37	88.59	87.97				88.28	0.44
7.23	100.40	96.04				98.22	3.08
8.10	110.50	106.70				108.6	2.69
8.97	122.40	121.60				122	0.57
9.84	144.40	135.60				140	6.22
10.70	166.70	150.60				158.65	11.38

Table A20. Left ventricular max DLVP from swim trained groups.

LAFP(mmHg)	Control	Control	Control	Mean	Std Dev
3.76	2171	2870	2957	2666.00	430.88
4.63	2371	3351	3202	2974.67	528.07
5.50	2751	3814	3408	3324.33	536.42
6.37	3225	4225	3727	3725.67	500.00
7.23	3663	4731	4248	4214.00	534.81
8.10	4114	4816	5214	4714.67	556.96
8.97	4524	4820	5943	5095.67	748.59
9.84	4728	4652	6678	5352.67	1148.40
10.70	4429	4513	7013	5318.33	1468.23

LAFP(mmHg)	Iso then E64c	Iso then E64c	Iso then E64c	Mean	Std Dev
3.76	3132	2909	3339	3126.67	215.05
4.63	3717	3398	3614	3576.33	162.80
5.50	4404	3978	4239	4207.00	214.80
6.37	5369	4490	4925	4928.00	439.51
7.23	6035	4838	5642	5505.00	610.15
8.10	6723	5101	6200	6008.00	827.87
8.97	7041	5584	6795	6473.33	779.95
9.84	7273	5437	7218	6642.67	1044.50
10.70	6981	5442	7168	6530.33	947.15

LAFP(mmHg)	E64c then Iso	E64c then Iso		Mean	Std Dev
3.76	2757	3260		3008.50	355.67
4.63	3040	3580		3310.00	381.84
5.50	3537	4153		3845.00	435.58
6.37	4224	4941		4582.50	507.00
7.23	4961	5550		5255.50	416.49
8.10	5664	6334		5999.00	473.76
8.97	6181	6747		6464.00	400.22
9.84	6724	7035		6879.50	219.91
10.70	6682	6932		6807.00	176.78

Table A21. Left ventricular +dP/dt from E64c and isoproterenol order trials.

LAFP mmHg	Control	Control	Control	Mean	Std Dev
3.76	1540	1661	1706	1635.67	85.85
4.63	1841	1949	2043	1944.33	101.08
5.50	2343	2061	2495	2299.67	220.22
6.37	2872	2245	3018	2711.67	410.69
7.23	3389	2621	3277	3095.67	414.87
8.10	3825	3388	3635	3616.00	219.12
8.97	4040	4045	3699	3928.00	198.34
9.84	3932	4562	3439	3977.67	562.89
10.70	3539	4911	3302	3917.33	868.66
LAFP mmHg	E64 then Iso	E64 then Iso	E64 then Iso	Mean	Std Dev
3.76	1882	1814	2233	1976.33	224.87
4.63	2306	2155	2538	2333.00	192.92
5.50	2742	2654	2766	2720.67	58.97
6.37	3254	3255	2928	3145.67	188.51
7.23	3978	3986	3515	3826.33	269.65
8.10	4474	4655	4395	4508.00	133.29
8.97	4694	5007	5057	4919.33	196.74
9.84	5030	4746	5467	5081.00	363.20
10.70	4956	4496	5577	5009.67	542.49
LAFP mmHg	Iso then E64c	Iso then E64c		Mean	Std Dev
3.76	1892	1669		1780.50	157.68
4.63	2132	1798		1965.00	236.17
5.50	2580	2215		2397.50	258.09
6.37	3160	2806		2983.00	250.32
7.23	3808	3539		3673.50	190.21
8.10	4491	4435		4463.00	39.60
8.97	4735	5014		4874.50	197.28
9.84	4873	5596		5234.50	511.24
10.70	4756	5515		5135.50	536.69

Table A22. Left ventricular -dP/dt from E64c and isoproterenol order trials.

LAFP mmHg	Control	Control	Control		Mean	Std Dev
3.76	61.13	72.64	70.25		68.01	6.07
4.63	69.10	79.10	81.38		76.53	6.53
5.50	81.37	84.50	91.42		85.76	5.14
6.37	93.94	92.09	104.20		96.74	6.52
7.23	106.60	103.40	113.20		107.73	5.00
8.10	117.20	121.70	121.80		120.23	2.63
8.97	127.00	137.40	123.40		129.27	7.27
9.84	130.90	153.80	121.90		135.53	16.45
10.70	126.10	157.10	118.10		133.77	20.60
LAFP mmHg	Iso then E64c	Iso then E64c	Iso then E64c		Mean	Std Dev
3.76	82.47	70.82	67.88		73.72	7.72
4.63	90.56	79.48	79.79		83.28	6.31
5.50	103.40	91.99	95.20		96.86	5.88
6.37	118.30	105.70	107.80		110.60	6.75
7.23	131.70	117.90	118.90		122.83	7.70
8.10	141.90	130.50	127.90		133.43	7.45
8.97	151.40	137.30	134.70		141.13	8.99
9.84	158.80	146.90	135.20		146.97	11.80
10.70	163.40	149.50	134.90		149.27	14.25
LAFP mmHg	E64c then Iso	E64c then Iso			Mean	Std Dev
3.76	74.34	71.34			72.84	2.12
4.63	82.73	80.46			81.60	1.61
5.50	92.47	92.53			92.50	0.04
6.37	106.50	106.30			106.40	0.14
7.23	117.40	121.00			119.20	2.55
8.10	131.40	135.00			133.20	2.55
8.97	138.20	144.50			141.35	4.45
9.84	146.70	156.40			151.55	6.86
10.70	148.90	161.10			155.00	8.63

Table A23. Left ventricular max DLVP from E64c and isoproterenol order trials.

LAFP mmHg	Control	Control	Control	Control	Control		Mean	Std Dev
3.76	2240	2558	2251	2141	3833		2604.60	704.28
4.63	2493	2728	2682	2460	4450		2962.60	839.53
5.50	3040	3062	3182	3172	5090		3509.20	885.98
6.37	3389	3589	3808	3566	5321		3934.60	789.16
7.23	3591	4178	4284	4308	5504		4373.00	696.57
8.10	4549	4848	4857	4730	5666		4930.00	429.80
8.97	4888	5258	5187	5180	5420		5186.60	192.86
9.84	5324	5403	5360	5330	6059		5495.20	316.72
10.70	5637	5603	5412	5515	6113		5656.00	269.92

LAFP mmHg	Iso	Iso	Iso				Mean	Std Dev
3.76	3494	3101	4033				3542.67	467.90
4.63	3788	3833	4844				4155.00	597.12
5.50	4306	4204	5490				4666.67	714.85
6.37	4961	4973	6217				5383.67	721.71
7.23	5771	5858	6741				6123.33	536.68
8.10	6113	7094	5799				6335.33	675.52
8.97	6333	7232	5920				6495.00	670.83
9.84	6676	7166	6259				6700.33	453.99
10.70	6312	7153	6113				6526.00	552.04

LAFP mmHg	E64c Iso	E64c Iso	E64c Iso				Mean	Std Dev
3.76	3481	2835	2764				3026.67	395.06
4.63	3840	3021	3056				3305.67	463.08
5.50	4521	3385	3371				3759.00	659.95
6.37	5141	3851	3799				4263.67	760.24
7.23	5982	4570	4822				5124.67	753.09
8.10	6362	6241	5687				6096.67	359.90
8.97	6686	6415	6047				6382.67	320.72
9.84	6296	6193	6088				6192.33	104.00
10.70	6314	6016	5866				6065.33	228.04

Table A24. Left ventricular +dP/dt 24 hours post injections

LAFP(mmHg)	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	961.5	1135	1233	1537		1216.63	241.29
4.63	1252	1388	1494	1927		1515.25	291.82
5.50	1773	1664	1799	2699		1983.75	480.41
6.37	2168	2056	2316	3439		2494.75	638.44
7.23	2486	2572	3095	3939		3023.00	667.34
8.10	2961	2875	3776	4362		3493.50	707.15
8.97	3476	3183	4196	4662		3879.25	673.40
9.84	3822	3422	4569	4817		4157.50	647.51
10.70	3910	3518	4590	4901		4229.75	629.60

LAFP(mmHg)	Iso	Iso	Iso	Iso	Mean	Std Dev
3.76	1500	1915	1499		1638.00	239.89
4.63	1733	2418	1779		1976.67	382.90
5.50	2039	2711	2238		2329.33	345.18
6.37	2419	3298	2837		2851.33	439.68
7.23	2920	3773	3270		3321.00	428.78
8.10	3435	4128	3857		3806.67	349.23
8.97	4201	4837	4323		4453.67	337.53
9.84	4166	4433	4544		4381.00	194.29
10.70	4230	4260	4279		4256.33	24.70

LAFP(mmHg)	E64c Iso	E64c Iso	E64c Iso	Mean	Std Dev
3.76	1415	1612	1654	1560.33	127.60
4.63	1557	2393	1956	1968.67	418.14
5.50	2093	3037	2322	2484.00	492.41
6.37	2383	3455	2766	2868.00	543.23
7.23	3291	3933	3147	3457.00	418.47
8.10	4268	4294	3645	4069.00	367.42
8.97	4557	4471	3887	4305.00	364.54
9.84	4604	4433	4153	4396.67	227.68
10.70	4500	4294	4079	4291.00	210.52

Table A25. Left ventricular -dP/dt 24 hours post injections

LAFP(mmHg)	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	51.41	47.86	55.04	57.85	78.02	58.04	11.79
4.63	59.47	51.35	66.53	66.69	87.36	66.28	13.36
5.50	67.47	59.77	76.66	77.05	97.29	75.65	14.06
6.37	77.97	70.55	89.47	88.56	106.6	86.63	13.64
7.23	87.26	81.57	100.1	103.2	115.6	97.55	13.47
8.10	99.43	91.95	108	103.1	126.2	105.74	12.85
8.97	109.6	100	115.5	121.8	128.6	115.10	11.02
9.84	118	106.7	118.8	125.3	142.1	122.18	12.99
10.70	125.5	113	119.1	124.2	150.9	126.54	14.48

LAFP(mmHg)	Iso	Iso	Iso	Mean	Std Dev
3.76	71.01	90.31	65.05	75.46	13.20
4.63	79.56	91.42	76.61	82.53	7.84
5.50	89.26	104.6	90.51	94.79	8.52
6.37	104.2	122.8	104.2	110.40	10.74
7.23	118.9	137.9	112.8	123.20	13.09
8.10	129.2	146.9	107.8	127.97	19.58
8.97	138.2	151.2	114.3	134.57	18.72
9.84	152.5	145	122.9	140.13	15.39
10.70	152.1	149.2	121.8	141.03	16.72

LAFP(mmHg)	E64c Iso	E64c Iso	E64c Iso	Mean	Std Dev
3.76	72.03	68.31	65.31	68.55	3.37
4.63	80.28	72.22	69.85	74.12	5.47
5.50	92.62	76.04	77.14	81.93	9.27
6.37	94.29	104.83	90.22	96.45	7.54
7.23	104.5	127	106.3	112.60	12.50
8.10	118.8	134.1	122.87	125.26	7.92
8.97	130.3	136.2	128.9	131.80	3.87
9.84	134.7	136.4	130.12	133.74	3.25
10.70	138	130.52	131.55	133.36	4.05

Table A26. Left ventricular max DLVP 24 hours post injections

LAFPmmHg	Control	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	2244	2558	2251	1941	2808	3065	2477.83	413.41
4.63	2493	2728	2682	2060	2983	3499	2740.83	482.09
5.50	2810	3062	3182	2972	3199	3926	3191.83	387.33
6.37	3189	3589	3808	3566	3713	4393	3709.67	395.71
7.23	3591	4178	4284	4308	3708	4767	4139.33	431.64
8.10	4149	4648	4657	4243	4276	5199	4528.67	392.34
8.97	4688	5058	5187	5380	4793	5340	5074.33	284.74
9.84	5124	5403	5360		5058	5689	5326.80	250.74
10.70	5537	5603	5412	5515	5426	5683	5529.33	103.68

LAFPmmHg	Iso	Iso	Iso	Iso			Mean	Std Dev
3.76	2730	2768	3597	2569			2916.00	462.12
4.63	3250	3089	4083	2655			3269.25	597.87
5.50	3565	3496	4758	2972			3697.75	754.80
6.37	4284	4034	5827	3424			4392.25	1022.43
7.23	4421	4615	6266	3852			4788.50	1036.85
8.10	4815	5249	7108	5708			5720.00	994.58
8.97	5383	5947	7369	6411			6277.50	840.35
9.84	5441	6910	6684	7228			6565.75	782.33
10.70	5652	7334	6421	7576			6745.75	882.63

LAFPmmHg	E64c Iso	E64c Iso	E64c Iso	E64c Iso	E64c Iso	E64c Iso	Mean	Std Dev
3.76	3281	2796	3242	2611	1484	3434	2808.00	720.54
4.63	3751	2795	3348	2807	1603	3869	3028.83	832.61
5.50	5026	3281	3935	3190	1808	4493	3622.17	1133.25
6.37	5609	4059	4703	3810	2582	5394	4359.50	1122.60
7.23	5726	4000	5348	5780	2891	6201	4991.00	1276.43
8.10	5041	4380	5579	5989	3499	6691	5196.50	1147.19
8.97	5207	4962	6258	6304	4150	6550	5571.83	947.66
9.84	5029	5338	5954	6093	4748	5719	5480.17	532.34
10.70	4963	5901	5089	6266	5150	5192	5426.83	527.27

Table A27. Left ventricular +dP/dt 72 hour post injections.

LAFPmmHg	Control	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	1770	1349	962	1135	1233	1537	1330.92	289.84
4.63	1879	1557	1252	1188	1494	1927	1549.50	307.70
5.50	1995	2237	1573	1364	1799	2699	1944.50	480.32
6.37	2469	2717	1938	1656	2316	3939	2505.83	797.57
7.23	2470	3208	2286	2072	3095	4366	2916.17	839.99
8.10	2944	3563	2961	2475	3776	4762	3413.50	809.71
8.97	3582	3586	3476	2893	4196		3546.60	462.79
9.84	4042	3733	3822	3222	4569	3725	3852.17	442.33
10.70	4556	3722	3910	3518	4590	4817	4185.50	535.91

LAFPmmHg	Iso	Iso	Iso	Iso			Mean	Std Dev
3.76	1463	1300	2230	1309			1575.50	442.70
4.63	1784	1707	2418	1266			1793.75	474.63
5.50	2004	2041	2854	1301			2050.00	634.98
6.37	2482	2562	3805	1430			2569.75	971.71
7.23	2809	3206	4270	1714			2999.75	1056.03
8.10	3267	3811	4753	3054			3721.25	758.10
8.97	3621	4374	4466	3920			4095.25	396.14
9.84	3957	4842	3895	4366			4265.00	437.76
10.70	4195	4928	3870	4919			4478.00	531.27

LAFPmmHg	E64c Iso	E64c Iso	E64c Iso	E64c Iso	E64c Iso	E64c Iso	Mean	Std Dev
3.76	1992	2030	1870	1031	870	2227	1669.93	571.40
4.63	2497	2046	1921	1342	843	2694	1890.57	697.97
5.50	2999	2450	2323	1548	926	3169	2235.75	859.86
6.37	3212	2998	2900	2031	1791	3708	2773.33	727.82
7.23	3253	2756	3573	2252	1935	4142	2985.17	830.48
8.10	3068	3200	4137	2893	2417	4147	3310.33	696.63
8.97	3261	3530	4761	3370	3513	3646	3680.17	546.22
9.84	3138	3679	4289	4058	4186	3084	3739.00	528.75
10.70	2986	4055	3643	4597	4425	2817	3753.83	739.14

Table A28. Left ventricular +dP/dt 72 hour post injections.

LAFPmmHg	Control	Control	Control	Control	Control	Control	Mean	Std Dev
3.76	61.60	63.18	51.41	47.86	55.04	57.85	56.16	5.91
4.63	66.11	73.39	59.47	51.35	66.53	66.69	63.92	7.57
5.50	70.74	83.22	67.47	59.77	76.66	77.05	72.49	8.29
6.37	82.20	96.40	77.97	70.55	89.47	88.56	84.19	9.22
7.23	84.29	103.90	87.26	81.57	100.10	103.20	93.39	10.12
8.10	96.45	116.50	99.43	91.95	108.00	103.10	102.57	8.76
8.97	105.90	121.60	109.60	100.00	115.50	121.80	112.40	8.79
9.84	113.70	129.00	118.00	106.70	118.80	110.10	116.05	7.84
10.70	123.30	127.50	125.50	113.00	119.10	125.30	122.28	5.37

LAFPmmHg	Iso	Iso	Iso	Iso			Mean	Std Dev
3.76	69.59	66.48	86.04	48.92			67.76	15.21
4.63	84.03	76.55	97.20	50.25			77.01	19.78
5.50	91.20	87.81	108.90	58.22			86.53	21.02
6.37	105.50	100.60	125.20	67.67			99.74	23.88
7.23	112.00	113.60	130.90	76.00			108.13	23.06
8.10	123.60	126.00	141.30	106.70			124.40	14.17
8.97	135.90	136.00	143.20	116.40			132.88	11.50
9.84	146.00	149.70	136.60	130.60			140.73	8.72
10.70	158.20	154.90	133.50	136.70			145.83	12.53

LAFPmmHg	E64c Iso	E64c Iso	E64c Iso	E64c Iso	E64c Iso	E64c Iso	Mean	Std Dev
3.76	71.07	63.06	78.18	59.94	34.91	71.43	63.10	15.26
4.63	83.56	65.39	81.44	68.06	37.96	80.41	69.47	17.16
5.50	104.80	75.26	91.12	76.04	44.14	91.35	80.45	20.93
6.37	117.90	89.43	103.70	87.50	62.02	106.00	94.43	19.46
7.23	122.70	87.70	114.30	105.10	70.49	118.70	103.17	20.29
8.10	118.30	101.00	119.10	110.70	81.43	128.90	109.91	16.77
8.97	123.20	109.50	131.40	115.30	98.51	128.20	117.69	12.41
9.84	122.70	111.90	135.50	116.70	112.00	120.10	119.82	8.81
10.70	121.90	121.40	134.00	123.40	120.50	112.80	122.33	6.82

Table A29. Left ventricular max DVLP 72 hour post injections.

Single Swim		Repeated Swimming		24hr Isoproterenol	
Condition	ATPase	Condition	ATPase	Condition	ATPase
Control	0.175	Control	0.18	Control	0.175
Control	0.183	Control	0.174	Control	0.183
Control	0.181	Control	0.2	Control	0.181
Control	0.191	Control	0.167	Control	0.191
		Control	0.161		
		Control	0.135		
Mean	0.18	Mean	0.17	Mean	0.18
Std Dev	0.01	Std Dev	0.03	Std Dev	0.01
Single Swim	0.132	Repeated Swim	0.179	Iso 24hr	0.169
Single Swim	0.157	Repeated Swim	0.177	Iso 24hr	0.163
Single Swim	0.148	Repeated Swim	0.157	Iso 24hr	0.16
Single Swim	0.172	Repeated Swim	0.193	Iso 24hr	0.172
Single Swim	0.12	Repeated Swim	0.18		
Single Swim	0.136	Repeated Swim	0.114		
Mean	0.14	Mean	0.17	Mean	0.17
Std Dev	0.02	Std Dev	0.03	Std Dev	0.01
E64c Swim	0.09				
E64c Swim	0.136				
E64c Swim	0.152			E64c Iso 24hr	0.149
E64c Swim	0.159			E64c Iso 24hr	0.185
E64c Swim	0.158	E64c R Swim	0.197	E64c Iso 24hr	0.181
E64c Swim	0.143	E64c R Swim	0.174	E64c Iso 24hr	0.166
Mean	0.14	Mean	0.19	Mean	0.17
Std Dev	0.03	Std Dev	0.02	Std Dev	0.02

Table A30. Left ventricular myofibrilar ATPase activity from swim and isoproternol groups.

Single Swim		Repeated Swim		24hr Iso		72hr Iso	
Condition	[cAMP]	Condition	[cAMP]	Condition	[cAMP]	Condition	[cAMP]
Control	557.30	Control	361.70				
Control	737.34	Control	454.17				
Control	457.08	Control	349.34				
Control	672.50	Control	423.88				
		Control	467.50				
		Control	528.10				
Mean	606.06	Mean	430.78				
Std Dev	124.13	Std Dev	67.56				
Swim	806.52						
Swim	882.92						
Swim	970.67						
Swim	1250.15						
Swim	1043.00	Swim	340.80	24hr Iso	1114.32	72hr Iso	638.51
Swim	1045.65	Swim	326.70	24hr Iso	774.80	72hr Iso	708.59
Swim	1064.21	Swim	651.85	24hr Iso	677.27	72hr Iso	399.05
Swim	1002.90	Swim	396.41	24hr Iso	842.70	72hr Iso	615.52
Swim	1131.20	Swim	416.20	24hr Iso	1202.40	72hr Iso	746.60
Swim	672.80	Swim	475.70	24hr Iso	1016.40	72hr Iso	500.95
Mean	987.00	Mean	434.61	Mean	937.98	Mean	601.54
Std Dev	165.07	Std Dev	119.27	Std Dev	205.35	Std Dev	130.53
						72hr E/Iso	747.01
E/AcSw	806.52			24hr E/Iso	636.32	72hr E/Iso	785.19
E/AcSw	306.48			24hr E/Iso	638.07	72hr E/Iso	784.83
E/AcSw	695.95	E/Chr Sw	485.73	24hr E/Iso	346.96	72hr E/Iso	633.86
E/AcSw	790.28	E/Chr Sw	313.18	24hr E/Iso	593.16	72hr E/Iso	551.29
Mean	649.81	Mean	399.46	Mean	553.63	Mean	700.44
Std Dev	234.02	Std Dev	122.01	Std Dev	139.34	Std Dev	103.86
		E/Chr con	487.19				
		E/Chr con	210.29				
		E/Chr con	456.60				
		E/Chr con	520.76				
		E/Chr con	403.76				
		Mean	415.72				
		Std Dev	122.63				

Table 31. Left ventricular [cAMP] from swim and isoproterenol groups.

	[ATP]	[ADP]	[AMP]	[TAN]		[ATP]	[ADP]	[AMP]	[TAN]
Control	4.51	1.56	0.43	6.50	E64c Control	3.32	1.58	0.42	5.32
Control	4.24	1.61	0.47	6.32	E64c Control	2.89	1.56	0.43	4.88
Control	4.01	1.62	0.42	6.05	E64c Control	4.45	1.47	0.42	6.34
Control	3.91	1.15	0.32	5.37	E64c Control	4.07	1.53	0.44	6.04
Control	4.43	1.43	0.46	6.32					
Control	3.92	1.41	0.39	5.72					
Mean	4.17	1.46	0.42	6.05	Mean	3.68	1.54	0.43	5.65
std dev	0.26	0.18	0.05	0.43	std dev	0.71	0.05	0.01	0.67
Iso 24 hrs	3.05	1.54	0.45	5.04	Iso/E 24hrs	3.67	1.35	0.40	5.42
Iso 24 hrs	2.47	1.30	0.38	4.15	Iso/E 24hrs	3.67	1.32	0.40	5.39
Iso 24hrs	2.75	1.50	0.48	4.73	Iso/E 24hrs	3.53	1.31	0.36	5.20
Iso 24hrs	2.39	1.19	0.33	3.91	Iso/E 24hrs	3.09	1.36	0.39	4.84
Iso 24hrs	3.19	1.17	0.35	4.71	Iso/E 24hrs	4.25	1.21	0.38	5.84
Iso 24hrs	3.02	1.38	0.38	4.78	Iso/E 24hrs	3.69	1.51	0.40	5.60
Iso 24hrs	3.36	1.48	0.41	5.25					
Mean	2.89	1.37	0.40	4.65	Mean	3.65	1.34	0.39	5.38
std dev	0.37	0.15	0.05	0.47	std dev	0.37	0.10	0.02	0.34
Iso 48hrs	2.88	1.38	0.48	4.24	Iso/E 48hrs	3.56	1.22	0.35	5.13
Iso 48hrs	3.30	1.52	0.42	5.24	Iso/E 48hrs	3.00	1.30	0.38	4.68
Iso 48hrs	3.00	1.25	0.36	4.61	Iso/E 48hrs	3.77	1.22	0.37	5.36
Mean	3.06	1.38	0.42	4.70	Mean	3.44	1.25	0.37	5.06
std dev	0.22	0.14	0.06	0.51	std dev	0.40	0.05	0.02	0.35
Iso 72hrs	4.74	1.86	0.40	6.73	Iso/E 72hrs	4.62	1.74	0.42	6.78
Iso 72hrs	4.17	1.21	0.51	5.89	Iso/E 72hrs	3.90	1.52	0.33	5.75
Iso 72hrs	2.75	1.46	0.44	4.65	Iso/E 72hrs	4.99	2.03	0.44	7.46
Iso 72hrs	3.34	1.30	0.32	4.96	Iso/E 72hrs	3.47	1.35	0.37	5.19
Iso 72hrs	3.74	1.43	0.38	5.55	Iso/E 72hrs	3.92	1.17	0.36	5.45
Iso 72hrs	2.87	1.42	0.38	4.67					
Mean	3.60	1.45	0.41	5.41	Mean	4.18	1.56	0.38	6.13
std dev	0.77	0.22	0.06	0.81	std dev	0.61	0.34	0.05	0.96
Swim	5.39	2.63	0.32	8.34	E64c/Swim	2.76	0.77	0.19	3.73
Swim	3.20	1.01	0.27	4.48	E64c/Swim	2.54	0.88	0.25	3.67
Swim	3.42	0.94	0.36	4.72	E64c/Swim	4.25	1.65	0.42	6.32
Swim	4.54	1.02	0.25	5.81	E64c/Swim	3.61	1.36	0.31	5.28
Swim	2.59	0.85	0.30	3.75	E64c/Swim	2.40	0.93	0.26	3.59
Mean	3.83	1.29	0.30	5.42	Mean	3.11	1.12	0.29	4.52
std dev	1.12	0.75	0.04	1.79	std dev	0.79	0.37	0.08	1.23

Table 32. Left ventricular adenylate concentrations from swim and isoproterenol groups.

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	[ATP]/[ADP]		[ATP]/[ADP]
Control	2.89	E64c Control	2.10
Control	2.63	E64c Control	1.85
Control	2.48	E64c Control	3.03
Control	3.40	E64c Control	2.66
Control	3.10		
Control	2.78		
Mean	2.88	Mean	2.41
std dev	0.33	std dev	0.53
Iso 24 hrs	2.89	Iso/E 24hrs	2.72
Iso 24 hrs	2.63	Iso/E 24hrs	2.78
Iso 24hrs	2.48	Iso/E 24hrs	2.69
Iso 24hrs	3.40	Iso/E 24hrs	2.27
Iso 24hrs	3.10	Iso/E 24hrs	3.51
Iso 24hrs	2.78	Iso/E 24hrs	2.44
Iso 24hrs	2.85		
Mean	2.88	Mean	2.74
std dev	0.30	std dev	0.43
Iso 48hrs	2.09	Iso/E 48hrs	2.92
Iso 48hrs	2.17	Iso/E 48hrs	2.31
Iso 48hrs	2.40	Iso/E 48hrs	3.09
Mean	2.22	Mean	2.77
std dev	0.16	std dev	0.41
Iso 72hrs	2.55	Iso/E 72hrs	2.66
Iso 72hrs	3.45	Iso/E 72hrs	2.57
Iso 72hrs	1.88	Iso/E 72hrs	2.46
Iso 72hrs	2.57	Iso/E 72hrs	2.57
Iso 72hrs	2.62	Iso/E 72hrs	3.35
Iso 72hrs	2.02		
Mean	2.51	Mean	2.72
std dev	0.55	std dev	0.36
Swim	2.05	E64c/Swim	3.57
Swim	3.18	E64c/Swim	2.90
Swim	3.63	E64c/Swim	2.57
Swim	4.45	E64c/Swim	2.66
Swim	3.04	E64c/Swim	2.59
Mean	3.27	Mean	2.86
std dev	0.88	std dev	0.42

Table 33. Left ventricular ATP/ADP ratios from swim and isoproterenol time course groups.

Condition	CPK U/L	Condition	CPK U/L	Condition	CPK U/L	Condition	CPK U/L
Control	500.20	6hr Iso	787.20	6hr Ec Iso	1205.40	6hr E64c	1004.50
Control		6hr Iso		6hr Ec Iso	1357.10	6hr E64c	389.50
Control	549.40	6hr Iso	545.30	6hr E Iso	832.30	6hr E64c	352.60
Control		6hr Iso		6hr E Iso	967.60	6hr E64c	328.00
Control	282.90	6hr Iso		6hr E Iso	1127.50	6hr E64c	635.50
Control	364.90	6hr Iso	1004.50	6hr E Iso		6hr E64c	
Mean	424.40	Mean	779.00	Mean	1097.98	Mean	426.40
Std Dev	122.40	Std Dev	229.70	Std Dev	204.40	Std Dev	141.70
12hr Control	184.50	12hr Iso	861.00	12hr E Iso	910.80		
12hr Control		12hr Iso	745.40	12hr E Iso	344.40		
12hr Control	389.50	12hr Iso	455.10	12hr E Iso	397.70		
12hr Control	492.00	12hr Iso	742.10	12hr E Iso	615.00		
12hr Control		12hr Iso	959.40	12hr E Iso	840.50		
Mean	355.30	Mean	752.60	Mean	621.70		
Std Dev	156.60	Std Dev	189.20	Std Dev	254.30		
		24hr Iso	643.70	24hr E Iso	434.60	24hr E64c	619.10
		24hr Iso	541.20	24hr E Iso	524.80	24hr E64c	377.20
		24hr Iso	430.50	24hr E Iso	807.70	24hr E64c	
		24hr Iso		24hr E Iso	672.40	24hr E64c	
		24hr Iso	487.90	24hr E Iso	906.10	24hr E64c	254.20
		24hr Iso	336.20	24hr E Iso	828.10	24hr E64c	315.70
		Mean	487.90	Mean	695.60	Mean	391.60
		Std Dev	115.60	Std Dev	185.60	Std Dev	159.80
48hr Control	516.60						
48hr Control	692.00						
48hr Control	660.00			48hr E Iso	959.40		
48hr Control	459.20	48hr Iso	528.90	48hr E Iso	660.10		
48hr Control	446.90	48hr Iso	766.70	48hr E Iso	684.70		
48hr Control	738.00	48hr Iso	770.80	48hr E Iso	754.40		
Mean	585.50	Mean	688.80	Mean	676.50		
Std Dev	126.50	Std Dev	138.50	Std Dev	229.50		
72hr Control	299.30	72hr Iso	217.30	72hr E Iso	426.40	72hr E64c	233.70
72hr Control	676.50	72hr Iso	754.40	72hr E Iso	196.80	72hr E64c	574.00
72hr Control	401.80	72hr Iso	373.10	72hr E Iso	155.80	72hr E64c	180.40
72hr Control	762.60	72hr Iso	537.10	72hr E Iso	393.60	72hr E64c	233.70
72hr Control	492.00	72hr Iso	143.50	72hr E Iso	323.90	72hr E64c	582.20
Mean	526.40	Mean	405.10	Mean	299.30	Mean	360.80
Std Dev	191.40	Std Dev	247.10	Std Dev	119.10	Std Dev	199.60

Table 34. Plasma CPK activity from isoproterenol time course study.

	Control		24hr Iso		24hr E Iso		72hr Iso		72hr E Iso	
Time	Mean	Std D	Mean	Std D	Mean	Std D	Mean	Std D	Mean	Std D
0(s)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	8.96	2.19	9.19	2.78	26.53	8.13	21.99	3.61	31.53	13.38
60	17.49	3.11	21.66	5.74	48.39	17.99	33.54	23.32	48.64	12.95
300	43.70	24.07	95.55	21.58	209.01	92.15	131.90	100.40	208.25	94.81
600	63.21	35.35	132.53	28.83	292.63	142.37	185.25	125.22	282.02	132.84
1800	75.99	34.51	180.31	31.34	383.27	188.33	248.51	148.36	357.04	137.58
	Control 1uM ryn		24hr Iso 1uM ryn		24hr E Iso 1uM ryn		72hr Iso 1uM ryn		72hr E Iso 1uM ryn	
Time	Mean	Std D	Mean	Std D	Mean	Std D	Mean	Std D	Mean	Std D
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	13.64	2.37	20.42	4.80	32.62	5.55	26.43	13.84	33.66	15.39
60	28.55	4.93	28.50	4.25	63.47	20.60	113.78	102.31	76.35	31.79
300	46.18	21.48	91.97	11.46	204.02	44.77	143.63	97.84	188.03	74.94
600	62.30	22.11	115.81	21.73	236.00	94.90	206.42	190.23	250.17	90.22
1800	74.81	23.83	155.60	26.68	294.44	149.94	227.92	153.51	315.28	102.64

Table 35. Ca^{2+} uptake of SR from isoproterenol study.

Alpha-actinin Time (hours)	Means Homogenate Control	Iso	E/Iso	E64c	Alpha-actinin Time (hours)	Std Dev Homogenate Control	Iso	E/Iso	E64c
6.00	0.00	-11.11	-8.24	-8.89	6.00	0.00	25.00	23.74	14.63
12.00	0.00	-13.64	-6.82		12.00	11.36	26.32	39.02	
24.00	0.00	-17.32	-10.61	-6.15	24.00	20.11	21.62	15.00	19.05
48.00	0.00	-17.39	-8.70		48.00	19.57	21.05	21.43	
72.00	0.00	-18.18	-6.82	-6.82	72.00	29.55	27.78	19.51	24.39

Alpha-actinin Time (hours)	Means Cytosolic Control	Iso	E/Iso	E64c	Alpha-actinin Time (hours)	Std Dev Cytosolic Control	Iso	E/Iso	E64c
6.00	0.00	10.87	-11.30	6.52	6.00	21.74	11.76	15.19	18.37
12.00	0.00	25.00	-6.25		12.00	12.50	13.33	6.67	
24.00	0.00	1.55	-12.95	-4.66	24.00	21.24	12.24	21.43	21.74
48.00	0.00	45.83	-8.33		48.00	20.83	22.86	13.64	
72.00	0.00	-7.84	-17.65	-19.61	72.00	29.41	19.15	19.05	29.27

Alpha-actinin Time (hours)	Means Fibril Control	Iso	E/Iso	E64c	Alpha-actinin Time (hours)	Std Dev Fibril Control	Iso	E/Iso	E64c
6.00	0.00	-25.27	0.98	-3.82	6.00	17.27	19.95	14.11	17.58
12.00	0.00	-22.73	-15.15		12.00	10.61	17.65	12.50	
24.00	0.00	-22.75	3.00	-7.30	24.00	16.53	20.00	20.00	18.52
48.00	0.00	-10.71	17.86		48.00	23.21	22.00	7.58	
72.00	0.00	-17.86	-1.79	10.71	72.00	16.07	23.91	16.36	19.35

Table 36. Alpha-actinin distribution within different fractions from isoproterenol time course study.

Desmin Time (hours)	Means Control	Iso	E/Iso	E64c	Desmin Time (hours)	Std Dev Control	Iso	E/Iso	E64c
6.00	0.00	-3.03	-0.84	-12.12	6.00	13.64	25.00	26.72	20.69
12.00	0.00	1.56	6.25		12.00	21.88	27.69	20.59	
24.00	0.00	5.60	7.20	-15.20	24.00	19.60	15.15	28.36	11.32
48.00	0.00	14.04	7.02		48.00	21.05	20.00	24.59	
72.00	0.00	-14.29	-23.81	-14.29	72.00	22.22	33.33	33.33	25.93

Desmin Time (hours)	Means Control	Iso	E/Iso	E64c	Desmin Time (hours)	Std Dev Control	Iso	E/Iso	E64c
		Cytosolic					Cytosolic		
6.00	0.00	7.89	4.55	0.00	6.00	11.84	10.98	15.39	9.21
12.00	0.00	8.11	-2.70		12.00	13.51	11.25	19.44	
24.00	0.00	27.52	14.09	-12.75	24.00	16.38	15.79	9.41	24.62
48.00	0.00	34.67	1.33		48.00	18.67	18.81	18.42	
72.00	0.00	5.48	5.48	1.37	72.00	21.92	18.18	14.29	21.62

Desmin Time (hours)	Means Control	Iso	E/Iso	E64c	Desmin Time (hours)	Std Dev Control	Iso	E/Iso	E64c
		Fibril					Fibril		
6.00	0.00	-29.74	-5.60	7.73	6.00	17.10	48.33	25.88	21.74
12.00	0.00	-19.51	-7.32		12.00	19.51	15.15	18.42	
24.00	0.00	-14.49	-4.72		24.00	18.52	28.57	25.64	
48.00	0.00	-9.52	-4.76		48.00	23.81	18.42	37.50	
72.00	0.00	-2.63	7.89	10.53	72.00	13.16	27.03	21.95	11.90

Table 37. Desmin distribution within different fractions from isoproterenol time course study.

Troponin-I Time (hours)	Means Homogenate Control	Iso	E/Iso	E64c
6.00	0.00	-6.17	-3.61	-7.41
12.00	0.00	-7.59	5.06	
24.00	0.00	-18.21	-4.15	-4.15
48.00	0.00	-24.32	-2.70	
72.00	0.00	-24.05	-12.66	-6.33

Troponin-I Time (hours)	Std Dev Homogenate Control	Iso	E/Iso	E64c
6.00	11.11	14.47	14.91	18.67
12.00	20.25	10.96	9.64	
24.00	14.06	18.75	16.00	8.00
48.00	13.51	19.64	18.06	
72.00	11.39	21.67	15.94	9.46

Troponin-I Time (hours)	Means Cytosolic Control	Iso	E/Iso	E64c
6.00	0.00	12.09	3.37	-2.20
12.00	0.00	28.24	11.76	
24.00	0.00	18.44	-0.56	-2.79
48.00	0.00	1.11	4.44	
72.00	0.00	0.00	-2.17	-1.09

Troponin-I Time (hours)	Std Dev Cytosolic Control	Iso	E/Iso	E64c
6.00	8.79	9.80	9.56	11.24
12.00	10.59	11.93	10.53	
24.00	9.50	11.32	11.24	12.64
48.00	7.78	13.19	4.26	
72.00	10.87	10.87	12.22	7.69

Troponin-I Time (hours)	Means Fibril Control	Iso	E/Iso	E64c
6.00	0.00	-12.32	-7.21	3.74
12.00	0.00	-34.38	-10.42	
24.00	0.00	-16.11	-9.65	-1.05
48.00	0.00	-18.95	-2.11	
72.00	0.00	-6.67	-6.67	0.00

Troponin-I Time (hours)	Std Dev Fibril Control	Iso	E/Iso	E64c
6.00	7.92	9.66	14.68	14.00
12.00	11.46	20.63	16.28	
24.00	11.08	12.82	15.48	14.13
48.00	14.74	7.79	15.05	
72.00	10.00	8.33	11.90	16.67

Table 38. Troponin-I distribution within different fractions from isoproterenol time course study.

APENDIX 3.

Supplementary graphs.
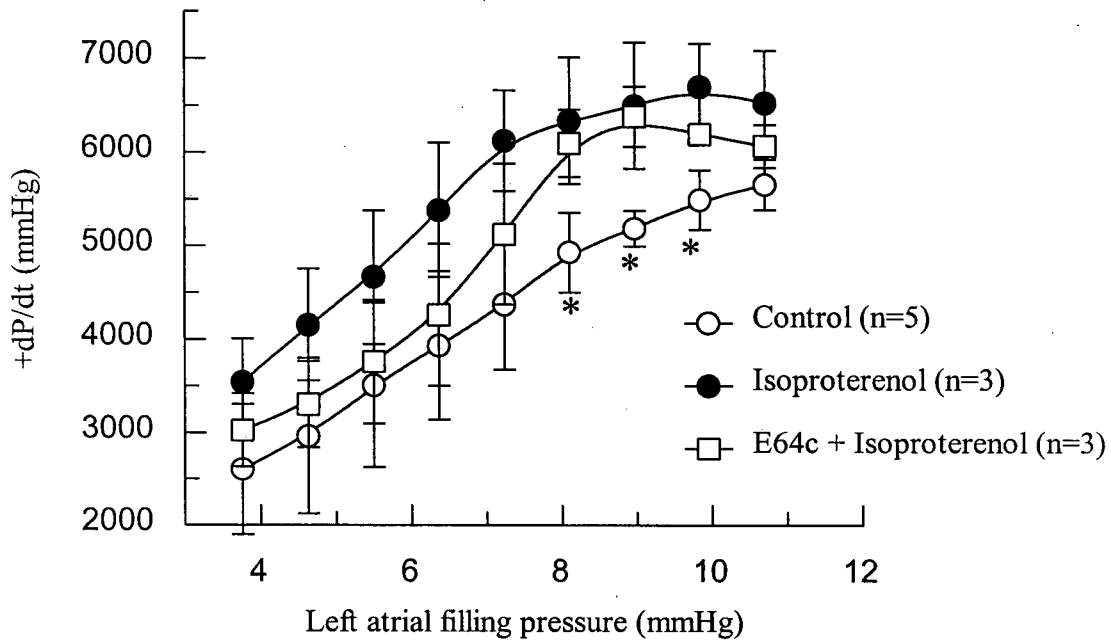
Figure A1. Effect of isoproterenol and E64c plus isoproterenol on the rate of rise of left ventricular pressure development (+dP/dt) over a range of left atrial filling pressures. Data was collected 24 hours after injections. Values shown are means $\pm$ standard deviation. * denotes significantly different from isoproterenol and E64c plus isoproterenol ($p<0.05$).
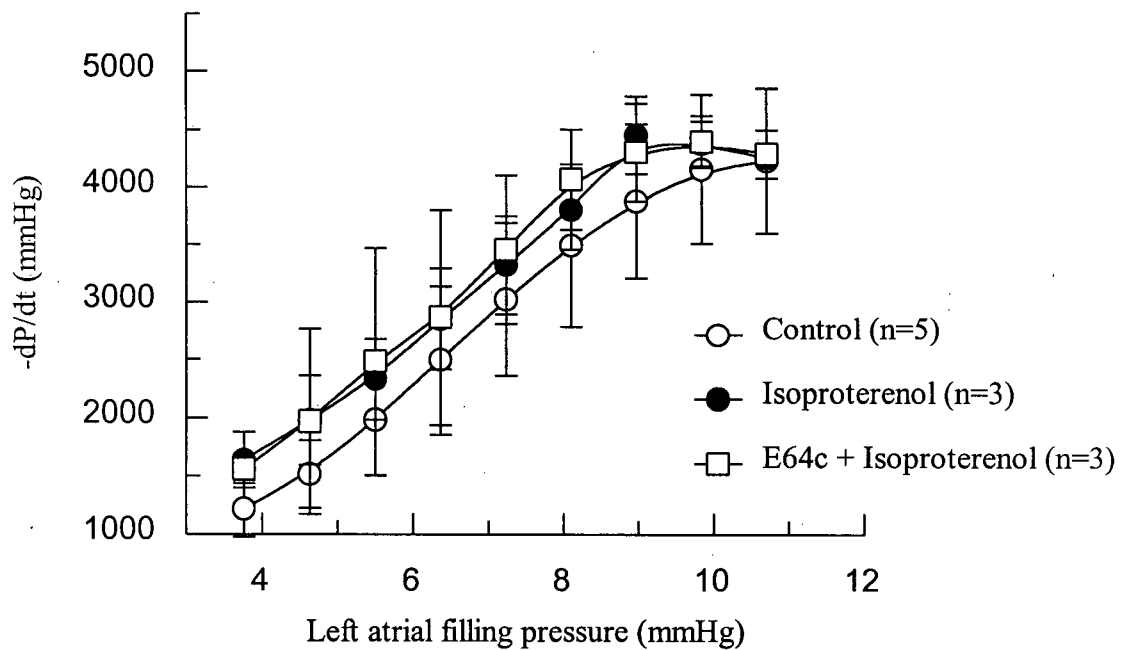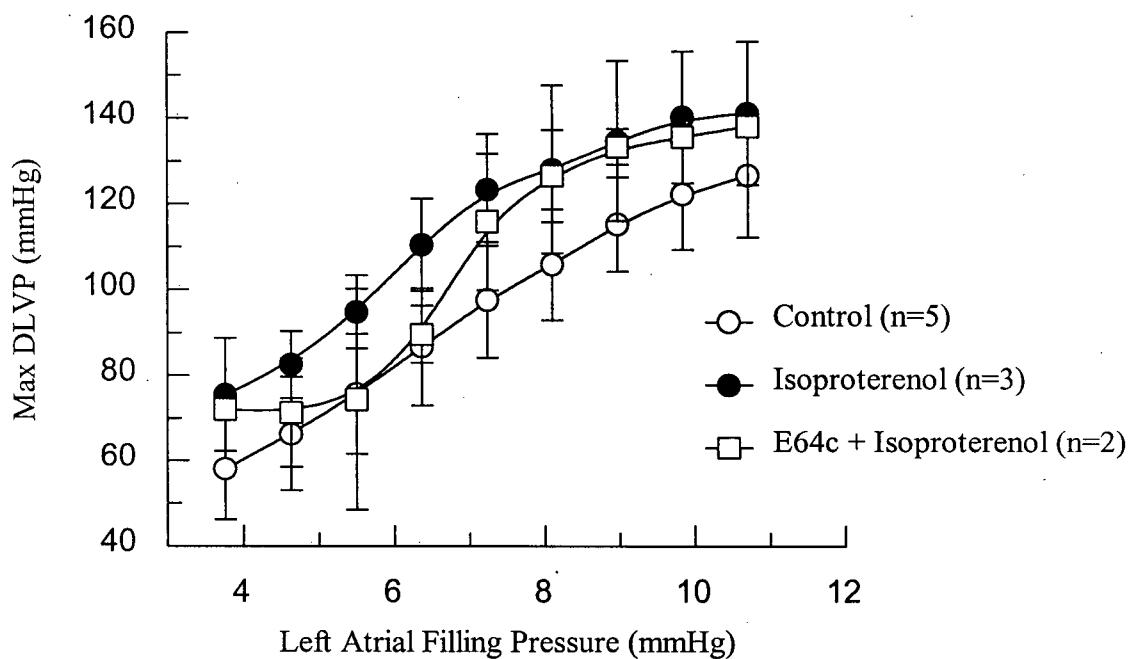
Figure A2. Effect of isoproterenol and E64c plus isoproterenol on the rate of decrease of left ventricular pressure (-dP/dt) over a range of left atrial filling pressures. Data was collected 24 hours after injections. Values shown are means ± standard deviation.

Figure A3. Effect of isoproterenol and E64c plus isoproterenol on the maximum developed left ventricular pressure (max DLVP) over a range of left atrial filling pressures. Data was collected 24 hours after injections. Values shown are means ± standard deviation.
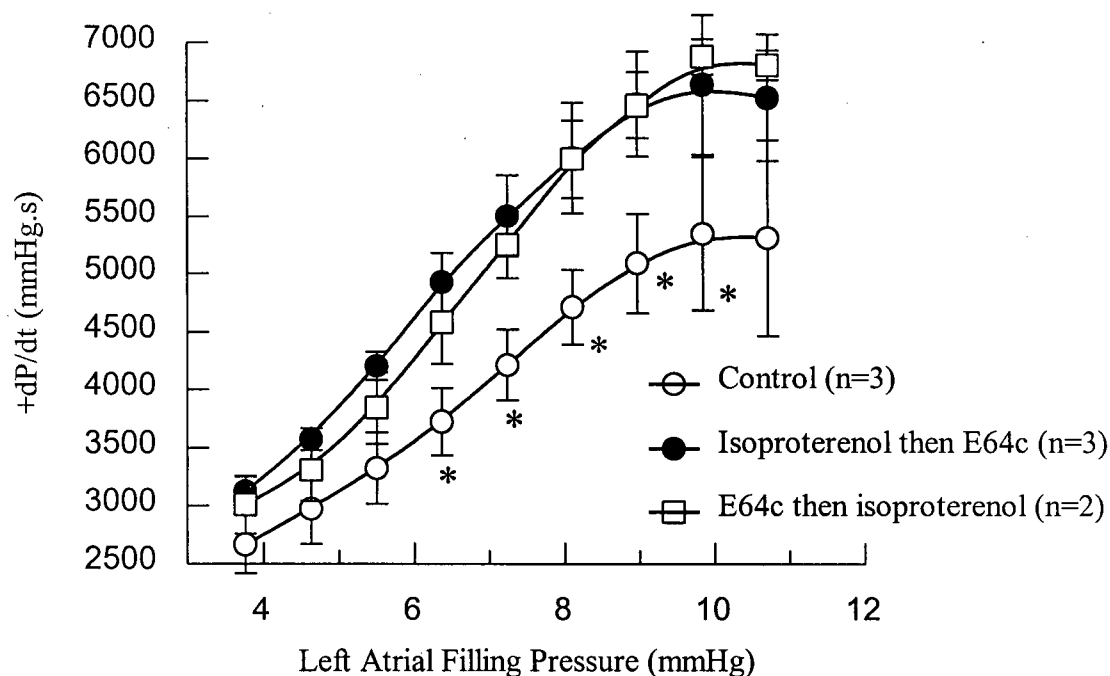
Figure A4. +dP/dt from E64c and isoproterenol order trials. Isoproterenol was given either 24 hours prior to E64c or 24 hours after E64c. Data was collected 72 hours after isoproterenol injection. * Denotes significantly different from both isoproterenol and isoproterenol plus E64c.
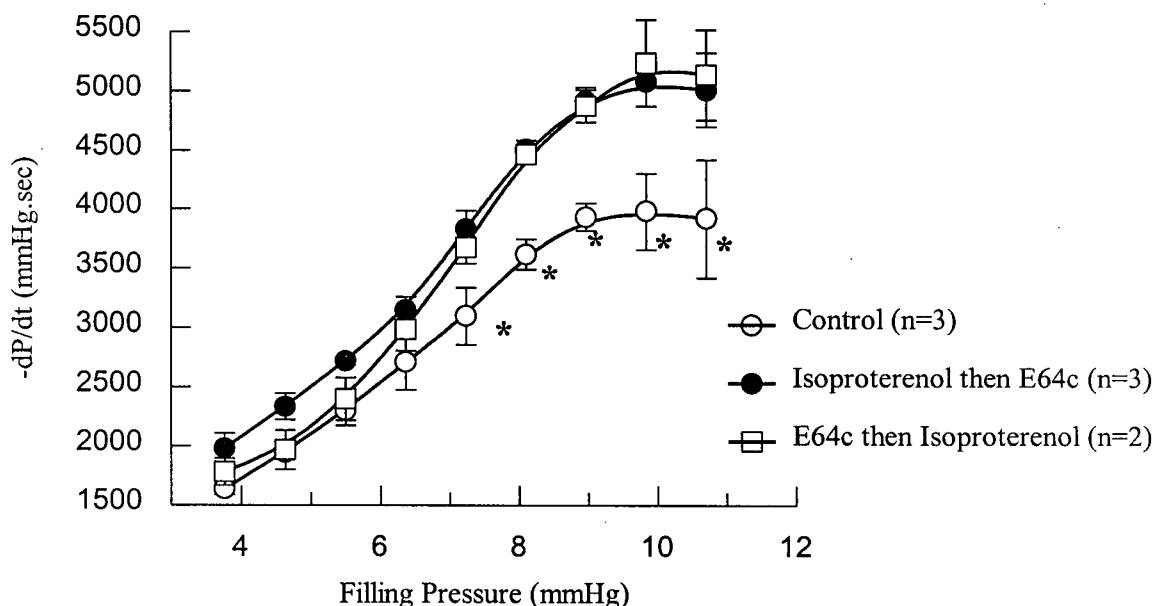

Figure A5. -dP/dt from E64c and isoproterenol order trials. Isoproterenol was given either 24 hours prior to E64c or 24 hours after E64c. Data was collected 72 hours after isoproterenol injection. * Denotes significantly different from both isoproterenol and isoproterenol plus E64c.
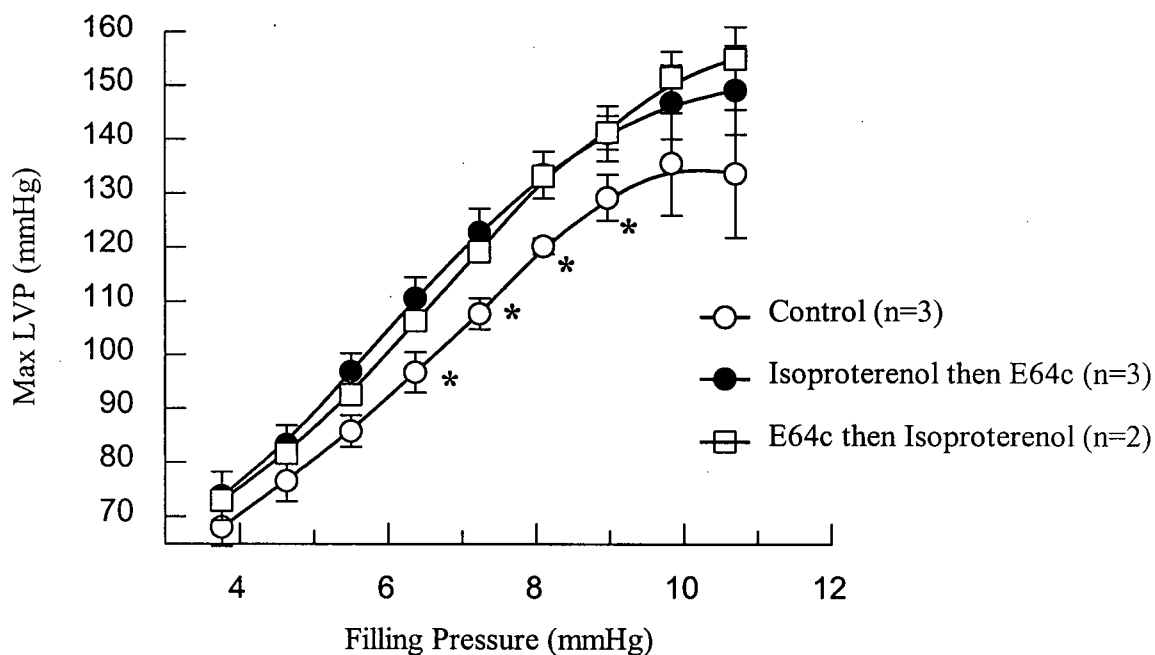
Figure A6. Max DLVP from E64c and isoproterenol order trials. Isoproterenol was given either 24 hours prior to E64c or 24 hours after E64c. Data was collected 72 hours after

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isoproterenol injection. * Denotes significantly different from both isoproterenol and isoproterenol plus E64c.
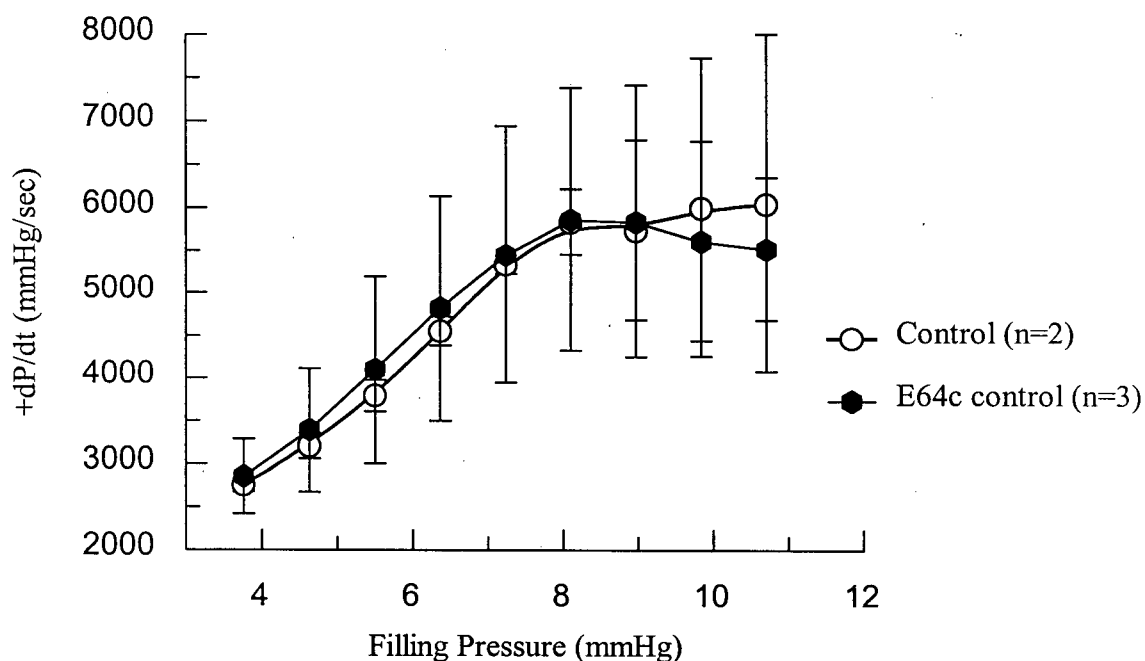
Figure A7. +dP/dt from control and E64c control animals. Data was collected 72 hours after isoproterenol injection.
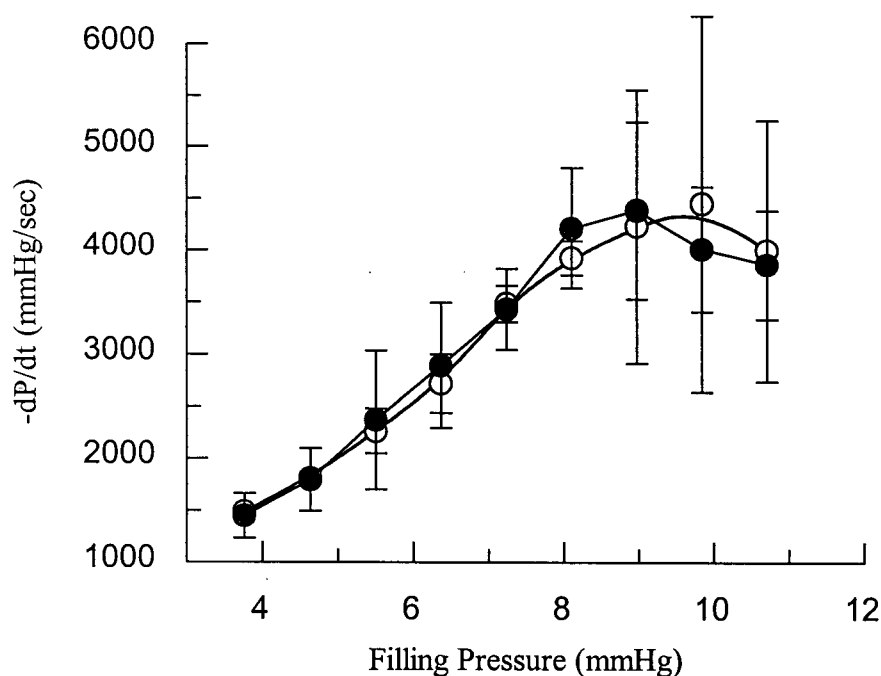
Figure A8. -dP/dt from control and E64c control animals. Data was collected 72 hours after isoproterenol injection.
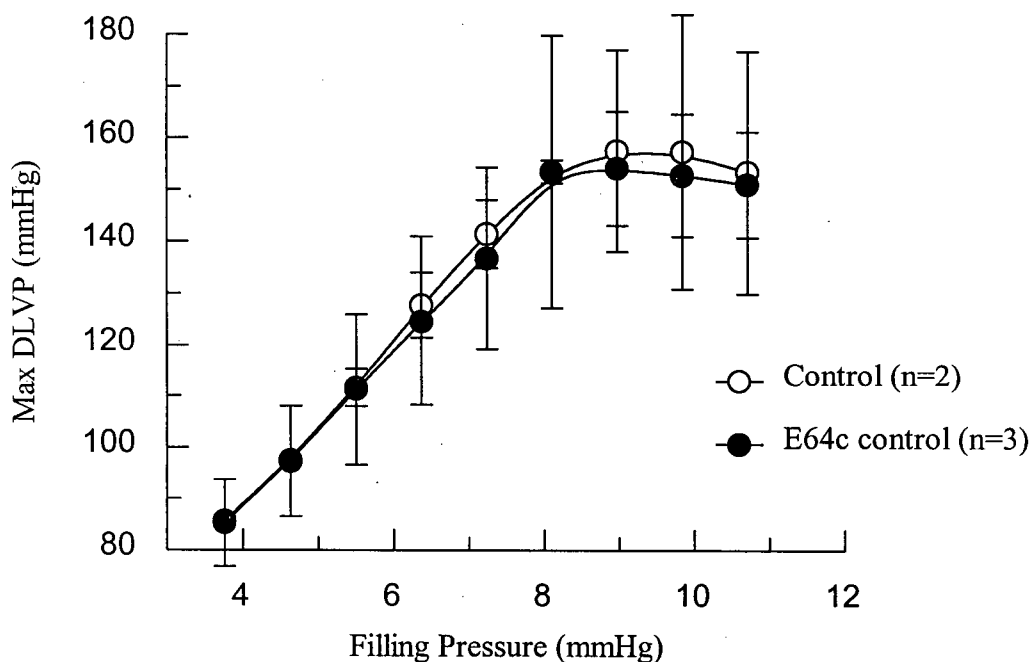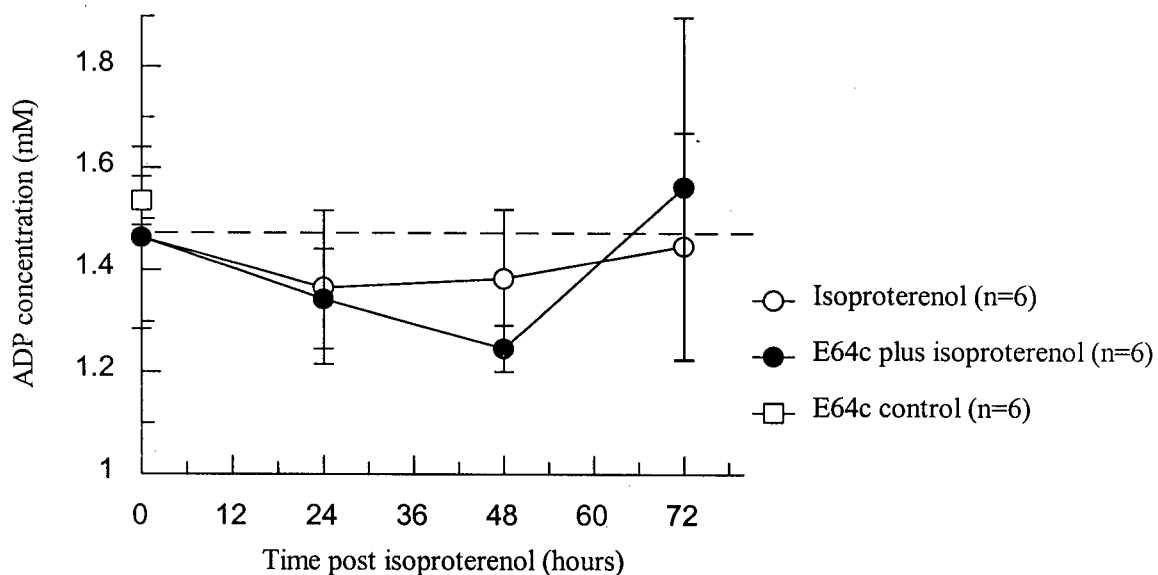
Figure A9. Max DLVP from control and E64c control animals. Data was collected 72 hours after isoproterenol injection.

Figure A10. Effect of isoproterenol and E64c plus isoproterenol on ADP concentration of the left ventricle over 72 hours following injections. Values shown are means $\pm$ standard deviation, control data only available at a zero time point and repreented by a dashed line.
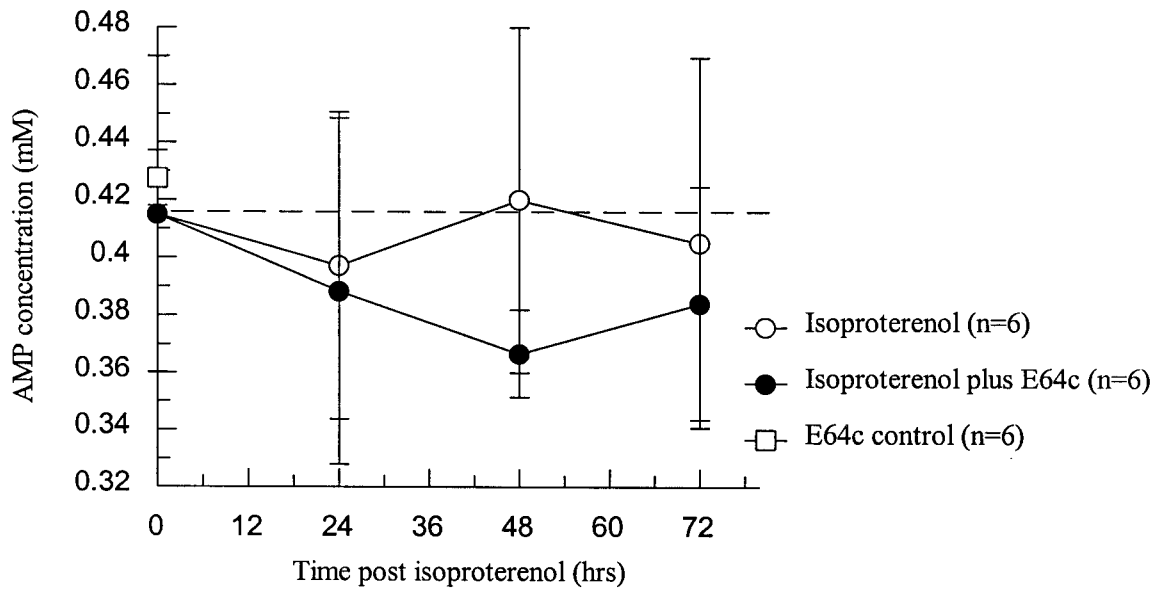
Figure A11. Effect of isoproterenol and E64c plus isoproterenol on AMP concentration of the left ventricle over 72 hours following injections. Values shown are means $\pm$ standard deviation, control data only available at a zero time point and represented by a dashed line.
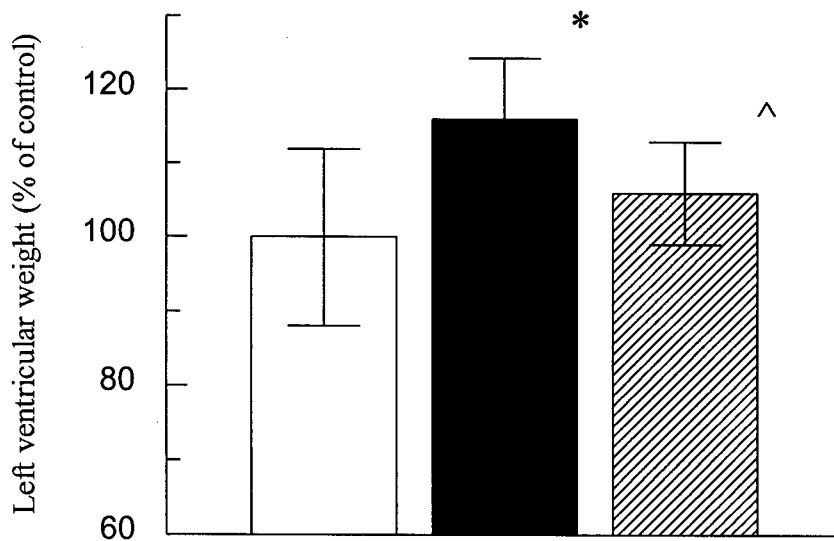

Figure A12. Left ventricular weight data from chapters 5&6 combined, comparing controls (open column, n=12), with 72 hours after isoproterenol (filled column, n= 15) and 72 hours after E64c and isoproterenol (hatched column, n=13). Data shown is mean percentage of control and standard deviation, * denotes significantly different from control (p<0.05), ^ denotes significantly different from isoproterenol (p<0.05).
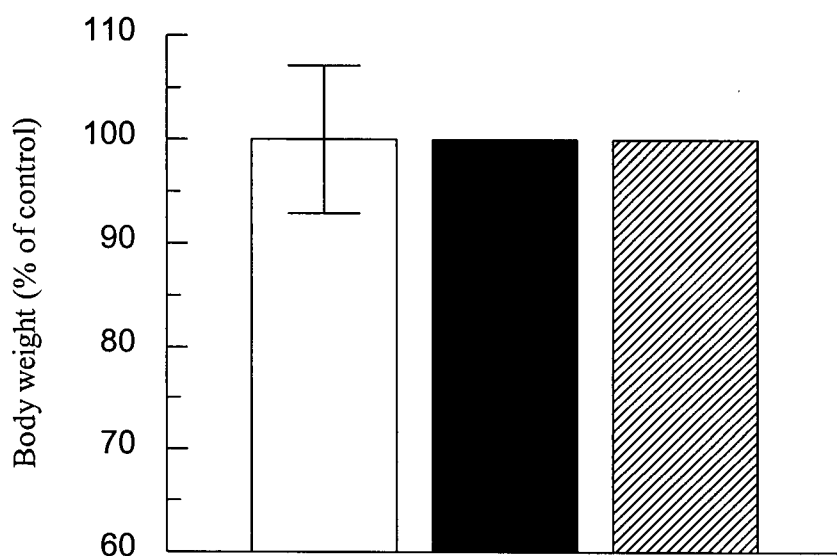
Figure A13. Body weight data from chapters 5&6 combined, comparing controls (open column, n=12), with 72 hours after isoproterenol (filled column, n= 15) and 72 hours after E64c and isoproterenol (hatched column, n=13). Data shown is mean percentage of control and standard deviation, no significant differences were found.
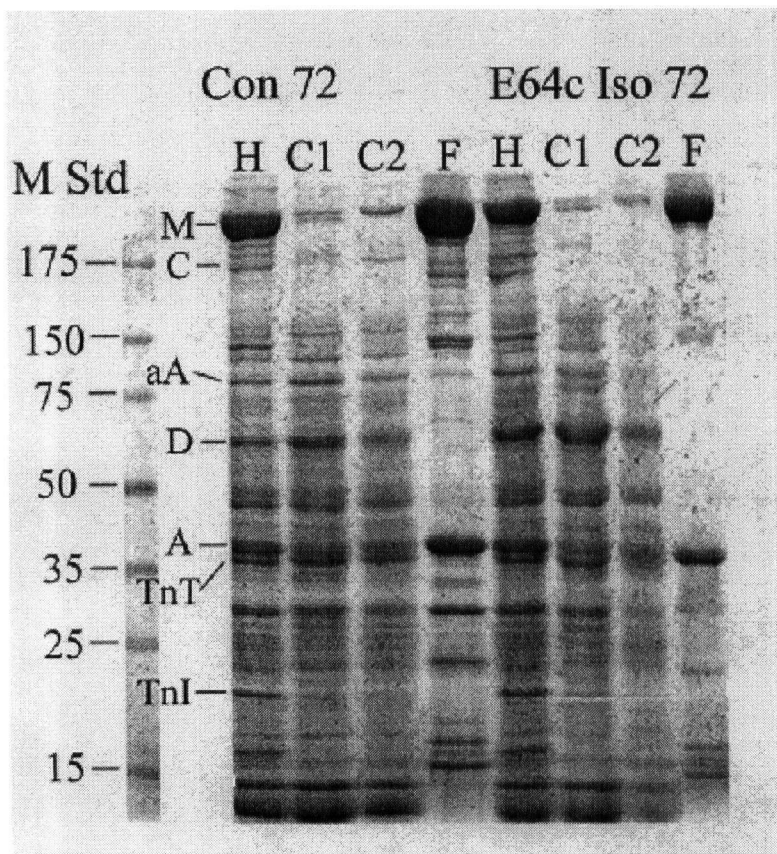
Figure A14. Representitve scan of a 5-15% gradient SDS-PAGE showing cardiac myofibrills. The left lane shows the molecular weight standards. Lanes are as follows H = homogenate fraction, C1 = 1st cytoplasmic fraction, C2 = 2nd cytoplasmic fraction, F = fibril fraction. Con 72 = fractions from control muscle at the 72 hour time point. E64c Iso 12 = fractions from E64c and isoproterenol treated muscle at the 12 hour time point. The bands of interest are labeled; M = myosin, A = actin, aA = alpha actinin, D = Desmin, TnI = troponin I.