UNIVERSITY OF CALGARY

# Conditions for Cardiac Muscle Myosin Regulatory Light Chain Phosphorylation

by

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# Abstract

To determine if phosphorylation of the myosin regulatory light chains (RLC) in intact cardiac preparations has a functional correlate, conditions must be established that allow predictable levels of myosin RLC phosphorylation. Therefore the objective of this study was to establish conditions that led to different levels of myosin RLC phosphorylation. The major findings were that stimulation between 1.0 and 4.0Hz produced moderate levels of myosin RLC phosphorylation (1.0Hz: 29.4% ± 3.0%; 2.0Hz: 27.1% ± 1.5%; 4.0Hz: 32.3% ± 4.0%) whereas 30 minutes of no stimulation produced low levels of myosin RLC phosphorylation (13.0% ± 0.9%). The addition of Okadaic acid (OA), which inhibits the myosin light chain phosphatase, resulted in high levels of myosin RLC phosphorylation (44.6% ± 2.0%). Now that predictable levels of myosin RLC phosphorylation have been obtained it is possible to look at functional correlates such as force, work and power.

# Preface

Chapter 3 of this thesis is based on the following manuscript:

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My family, who are all so important to me and without whom I would not have had the courage to do this.

Aron, my husband and best friend who has been my biggest supporter and encouraged me to be best that I can be.

# Dedication

**This thesis is dedicated to:**

***Aron, who means the world to me and who is my best friend. Thank-you for all the support, love and happiness you have given me.***

***My mom and dad, who taught me that I can do anything I set my mind on doing, and that hard work is not something to be afraid of.***

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# List of Abbreviations

| | |
|---|---|
| $\frac{1}{2}R_t$ | half relaxation time |
| **ANOVA** | analysis of variance |
| **CaM** | calmodulin |
| $C_t$ | contraction time |
| **CICR** | calcium induced calcium release |
| **DMSO** | dimethylsulfoxide |
| $dP \cdot dt^{-1}_{min}$ | peak rate of relaxation |
| **DT** | developed tension |
| $DT \cdot Ct^{-1}$ | average rate of tension development |
| $I_k$ | potassium delayed rectifier current |
| $I_{k1}$ | potassium inward rectifier current |
| $I_{to}$ | potassium transient outward current |
| **IEF** | isoelectric focussing |
| **LC** | light chains of myosin (essential and regulatory) |
| **MLCK** | myosin light chain kinase |

| | |
|---|---|
| **MLCP** | myosin light chain phosphatase |
| **OA** | okadaic acid |
| **PAGE** | polyacrylamide gel electrophoresis |
| **RLC** | regulatory light chains of myosin or light chain 2 |
| **RyR** | ryanodine receptor |
| **SEM** | standard error of the mean |
| **SR** | sarcoplasmic reticulum |
| **TEMED** | tetramethylethylenediamine |
| $\mathbf{V_{max}}$ | maximal velocity |

# Chapter 1

## Introduction

The Frank-Starling law of the heart tells us that stroke volume will increase with increased filling of the heart ventricles. The atria may play a role in increasing stroke volume if the amount of blood that passes through them into the ventricles is increased. Therefore stroke volume may be enhanced by a stronger contraction of the atrium and it has been reported that the importance of atrial contraction is greater in old age (Bryg et al. 1987). A stronger contraction of the atrium could be obtained by either enhanced calcium transients, or by increased sensitivity to calcium.

A potential mechanism by which increased sensitivity of the myofilaments to calcium could be obtained is phosphorylation of the cardiac myosin regulatory light chains (RLC). Two enzymes modulate phosphorylation of the myosin RLC: myosin light chain kinase (MLCK) and, myosin light chain phosphatase (MLCP). If cardiac muscle can regulate the level of myosin RLC phosphorylation then it seems very likely that this regulation would play a role in modulation of cardiac contractility.

In cardiac muscle, the functional significance of myosin RLC phosphorylation is unclear but it has been shown that RLC phosphorylation does alter calcium sensitivity in skinned ventricular muscle preparations (Morano et al 1985, Sweeney & Stull, 1986, Moore et al. 1991). However a functional correlate of this observation has not been confirmed in an intact preparation.

In comparison to cardiac muscle, the functional significance of skeletal muscle myosin RLC phosphorylation is becoming increasingly clear. Myosin RLC phosphorylation in skeletal muscle is thought to produce one major change: potentiation of contractile force. Myosin RLC phosphorylation causes an enhancement of calcium sensitivity which then augments isometric force development during submaximal activation. It is now well known that, in skeletal muscle, myosin RLC phosphorylation is correlated to the degree of activity-dependent potentiation of isometric twitch tension (Manning & Stull, 1979, Stull et al. 1985, Grange et al. 1995). It seems logical then to expect that RLC phosphorylation in cardiac muscle would also have a positive inotropic effect.

In summary, we know that myosin RLC phosphorylation enhances force production in skinned cardiac fibres (Sweeney & Stull, 1986, Moore et al. 1991) and, therefore, by association it is believed that the same must happen in intact preparations. The observed changes and functional consequences for ventricular muscle (skinned cardiac fibres) would logically be thought to occur in atria as well. To facilitate the systematic study of the functional consequences of phosphorylation in intact atria, it is therefore important to establish conditions for obtaining different levels of myosin RLC phosphorylation.

The next section is a review of literature that provides the background information required for understanding the rationale of the experiments conducted. In the review of literature, many fundamental concepts pertaining to both cardiac and skeletal muscle are examined.

It should be pointed out that there are several reasons for selecting the rat atrium for study. Many of these will be evident in the background material to follow. However, it should be kept in mind that the primary consideration is that this is simply a model of excitation-contraction coupling and contractile response in cardiac muscle. For this reason, there is no attempt to emulate in vivo conditions in this preparation.

# Chapter 2

## Review of Literature

The purpose of the proposed research is to establish conditions that will produce different levels of myosin RLC phosphorylation. This will facilitate subsequent research on the functional correlates (force, work, and power) of RLC phosphorylation in atria. To better understand how myosin RLC phosphorylation can be altered and how it can have a functional consequence it is necessary to examine results that have been obtained concerning the regulation of myosin RLC phosphorylation, contractile force development, and the factors that modify force development. In order to do this, prior research on skinned cardiac fibres must be compared with what is known about skinned skeletal muscle fibres and the mechanisms that regulate force production.

## Contractile Force Development

### *Cardiac Muscle versus Skeletal Muscle*

Since much more is known about the functional consequences of myosin RLC phosphorylation in skeletal muscle compared to cardiac muscle it is important to consider their similarities and differences.

In cardiac muscle, activation begins with the generation of an action potential at the sinoatrial node located in the right atria. The action potential then makes its way across the atria to the atrioventricular node before coursing down the ventricular septum through the bundle branches and Purkinje system to individual cardiac cells. Individual cardiac cells (atrial or ventricular) are activated by a sequence of events referred to as excitation-contraction coupling. As the action potential is propagated along the sarcolemma to the t-tubules, the voltage dependent calcium channels (Dihydropyridine receptor or L-type calcium channel) open and allow calcium to enter the cell. This calcium then causes the opening of the ryanodine receptor (RyR) calcium channels in the sarcoplasmic reticulum (SR) by a process that is known as calcium induced calcium release (CICR). The opening of the RyR channels allows a rapid influx of calcium to the cytosol. The calcium released from the SR then binds to troponin C located on the thin filament. This in turn causes a conformational change in the thin filament allowing for the interaction of actin and myosin. With the thin filament conformational change, that results from calcium binding to troponin C the myosin head can attach to actin and begin the process of muscle contraction. For

relaxation to occur, calcium must be pumped back out of the cytosol into both the SR via the $Ca^{2+}$ATPase and into the extracellular space via the $Na^{+}/Ca^{2+}$ exchanger or the plasma membrane $Ca^{2+}$ pump (Ebashi, 1991).

In contrast to cardiac muscle, calcium from the extracellular space is not required for contraction in skeletal muscle. In skeletal muscle, release of acetylcholine from the nerve ending results in an increased permeability of the muscle plasmalemma to $Na^{+}$ and $K^{+}$, this results in an end plate potential that subsequently gives rise to an action potential. The action potential then propagates in both directions towards the ends of the muscle fibre causing depolarization. This depolarization of the cell membrane then results in activation of the voltage dependent calcium channels in the transverse tubules that in turn activate the ryanodine receptors located on the SR that release calcium into the cytosol. The calcium then diffuses through the intracellular space to bind to troponin C in the same fashion as it does in cardiac muscle. The calcium is subsequently sequestered into the SR as it is in cardiac muscle (Ebashi, 1991).

*Primary Mechanisms Underlying Force Development*

Calcium is essential for contractile signaling in both skeletal and cardiac muscle. The primary determinants of force development include calcium concentration and the calcium sensitivity of the filaments.

Increased force production can occur as a result of higher calcium concentrations and/or greater calcium sensitivity. In cardiac muscle at 30°C, the

cytosolic calcium concentration during systole is only 1μM (Allen & Kurihara, 1980). This calcium concentration produces less than 50% of maximal force, showing that cardiac muscle typically contracts at a submaximal level. An increase in the contractile response can occur as the result of changing either the calcium transients or the contractile protein sensitivity (Morano & Ruegg, 1986).

A leftward shift in the force-calcium relationship indicates increased sensitivity to calcium, and a rightward shift in the force-calcium relationship indicates a decrease in sensitivity to calcium. Shifting the force-calcium curve to the left means that, at any given submaximal calcium concentration, more force will be produced as compared to the original force-calcium curve (Figure 1). Two mechanisms for increased calcium sensitivity have been proposed. Increased calcium sensitivity can result from increased calcium binding with troponin C or an alteration in cross-bridge kinetics. Cardiac skinned fibre experiments have shown that enhanced phosphorylation of the RLC significantly increases the calcium sensitivity (Morano et al. 1985).

As discussed below, skeletal muscle experiments have also shown that myosin RLC phosphorylation increases calcium sensitivity. As more research has been conducted on the role of myosin RLC phosphorylation in skeletal muscle then in cardiac muscle, it is important to review that literature here.

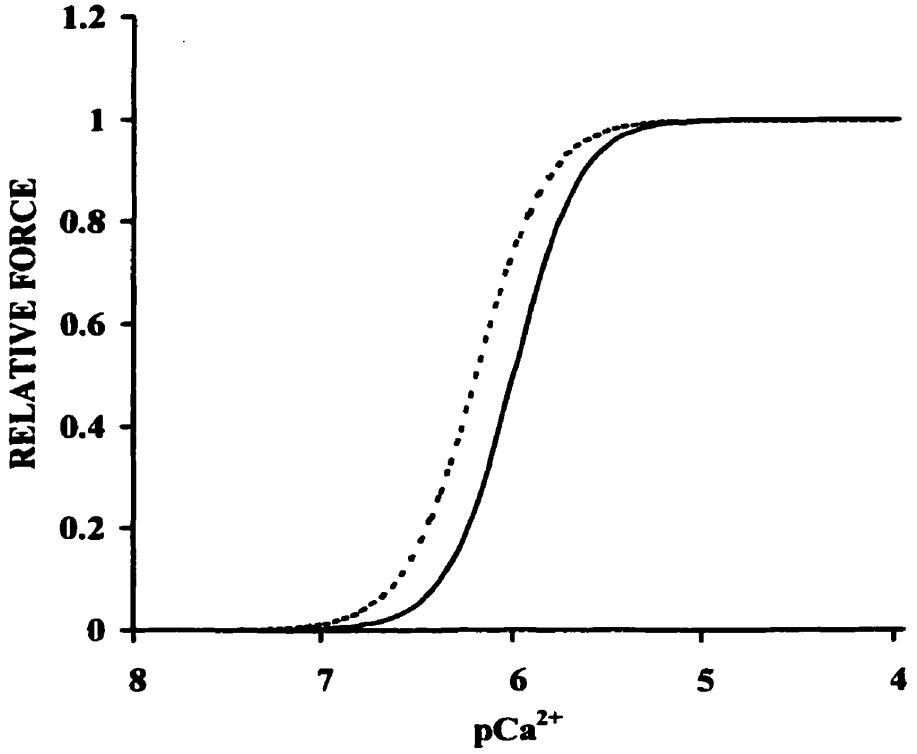


**Figure 1.** Force-calcium curve shown as the relative force versus $pCa^{2+}$ where the $pCa^{2+}$ is the –log of the calcium concentration. The curve on the right indicates the force response of the muscle under normal conditions. The curve to the left is the force response with an increase in calcium sensitivity. Sensitivity can be quantified by the half-maximal calcium concentration on the force-calcium curve.

## Skeletal Muscle Comparison

### *Staircase and Posttetanic Potentiation in Skeletal Muscle*

In skeletal muscle, a progressive enhancement of twitch force can be observed during the initial phase of repetitive low frequency stimulation. This is called staircase potentiation. Twitch force enhancement can also be seen following a tetanic stimulation and is referred to as posttetanic potentiation (Rankin et al. 1988). A positive correlation between the amount of force potentiation in fast-twitch skinned skeletal muscle preparations and the level of myosin RLC phosphorylation has been observed (Metzger et al. 1989, Persechini et al. 1985). This relationship is not as well defined in slow-twitch muscle, perhaps due to slower rates of change in myosin RLC phosphorylation (Moore & Stull, 1984).

### *Whole and Skinned Skeletal Muscle Experiments*

In whole skeletal muscle preparations Palmer & Moore (1989) found that augmentation of isometric tension was correlated with myosin RLC phosphorylation and concluded that this occurred because the contractile element became more sensitive to calcium. In skinned skeletal muscle preparations, phosphorylation of the myosin RLC causes an increase in myofilament calcium sensitivity (Sweeney & Stull 1986, Sweeney & Stull 1990, Metzger et al. 1989) which can be seen as a leftward shift in the force-calcium relationship (Figure 1). These findings then suggest that

RLC phosphorylation affects contractility by increasing the calcium sensitivity and explains the correlation between RLC phosphorylation and the level of isometric twitch potentiation (Manning & Stull 1982, Moore & Stull 1984).

*Proposed Mechanism of Enhanced Tension Development*

Enhancement of tension development as a result of cardiac myosin RLC phosphorylation and subsequent changes to calcium sensitivity could be the result of two mechanisms. First the enhanced tension could be the result of an increased number of active actin-myosin cross-bridges and second, more tension could be produced by each attached cross-bridge. If this second mechanism were true it would be expected that a proportional increase in tension would occur at all calcium concentrations. This is not the case because maximum force is not altered by myosin RLC phosphorylation. Therefore it is more likely that the mechanism of force enhancement is the result of the attachment of more cross-bridges (Sweeney & Stull 1990).

*Two-State Cross-Bridge Model*

Sweeney & Stull (1990) utilized a two-state cross-bridge model to explain the possible mechanism by which RLC phosphorylation may enhance isometric force development. This two-state model considers the rate at which the cross-bridges cycle (cross-bridge kinetics) and is a function of $f$ (rate of cross-bridge attachment)

and $g$ (rate of cross-bridge detachment). Cross-bridge kinetics ($f$ and $g$) will determine the proportion of active cross-bridges in a strongly bound state during steady-state conditions according to the following equation (1).

$$\text{Force} = f / (f + g) \qquad (1)$$

Increases in force associated with increased myosin RLC phosphorylation were determined to be proportional to increased fibre stiffness confirming that more cross-bridges were attached. The attachment of more cross-bridges would require either an increase in $f$ or a decrease in $g$. If $g$ decreased, the energy cost to maintain force (ATPase activity) would then not increase in proportion to force. Sweeney & Stull's (1990) observations that there was no change in the relationship between ATPase activity and force development with myosin RLC phosphorylation suggests that the reverse rate constant ($g$) is unchanged with RLC phosphorylation.

Using submaximal calcium concentrations Persechini et al. (1985) found that myosin RLC phosphorylation elevated isometric tension but that phosphorylation did not affect maximal shortening velocity. This is consistent with Sweeney & Kushmerick (1985) who used a skinned rabbit psoas muscle fibre to show that phosphorylation of the myosin RLC did not affect shortening velocity at maximal calcium concentrations. Therefore the time during which the actin-myosin cross-bridge is attached probably does not change with phosphorylation of the myosin RLC (Sweeney & Stull 1986). Slowing the detachment (increasing g) would decrease

maximal velocity ($V_{max}$) because $V_{max}$ is limited by the rate of detachment of the cross-bridges.

If the reverse rate constant (*g*) does not change with variation in calcium levels or phosphorylation of the myosin RLC then any changes in force development must be due to the forward rate constant (*f*). Therefore by increasing *f* more of the cross-bridges would be producing force at steady state (Sweeney & Stull 1990) and phosphorylation of the myosin RLC must increase the probability of cross-bridges entering the force generating state.

Grange et al. (1995) showed that, in a whole skeletal muscle preparation, increased myosin RLC phosphorylation occurred along with augmentation of force (potentiation) and increases in both the submaximal velocity of shortening and the displacement of twitch isotonic contractions. The power output and amount of work produced were also augmented with the rise in myosin RLC phosphorylation. If cardiac muscle displays the same characteristics as skeletal muscle then it would be expected that the rate of force development, work and power output would also be significantly increased with augmentation of myosin RLC phosphorylation. These changes to the power output and work per beat in cardiac muscle as the result of myosin RLC phosphorylation would be representative of modulation of muscle function.

**Regulation of Myosin RLC Phosphorylation**

The myosin RLC in striated muscle is a 20,000-dalton protein that can be phosphorylated or dephosphorylated. Myosin RLC is phosphorylated by the enzyme MLCK which, is regulated by calcium binding to calmodulin and the binding of the calcium-calmodulin complex to MLCK (Kennelly et al. 1989). Therefore any increase in cytosolic calcium concentration (muscle activation) would be expected to increase the formation of the calcium-calmodulin complex and thus augment MLCK activity. Activation of the MLCK causes the breakdown of adenosine triphosphate (ATP) and allows the terminal phosphoryl group of the ATP to bind with the myosin RLC thus phosphorylating it. Subsequent decreases in calcium concentration (muscle relaxation) cause the calcium-calmodulin complex to dissociate and MLCK becomes inactive. The enzyme MLCP is responsible for cleaving off the phosphoryl group from the myosin RLC (dephosphorylation). How or if this enzyme is regulated in striated muscle is unknown (Morgan et al. 1976). Figure 2 summarizes the mechanism by which myosin RLC phosphorylation and dephosphorylation is mediated by the enzymes MLCK and MLCP.

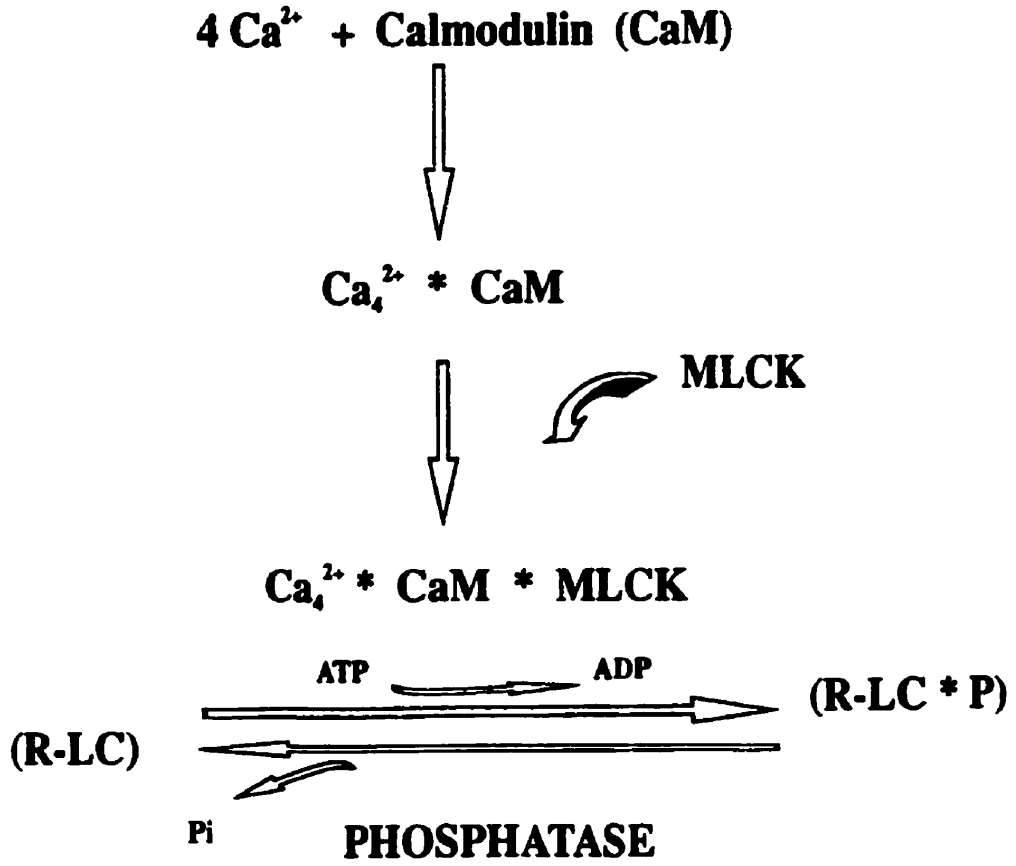


**Figure 2.** Phosphorylation of the myosin RLC by calcium activated MLCK followed by dephosphorylation of the myosin RLC by the enzyme MLCP (Tubman 1996, as modified from Stull et al. 1990).

## Phosphorylation of Myosin RLC in Cardiac Muscle

### *Cardiac Skinned Fibre Experiments*

Morano et al. (1985, 1986) have shown that addition of myosin light chain kinase (MLCK) to a chemically skinned pig cardiac fibre increases the level of myosin RLC phosphorylation and calcium sensitivity of contractions; where calcium sensitivity is the half maximal calcium concentration on the force-calcium curve (Figure 1). Increases in calcium sensitivity means that at any given submaximal calcium concentration greater force can be produced. In experiments performed by Sweeney & Stull (1986) it was shown that in cardiac muscle maximal force production does not increase with augmented myosin RLC phosphorylation, therefore, myosin RLC phosphorylation must not play a role at maximal calcium concentrations.

Morano et al. (1985, 1986) suggest that changes to calcium sensitivity as a result of myosin RLC phosphorylation may play a key role in modulation of cardiac contractility; however, the functional consequence of myosin RLC phosphorylation in intact cardiac muscle is unknown. To ascertain whether any functional consequence exists, circumstances must be found that produce predictable levels of myosin RLC phosphorylation in intact cardiac muscle preparations. To achieve this goal it is first necessary to understand what factors modulate myosin RLC phosphorylation.

*Importance of Calcium Transients in Activating MLCK Activity*

One factor that may modulate myosin RLC phosphorylation is the calcium transient. Calcium transients can be altered with changes to stimulation frequency. Larger calcium transients would provide more calcium for binding with calmodulin and thus activating MLCK, whereas the opposite (smaller transients) should produce less MLCK activation.

*Beat to Beat Regulation of Myosin RLC Phosphorylation*

It has been suggested that myosin RLC phosphorylation plays a regulatory role in the beat to beat response of the heart (Sayers & Barany 1983). Cooling turtle hearts to 5°C reduces the heart rate to 5-8 beats per minute so that the muscle can easily be frozen in either systole or diastole. Using this method Sayers and Barany (1983) determined that hearts frozen in systole contained more phosphorylated myosin RLC (75.1%) as compared to hearts frozen in diastole (67.4%). Since the intracellular calcium concentration changes on a beat to beat basis and since MLCK is calcium dependent (Pires et al. 1974) it is possible that cardiac myosin RLC phosphorylation and dephosphorylation also changes on a beat to beat basis. If this were the case, the rate of phosphorylation and dephosphorylation would have to be relatively fast.

According to Manning and Stull (1979) it takes only a few seconds to increase the phosphate content of the myosin RLC in skeletal muscle. Since skeletal muscle has considerably more MLCK than cardiac muscle it seems unlikely that

myosin RLC in mammalian cardiac muscle would phosphorylate in a beat to beat manner (High & Stull 1980).

Cardiac muscle of rodents contains approximately 9 times to 20 times less MLCK than either rat slow twitch soleus or fast twitch extensor digitorum longus muscles respectively (Fitzsimons et al. 1989), thus it would be expected that a longer period of time is required for augmentation of myosin RLC phosphorylation. It would also be expected that the relationship between the rate of MLCK activation and inactivation as well as the kinetics of both calcium release and sequestration would influence the amount of cardiac myosin RLC phosphorylation obtained.

*Frequency Dependence of Cardiac Muscle Myosin RLC Phosphorylation*

Several attempts have been made to obtain different levels of myosin RLC phosphorylation (Silver et al. 1986, Fitzsimons et al. 1990, and Stull et al. 1985). Most of these studies have changed the frequency of activation to see if the result is a change in the myosin RLC phosphorylation level.

If there is a functional consequence of myosin RLC phosphorylation similar to that suggested by skinned fibre experiments, then the level of myosin RLC phosphorylation must be regulated over a substantial range. Fitzsimons et al. (1990) found that the level of cardiac RLC phosphorylation went from 33% at rest to 39% following 10 minutes of exercise at a heart rate 530 to 550 beats/minute in the rat.

Silver et al. (1986) using a rabbit whole cardiac muscle preparation showed that a linear relationship ($r=0.96$) could be found between contraction frequency and

myosin RLC phosphorylation. In this study, myosin RLC phosphorylation levels changed from 12% to 38% as heart rate increased from 0 to 126 beats per minute. It was also determined that the contractile properties: developed tension, maximal rate of tension development, and maximal rate of relaxation generally increase with an increase in stimulation rate (Silver et al. 1986).

The rabbit septal preparation used by Silver et al. (1986) was allowed to equilibrate for 60 minutes at 42 beats per minute before beginning any intervention after which it was determined that 30 minutes was required to establish steady-state values for myosin RLC phosphorylation. Fitzsimons et al. (1990) rationalized the shorter experimental duration (10 minutes) they needed by suggesting that the frequency they utilized (530 to 550 beats/minute) is 90% of maximum for the rat whereas Silver et al. (1986) used a frequency of less than 50% of maximum in the rabbit. One major difference between the studies by Silver et al. (1986) and Fitzsimons et al. (1990) is that Fitzsimons did not allow the muscle to reach steady state values.

The results of Fitzsimons et al. (1990) are consistent with the results of others where longer periods of time are required to significantly increase cardiac myosin RLC phosphorylation (Silver et al. 1986, Stull et al. 1985). This time frame is significantly longer than that of skeletal muscle in which only a few seconds are required to induce substantial changes in myosin RLC phosphorylation (Moore & Stull, 1984, Moore et al. 1985). The difference between cardiac and skeletal muscle is thought to be due to the high activity of both MLCK and MLCP in skeletal muscle and very low activity of these enzymes in cardiac muscle (Stull et al. 1982, Stull et al.

1985). In cardiac muscle, the activities of both enzymes are similar which, means that changes in myosin RLC phosphorylation are obtained very slowly.

Results obtained from isolated working heart preparations (High & Stull 1980, Jeacocke & England 1980, Silver et al. 1986) have all failed to explicitly demonstrate a relationship between myosin RLC phosphorylation and increased cardiac contractility with the exception of a study by Kopp & Barany in 1979. Kopp and Barany (1979) used a cannulated rat heart preparation with the addition of either positive of negative inotropic agents (e.g. isoproterenol or $MgCl_2$ respectively) and found that under these conditions only active tension was positively correlated ($r = 0.96$) to increased myosin RLC phosphorylation (3.5% to 17%). Other investigators preparations (High & Stull 1980, Jeacocke & England 1980, Silver et al. 1986) most likely did not duplicate the relationship observed by Kopp and Barany (1979) because the other investigators did not use as wide a range of positive and negative inotropic agents on the muscle preparations.

Fitzsimons et al. (1990) reported that augmented peak left ventricular pressure was associated with myosin RLC phosphorylation and may aid in the modulation of force development in cardiac muscle. This could occur if myosin RLC phosphorylation increases calcium sensitivity, as it should then impact force production during the contractile phase of the cardiac cycle. This shift would become very important at elevated heart rates (i.e. exercise) as phosphorylation of the myosin RLC could then enhance maximal rates of ventricular pressure development to maintain or augment stroke volume.

*Ryanodine Receptor Effects on Cardiac Calcium Concentrations*

The calcium required for contraction in cardiac muscle comes from both the SR and the extracellular spaces. There are differences between action potentials for rats and rabbits that lead to differing levels of contribution to the cytosolic calcium by the SR and extracellular space. In ventricular muscle the action potential begins when the $Na^+$ channels open, allowing the entry of $Na^+$ into the cell, thus generating the upstroke phase. This rapid entry of $Na^+$ into the cell is followed by an efflux of $K^+$ via the transient outward current ($I_{to}$) generating an early but partial repolarization. The next phase of the action potential is the plateau where the influx of $Ca^{2+}$ is balanced by the efflux of $K^+$ through the delayed rectifier current ($I_k$), the inward rectifier current ($I_{k1}$) and $I_{to}$. During the early repolarization phase of the action potential the efflux of $K^+$ through the $I_k$, $I_{k1}$, and $I_{to}$ channels is favoured over the influx of $Ca^{2+}$. Final repolarization then occurs as the result of the $i_k$ and $i_{k1}$ channels removing $K^+$ from the cytosol at a slightly faster rate than the same channels can add $K^+$ to the cytosol resulting in a return to the resting membrane potential (Giles 1989).

There are fundamental differences in the action potentials of rat and rabbit cardiac muscle (Yuan et al. 1996). In rat cardiac muscle, the action potential is of very short duration with a small plateau phase when the L-type calcium channels are usually open. This short duration plateau phase only allows small amounts of calcium to enter the cell and therefore rat cardiac muscle is more dependent on the sarcoplasmic reticulum for calcium. In comparison, the action potentials of rabbit cardiac muscle have a longer duration plateau. The longer duration means the L-type

calcium channels are open longer and therefore play a greater role in increasing intracellular calcium concentrations (Yuan et al. 1996).

In the rabbit, more calcium is provided by opening of the L-type calcium channels in the cell membrane although the SR is still a major calcium contributor. Addition of ryanodine (which inhibits SR function) to rabbit cardiac muscle does not significantly alter the calcium transients or developed tension because of the contribution of calcium by the L-type calcium channels (Sutko & Willerson, 1980). The opposite is true for rat cardiac muscle in that the short action potential does not allow the L-type calcium channels to significantly contribute to cytosolic calcium concentrations but allows the SR to be the major calcium contributor. Addition of ryanodine to rat cardiac muscle thus causes significant reductions in the calcium transients and force production (Sutko & Willerson, 1980). Fabiato (1982) also determined that the CICR mechanism of adult rat cardiac muscle was more developed than that of adult rabbit cardiac muscle. This fact contributes to greater SR dependence in the rat cardiac muscle.

*Effect of Isoproterenol on Myosin RLC Phosphorylation*

Regulation of myosin RLC phosphorylation may occur in vivo by hormonal mechanisms. Two attempts with the beta-agonist Isoproterenol, which has been shown to enhance force development, have been negative. Both Silver et al. (1986) and High and Stull (1980) added isoproterenol (3μM, and 0.1μM respectively) to the tissue bath for 30 seconds and observed no changes to the level of myosin RLC phosphorylation

although developed tension did significantly rise in the rat cardiac preparations. High and Stull (1980) also observed an increase in the maximum rate of rise of left ventricular pressure with the addition of isoproterenol whereas Silver et al. (1986) observed increases in developed tension, peak rate of force development, and peak rate of relaxation. The isoproterenol was left in the bath for only 30 seconds, as this was the point where the maximal inotropic response was observed yet both studies state that long periods of time are required for changes to phosphorylation of the myosin RLC to occur. These studies do not provide evidence for the lack of hormonal control of myosin RLC phosphorylation.

**Summary**

A wide range of times and a variety of preparations have been used to investigate myosin RLC phosphorylation (10-minute to 60-minute equilibration plus 30 minute intervention) most of which resulted in small changes to myosin RLC phosphorylation levels. A small range of 33 to 39% myosin RLC phosphorylation was reported in an in vivo rat heart (Fitzsimons et al. 1990) whereas a substantial range of 12 to 38% myosin RLC phosphorylation was reported in a whole rabbit heart preparation (Silver et al. 1986). Therefore it seems appropriate to measure RLC phosphorylation following extended periods of stimulation to determine to what extent rat cardiac myosin RLC becomes phosphorylated.

It has been difficult to find compelling evidence for a functional consequence of myosin RLC phosphorylation in intact cardiac preparations. This is probably

because changes in condition intended to (or expected to) alter myosin RLC phosphorylation, also affects calcium transients. Any change in contractile response could then just as easily be ascribed to the altered calcium transients as to the change in myosin RLC phosphorylation.

Phosphorylation of the myosin RLC in skeletal muscle results in potentiation. In cardiac muscle it is thought that myosin RLC phosphorylation may lead to enhanced cardiac contractility. The experiments by Fitzsimons et al. (1989, 1990) show that myosin RLC phosphorylation of cardiac muscle may have a significant impact on cardiac contractility. Changes in the level of calcium sensitivity as a result of RLC phosphorylation have been recorded in skinned skeletal muscle preparations and in skinned cardiac preparations. Therefore it is important to determine values for myosin RLC phosphorylation following set periods of stimulation so that functional correlates such as enhanced force, work, and power can be detected.

## Purpose

This thesis reports three distinct sets of experiments, all of which establish standard conditions that provide different known levels of RLC phosphorylation. The first experiments were done to determine the level of regulatory light chain phosphorylation in a left atrial preparation following either 15 minutes at a frequency of 4.0Hz or 30 minutes at frequencies of 1.0Hz, 2.0Hz, or 4.0Hz. The second set of experiments was done to determine the effect of okadaic acid on the level of regulatory light chain phosphorylation in the left atrial preparation. The third set of experiments was done to determine if either 1 or 30 minutes of rest following 30 minutes of stimulation at 4.0Hz would be sufficient to significantly reduce regulatory light chain phosphorylation.

## Hypothesis

It was hypothesized that left atrial RLC phosphorylation would be proportional to the frequency of stimulation and okadaic acid would enhance the level of phosphorylation of the RLC through inhibition of myosin light chain phosphatase. It was also hypothesized that a rest period of more than one-minute would be required to significantly reduce phosphorylation of the regulatory light chains.

# Chapter 3

## Conditions for Atrial Myosin Light Chain Phosphorylation

The contents of the introduction of this chapter are slightly redundant, as this chapter was submitted to a scientific journal. For a very in depth presentation of the methodology used please see Appendix A and B.

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### Introduction

The phosphorylation and dephosphorylation of proteins in striated and smooth muscle is important in controlling many processes in muscle such as ion flux, the contractile machinery, or metabolism (Shabb & Corbin, 1992). In particular the contractile machinery in cardiac muscle could be affected by changes in phosphorylation of the myosin regulatory light chains (Morano et al. 1986).

There are two major regulatory mechanisms that may be involved in the modulation of contractile activity in cardiac muscle: changing either the calcium transient or the calcium sensitivity. Changing the calcium transient would alter the amount of calcium available in the cytosol for binding to troponin thus altering the number of cross-bridges cycling. An increase in the calcium sensitivity would allow more force production to occur with the same level of calcium present (a leftward

shift in the force-calcium curve). According to several papers by Morano et al. (1985, 1986a, 1986b, 1988) and Sweeney and Stull (1986) such a leftward shift in the force-calcium relationship is obtained when the regulatory light chains (RLC) of myosin are phosphorylated.

Though changes have been observed in the level of calcium sensitivity in skinned fibre experiments there is still a need for these observations to be confirmed in intact preparations. In order to find such a functional correlate for RLC phosphorylation in intact preparations, there is a need to establish conditions that result in predictable levels of myosin RLC phosphorylation.

Myosin RLC phosphorylation is regulated by the activity of two enzymes: myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). MLCK is responsible for phosphorylation and is activated by binding a calcium-calmodulin complex whereas MLCP is responsible for dephosphorylation. How MLCP is regulated in cardiac muscle is unknown.

Given that MLCK is calcium dependent it would be anticipated that increasing the frequency of stimulation (frequency of calcium transients) would result in increased MLCK activity and therefore higher levels of myosin RLC phosphorylation. Several investigators have looked at myosin RLC phosphorylation with different stimulation frequencies (Fitzsimons et al. 1989, Moore et al. 1991, and Silver et al. 1986). The study by Fitzsimons et al. (1989) revealed that increasing the heart rate from 315 to 583 beats/minute in exercising rats resulted in a limited increase in RLC phosphorylation from 33% to 41%. Moore et al. (1991) found in isolated perfused rat heart that RLC phosphorylation levels were 29% when the heart rate was 225

beats/minute and 35% when the heart rate was >400 beats/minute. A study by Silver et al. (1986) that used a rabbit ventricular preparation showed a linear relationship between myosin RLC phosphorylation levels and stimulation frequency over the range of 0 to 2.1Hz (0 to 126 beats/min). This range of stimulation frequencies resulted in myosin RLC phosphorylation levels as low as 12% at 0Hz to as high as 38% at 2Hz. These observations confirm that myosin RLC phosphorylation can be regulated over a substantial range by variations in the stimulation frequency. However, it would be useful for studying functional correlates if a broader range of myosin RLC phosphorylation could be obtained. An alternative to looking at changes in stimulation frequency would be to inhibit the enzyme, MLCP.

The toxin okadaic acid (OA) is a known MLCP inhibitor that has been shown to enhance force production in isolated cardiac muscle (Lee et al. 1991). Inhibition of MLCP by OA should also result in changes to the myosin RLC phosphorylation levels. The studies by both Bialojan et al. (1988) and Siegman et al. (1989) have shown that addition of OA results in enhanced myosin RLC phosphorylation in smooth muscle. Okadaic acid has been used on cardiac preparations but whether changes in myosin RLC phosphorylation levels occur has yet to be determined. Given that OA causes an increase in RLC phosphorylation in smooth muscle, it is reasonable to expect the same for cardiac muscle.

The purpose of this paper was to establish conditions that lead to different levels of myosin RLC phosphorylation in cardiac muscle. This was done by looking at the level of myosin RLC phosphorylation following stimulation with a range of frequencies and by inhibiting MLCP with OA.

## Methods

### *Muscle Preparation*

The left atrium was dissected from the heart of an anaesthetised female Sprague-Dawley rat (220- 320g) and horizontally mounted in a tissue bath superfused with Tyrodes solution. The composition of the Tyrodes solution was 137 mM NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 23.3 mM $NaHCO_3$, 1.78 mM $NaH_2PO_4$, 0.5 mM $MgCl_2$, and 5.6 mM dextrose. This solution was equilibrated with 95% $O_2$ and 5% $CO_2$ to give an extracellular pH of 7.40. The atrio-ventricular ring end of the atrium was attached to a force transducer with a silk ligature (size 4-0) and the free end was fastened under a spring-loaded clip. The temperature of the bath was monitored throughout the experiment with a probe in the muscle bath and maintained at a constant temperature of 32.5°C ± 0.5°.

### *Contraction Experiments*

The length of the atrium was adjusted in the muscle bath by moving the force transducer forward or backward to the position where maximal isometric developed tension was obtained. This was considered to be the optimal length. The atrium was stimulated with suprathreshold square pulses of 0.5ms duration (Grass model S88, Grass Instruments, Quincy Mass. USA). Following surgery, the atrium underwent a

45-minute equilibration period during which it was continuously stimulated at 0.2Hz. Following the equilibration period the stimulation frequency was set at: 4.0Hz for 15 minutes ($4.0Hz_{short}$) or 1.0Hz, 2.0Hz, or 4.0Hz for 30 minutes. The muscle was frozen following 15 minutes for the 4.0Hz data only because a) this was the only frequency that resulted in changes to myosin RLC phosphorylation levels and b) we wanted to establish whether or not myosin RLC phosphorylation following 30 minutes at 4.0Hz was at steady-state. Since cardiac muscle contains a calcium-calmodulin dependent MLCK that specifically phosphorylates the myosin RLC (Wolf & Hofmann, 1980) it was anticipated that more frequent activation of the atrium would result in a higher degree of RLC phosphorylation.

A muscle twitch was recorded at the end of the equilibration period and at 2, 15, and 30 minutes postequilibration. The twitches were recorded to observe the contractile parameters over the duration of the experiment. At the end of the stimulation period, the Tyrodes solution was removed from the muscle bath and the atrium frozen with pre-chilled isopentane and stored for biochemical analysis.

Twitches were analyzed for the following contractile properties using an Excel macro: contraction time ($C_t$), half relaxation time (1/2 $R_t$), developed tension (DT), average rate of tension development ($DT \bullet C_t^{-1}$), and peak rate of relaxation ($dP \bullet dt^{-1}_{min}$). These contractile properties are important because, with RLC phosphorylation, it would be expected that $C_t$ does not change but the rates $DT \bullet C_t^{-1}$ and $dP \bullet dt^{-1}_{min}$ would become faster.

Statistical analysis of this data was performed with the program Statistica (Statsoft, Inc. Tulsa, Ok) and a statistical significance level of $p<0.05$ was utilized for

all comparisons. The 30-minute trials that were recorded at three separate frequencies (1.0Hz, 2.0Hz, & 4.0Hz) were analyzed using a two-way analysis of variance (time, frequency) with repeated measures (time). If there was a significant interaction the data were then analyzed with a one-way analysis of variance looking for simple main effects. This was followed by posthoc analysis using the Scheffe test. If there was no significant interaction but there was a significant time or frequency effect, then the Scheffe test was performed on the data to locate the significant effects.

The RLC phosphorylation data were analyzed using a one-way analysis of variance between groups. After analysis of this data was complete, another set of experiments was conducted in an attempt to obtain a broader range of RLC phosphorylation. Okadaic Acid, a known protein phosphatase inhibitor (Bialojan & Takai, 1988) was used to see if higher levels of RLC phosphorylation could be obtained. Alternatively stimulation was stopped for a period of either 1 minute or 30 minutes following equilibration to determine if low levels of RLC phosphorylation were attainable.

*Flow versus No Flow Experiments*

It became apparent that the cost of experiments with OA in the flowing Tyrodes solution would be significant. Therefore, as a possible solution to this problem we stopped the flow of Tyrodes solution during exposure to OA. Before doing the experiments with the OA, a series of experiments was completed to

determine if turning the flow of Tyrodes solution off prior to the addition of OA would negatively impact the muscle preparation.

For these experiments, the muscle was excised from the rat and placed in the tissue bath, then given a 45-minute equilibration period with a constant flow of oxygenated Tyrodes solution. Following the equilibration period the flow of Tyrodes solution was turned off and 95% $O_2$ and 5% $CO_2$ was delivered directly into the bath so that the muscle received a continuous supply of oxygen for the duration of the experiment. The muscle was then stimulated at 1.0Hz for 30 minutes with contractions being recorded after 2, 15, 20, 25, and 30 minutes. These twitches were analyzed and compared to twitches collected from muscles that had a constant flow of Tyrodes over the duration of the experiment to determine if stopping the flow of Tyrodes solution had a negative impact on the contractile properties of the muscle.

*Okadaic Acid (OA) Preparation*

In an attempt to obtain high levels of RLC phosphorylation, a set of experiments was done with the addition of the phosphatase inhibitor, OA. Again the atrium underwent the 45-minute equilibration period at 0.2Hz before the stimulation frequency was changed to 1.0Hz. Once the stimulation frequency was changed to 1.0Hz the flow of Tyrodes solution was stopped and the bath was bubbled with 95% $O_2$ and 5% $CO_2$. The atrium was left contracting in the oxygenated Tyrodes solution at a frequency of 1.0Hz for 15 minutes following the equilibration period. During this 15-minute period, twitch contractions were recorded at 2 and 15 minutes. After the

15-minute contraction was recorded the 2 mL of Tyrodes solution in the bath was removed and 2 mL of fresh Tyrodes solution containing either a) 0.25% Dimethylsulfoxide (DMSO) or b) the DMSO plus OA (final concentration 62μM) was added to the bath.

Once the Tyrodes solution containing either DMSO or DMSO + OA was added to the bath the muscle was stimulated at 1.0Hz for an additional 10 minutes (from 15 to 25 minutes). In total, each atrium in this protocol was stimulated for 25 minutes at 1.0Hz and contractions were recorded at 2, 15, 20, and 25 minutes. The addition of either DMSO or DMSO + OA was done at the 15 minute mark and lasted for only 10 minutes because the maximal level of developed tension had been reached within this time. The solutions containing DMSO and DMSO + OA were added after 15 minutes and not directly following the equilibration period so that data collection could be done at the 2 and 15 minute marks and compared to control values of muscles stimulated at 1.0Hz for 30 minutes. These twitches were analyzed for the same contractile properties as described previously. Following the 25 minutes of stimulation, the Tyrodes solution was removed from the bath and the atria were quick-frozen with chilled isopentane and stored at –70°C for biochemical analysis.

To determine if a basal level of RLC phosphorylation was always present, a set of 5 experiments was done in which the atria remained in the muscle bath with no stimulation (0Hz) for either 1 minute or 30 minutes. This set of experiments followed the protocol as described before where the equilibration period was followed by 30 minutes at 1.0Hz. Following the 30 minutes at 1.0Hz the atrium was left for 30

minutes with no stimulation (0Hz) before the muscle was frozen for biochemical analysis.

*Myosin RLC Phosphorylation Analysis*

The frozen muscle samples were analyzed to determine the magnitude of RLC phosphorylation. The protocol utilized for analyzing the amount of myosin RLC phosphorylation is a slightly modified version of the protocol utilized by both Moore and Stull (1984) and Stuart et al. (1987). Briefly, the muscle samples were pulverized then homogenized with a kinematic Polytron (Brinkmann Instruments, Rexdale Ont. Canada) and centrifuged at 7500 rpm at 0°C. The supernatant was applied to a pyrophosphate polyacrylamide tube gel and electrophoresed for 3 hours at 90V. These gels were then stained with 0.15% Coomassie Blue (R-250, Sigma Chemical Co., St. Louis, Mo), 50% methanol, and 10% acetic acid solution so the myosin band could be identified. Once identified, this band was excised from the gel.

The excised myosin band was denatured then loaded onto an isoelectric focussing (IEF) polyacrylamide gel to separate the phosphorylated from the non-phosphorylated RLC. The IEF polyacrylamide gel was electrophoresed at a constant power of 5W for 3 hours. A silver stain was used to label the light chain bands within the gel.

To determine the level of phosphorylation of the myosin light chains the gels were scanned using a program called UN-ScanIt-Gel version 5.1 (Silk Scientific Corporation, Orem, Utah). This program analyzed each sample individually with a

vertical lane analysis. Vertical lane analysis identified all of the bands found in the lane of each sample and then determined the density of each band before plotting the densities from one end of the lane to the other. The area under the curve representing each band was then calculated. To determine the total percent of phosphorylation, the area that represents the phosphorylated RLC was divided by the sum of the non-phosphorylated RLC, and phosphorylated RLC then multiplied by 100.

Similar to the first series of experiments, these were also analyzed with Statistica and a statistical significance level of $p<0.05$ was accepted for all comparisons. Twitches recorded during the 30 minutes at 1.0Hz followed by 30 minutes of no stimulation, and the twitches recorded during the 25 minutes at 1.0Hz with either DMSO or DMSO + OA added after 15 minutes were analyzed using a 2-way analysis of variance (time, group) with repeated measures (time). If there was a significant interaction, the data were then analyzed with a one-way analysis of variance for simple main effects. This was followed by posthoc analysis using the Scheffe test. If there was no significant interaction but there was either a significant time or frequency effect, then the Scheffe test was performed on the data to find the significant difference. The RLC phosphorylation data were analyzed using a one-way analysis of variance between groups followed by a Scheffe test for posthoc analysis.

**Results**

Initial experiments were done to determine if the three different stimulation frequencies (1.0, 2.0, & 4.0Hz) altered the RLC phosphorylation levels in a left

atrium preparation. Contractile properties were also measured. Each group in this section of results contained 10 atria.

*Developed Tension*

Figure 3 shows developed tension calculated as a percent of maximum (end of equilibration) versus time (min). Each of the three frequencies is represented and followed for either 15 or 30 minutes from the point where frequency was changed following equilibration. Developed tension decreased when frequency was increased from 0.2Hz (100%) to any of the test frequencies. Using a 2-way (frequency, time) repeated measures (time) ANOVA on the experiments lasting 30 minutes revealed no significant interaction ($p=0.09$) and no statistically significant time effect ($p=0.28$). A statistically significant frequency effect ($p=0.0009$) was found. The average values for the developed tension as a percent of maximum obtained at 1.0Hz were significantly higher ($p=0.007$) than the developed tensions recorded at either 2.0 or 4.0Hz. Also a comparison was done at 15 minutes on the developed tension as a percent of maximum on both the $4.0Hz_{short}$ data and the 4.0Hz data which revealed no significant differences ($p=0.6$).

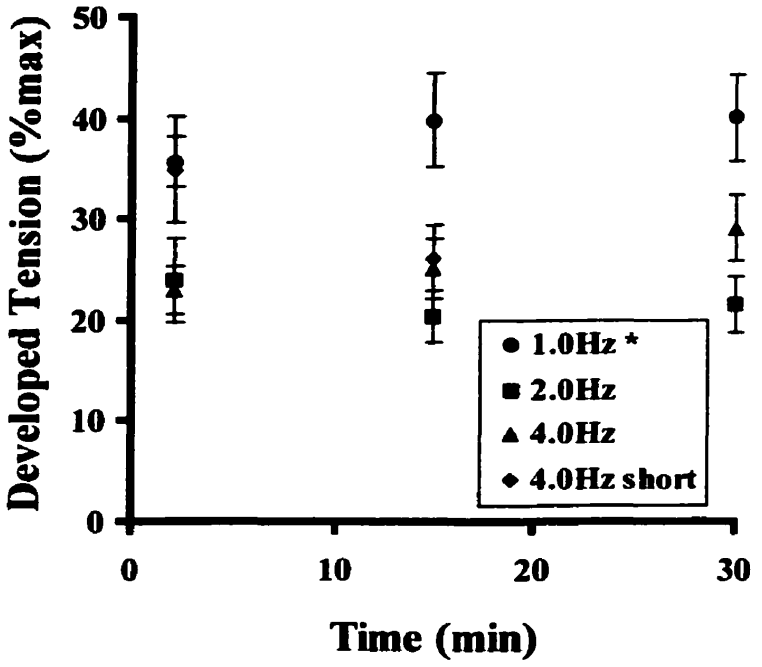


**Figure 3.** Mean developed tension as a percent of maximum ± SEM as a function of time for each stimulation frequency over a course of 15 ($4.0Hz_{short}$) or 30 minutes (1.0, 2.0, 4.0Hz). The asterisk represents the significantly higher average values at the frequency of 1.0Hz as compared to the other frequencies.

*Contraction Time*

The contraction time as a percent of maximum (end of equilibration) versus time (min) is presented in Figure 4 for the twitches over 15 or 30 minutes. A two-way (time, frequency) repeated measures (time) ANOVA revealed no significant interaction between frequency and time ($p=0.09$). This same analysis revealed no statistically significant time effect ($p=0.2$) and a statistically significant main effect of frequency ($p=0.001$). Since a significant frequency effect was found, a Scheffe test on the data was performed. This posthoc analysis revealed that the average contraction time as a percent of maximum for the 4.0Hz data was significantly shorter than the contraction time at the frequencies of 1.0Hz ($p=0.02$) or 2.0Hz ($p=0.002$). A comparison of the contraction times as a percent of maximum was also done between the $4.0Hz_{short}$ data and 4.0Hz data at 15 minutes and revealed no significant differences ($p=0.9$).

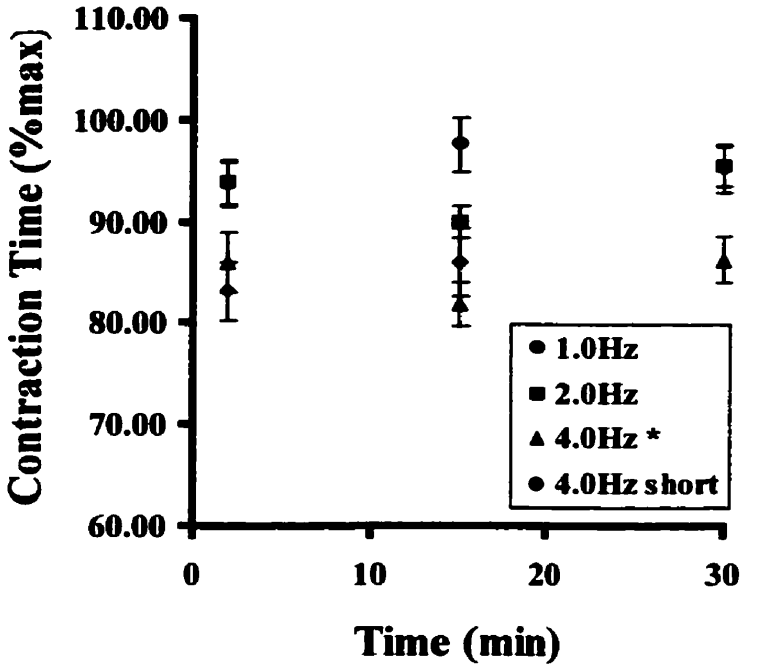


**Figure 4.** Mean contraction time as a percent of maximum ± SEM versus time for each stimulation frequency over a course of 15 ($4.0Hz_{short}$) or 30 minutes (1.0, 2.0, & 4.0Hz). The average contraction times as a percent of maximum at 4.0Hz was significantly shorter (*) than that at 1.0Hz and 2.0Hz.

*Average Rate of Force Development/Peak Rate of Relaxation*

Figure 5a shows the average rate of force development as a percent of maximum (end of equilibration) plotted against time (min). There was no significant interaction between the three stimulation frequencies for the 30 minute experiments ($p=0.5$) as ascertained by a 2-way (time, frequency) repeated measures ANOVA. There was also no significant time effect ($p=0.11$) but there was a significant frequency effect ($p=0.003$). A Scheffe test on the frequency data revealed that the average values for the 1.0Hz data were significantly different from the average values of both the 2.0Hz ($p=0.0003$) and 4.0Hz ($p=0.006$) data. The average values of the 2.0Hz data were not significantly different from those at 4.0Hz ($p=0.6$).

The graph representing the peak rate of relaxation as a percent of maximum (end of equilibration) versus time (min) is shown in Figure 5b. According to a 2-way (time, frequency) repeated measures (time) ANOVA there was no significant interaction between frequency and time ($p=0.12$). There was also no significant time effect ($p=0.4$) but there was a significant frequency effect ($p=0.003$). The Scheffe test performed on the frequency data revealed that the average values for the 1.0Hz data were significantly different from the average values for both the 2.0Hz and 4.0Hz ($p<0.0001$) but the average values of the 2.0Hz data were not significantly different from the 4.0Hz data ($p=0.8$).

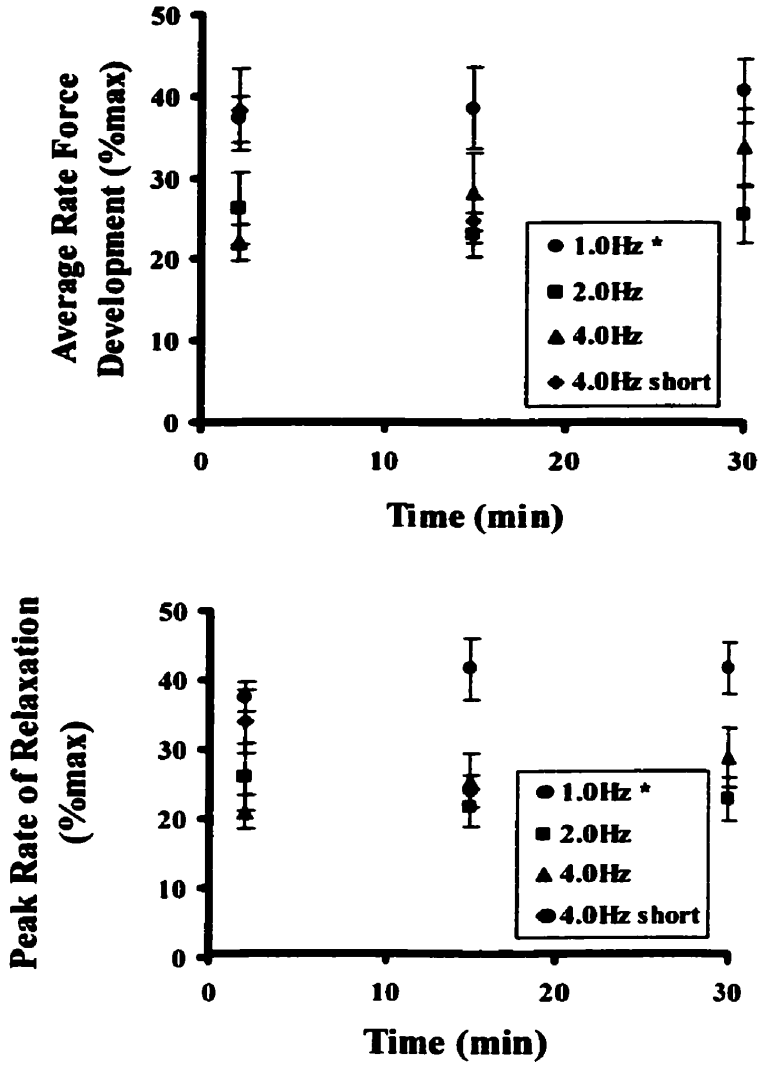


**Figure 5. a)** Average rate of force development as a percent of maximum (equilibration) ± SEM for each stimulation frequency versus time (min) for the duration of either 15 ($4.0Hz_{short}$) or 30 minutes (1.0, 2.0, 4.0Hz). The average 1.0Hz values are significantly different from the average values of both 4.0Hz and 2.0Hz (*) but the average values of the 2.0Hz data were not different from the 4.0Hz data. **b)** Peak rate of relaxation as a percent of maximum (equilibration) ± SEM as a function of time (min). The average 1.0Hz values are significantly different from the average values of both the 2.0Hz and 4.0Hz (*) data but the 2.0Hz data is not different from the 4.0Hz data.

*RLC Phosphorylation*

The level of RLC phosphorylation was determined using a pyrophosphate polyacrylamide gel followed by isoelectric focussing. This allowed quantification of the myosin regulatory light chain phosphorylation. When the light chains become phosphorylated the protein migrates to a region further down the gel so distinct bands can be identified (Figure 6).

The gels from these samples revealed a fourth band directly below the monophosphorylated RLC. This fourth band most likely represents diphosphorylation of the myosin RLC. The appearance of this fourth band is not consistent throughout all of the samples. Table 1 gives the percent phosphorylation at each stimulation frequency ± the standard error of the mean (SEM) as determined from vertical lane analysis (Un-Scan-IT) as well as the regulatory light chains as a percent of the total light chains ± SEM. A 1-way ANOVA revealed that there were significant differences in the percent of RLC phosphorylation for 1.0, 2.0, and 4.0Hz data ($p=0.04$). Posthoc analysis using the Scheffe test revealed that the percent of myosin RLC phosphorylation following 2.0Hz was lower than the percent of myosin RLC phosphorylation following 4.0Hz ($p=0.05$). Phosphorylation after 1.0Hz stimulation was not different from either 2.0Hz or 4.0Hz ($p>0.22$). Analysis of the equilibration values compared to both the $4.0Hz_{short}$ and 4.0Hz data revealed that the percent of myosin RLC phosphorylation following $4.0Hz_{short}$ was significantly higher than the percent of myosin RLC phosphorylation following the equilibration period ($p=0.05$).

The percent of myosin RLC phosphorylation following 30 minutes at 4.0Hz was not different from either the equilibration or the $4.0Hz_{short}$ data ($p>0.31$).

The purpose of these experiments was to establish conditions that provide different levels of myosin RLC phosphorylation. In contrast to what was expected stimulation at the three frequencies (1.0, 2.0, & 4.0Hz) did not result in three different levels of myosin RLC phosphorylation. Therefore another series of experiments was required, in which lower and higher values of myosin RLC phosphorylation could be obtained. The protocol utilized in the next series of experiments dealt with two frequencies (0Hz & 1.0Hz) and the addition of the phosphatase inhibitor, Okadaic Acid during 1.0Hz stimulation. Okadaic acid was utilized because it has been shown to have a positive inotropic effect on cardiac muscle preparations (Lee et al. 1991) and augment myosin RLC phosphorylation in smooth muscle preparations (Bialojan et al. 1988, Siegman et al. 1989). It was expected that the addition of OA to the muscle bath would enhance myosin RLC phosphorylation and increase force production in the atria as well.

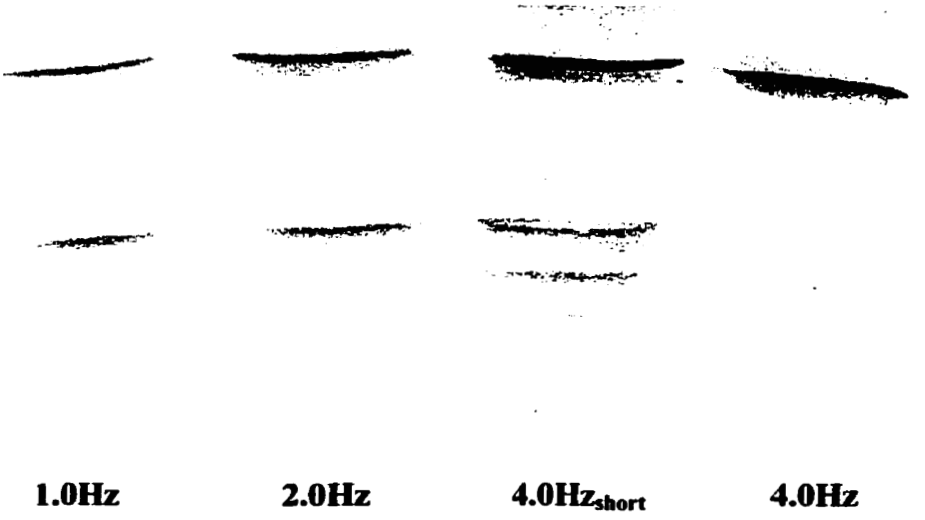


**Figure 6.** Representative samples from gels for 1.0Hz, 2.0Hz, 4.0Hz$_{short}$, and 4.0Hz from left to right respectively. The top band is the essential myosin light chains, the second band is the non-phosphorylated myosin RLC, the third band is mono-phosphorylated myosin RLC and the fourth band is diphosphorylation of the myosin RLC.

**Table 1.** Both the myosin light chain phosphorylation levels and the RLC as a percent of the total light chains ± SEM for each of the three stimulation frequencies: 1.0, 2.0, and 4.0Hz over 30 minutes and 4.0Hz over 15 minutes are shown. For statistical analysis two comparisons were made. First the equilibration, 1.0Hz, 2.0Hz, and 4.0Hz RLC phosphorylation levels were compared then the equilibration $4.0Hz_{short}$, 4.0Hz RLC phosphorylation data was compared. The average values represent the percent of RLC phosphorylation as determined by vertical lane analysis using the program Un-Scan-it. To determine percent phosphorylation the area beneath the curves that represented phosphorylated light chains was divided by the total area for all light chains (both phosphorylated and non-phosphorylated) then multiplied by 100.

| | | % RLC | | RLC as % | |
|---|---|---|---|---|---|
| | *n* | Phosphorylation | | of all LC | |
| **Equilibration** | **10** | **27.15** | **±2.88** | **50.81** | **±1.23** |
| **1.0Hz** | **9** | **29.35** | **±3.01** | **44.89** | **±2.21** |
| **2.0Hz** | **10** | **27.05 *** | **±1.48** | **46.03** | **±1.99** |
| **4.0Hz** | **10** | **32.27** | **±4.01** | **47.73** | **±2.04** |
| **$4.0Hz_{short}$** | **11** | **39.02 **** | **±2.24** | **42.45** | **±1.65** |

* Significantly different from the 4.0Hz (p=0.05)
** Significantly different from equilibration (p=0.05)

*Flow versus No Flow*

As hypothesized there were no differences in the level of developed tension when the flow was stopped as compared to when the flow of Tyrodes solution was maintained. A two-way (group, time) repeated measures (time) ANOVA determined that there was no significant interaction ($p>0.09$) or main effects for the developed tension as a percent of maximum when comparing the flow to the no flow groups. There were also no significant interactions ($p>0.5$) or main effects for the contraction times as a percent of maximum for this same data. Therefore stopping the flow of Tyrodes solution with the addition of $O_2$ and $CO_2$ to the tissue bath did not hinder the muscle preparation.

*Developed Tension-OA*

Addition of Okadaic Acid to the tissue bath at the 15-minute mark caused the developed tension to increase over the following 10 minutes of the experiment. Figure 7 presents the developed tension as a percent of maximum (end of equilibration) for the 1.0Hz group (n=9), the OA + DMSO group (n=5) and the DMSO group (n=5). The developed tension is very similar between the three groups for the first 15 minutes of the experiment. The 1.0Hz and DMSO groups were not statistically different for the duration of the experiments, which was as expected as both were stimulated at 1.0Hz and no difference was expected. Once the DMSO + OA had been added to the muscle bath (t = 15min) there was an increase in developed

tension over time. According to a two-way (group, time) repeated measures (time) ANOVA there was a statistically significant interaction between the two groups ($p=0.03$). Further statistical analysis using a one-way ANOVA to check for simple main effects determined that addition of OA caused an increase in developed tension which became statistically significant at 25 minutes ($p=0.02$). Though an apparent increase in developed tension is seen at 20 minutes it is not statistically significant ($p=0.07$).

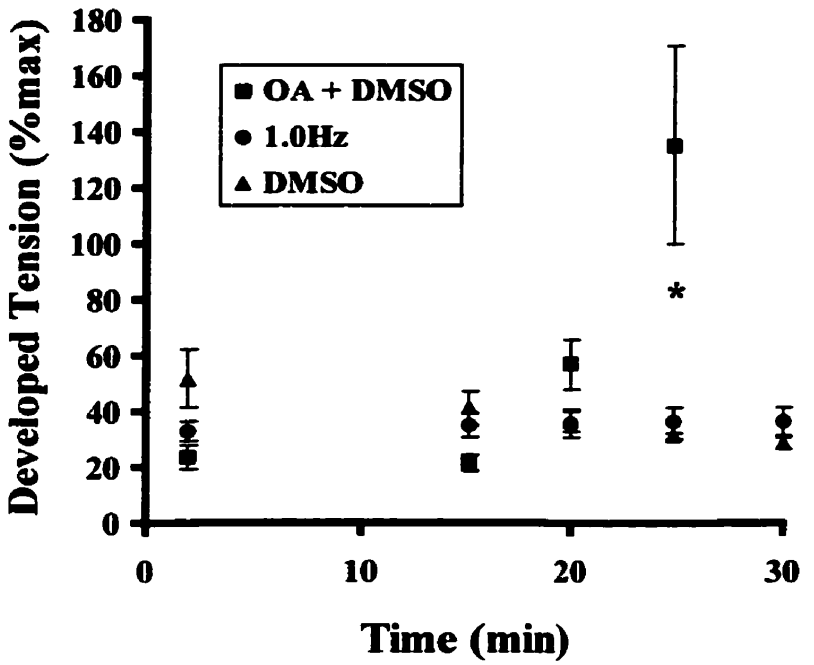


**Figure 7.** Mean values for developed tension as a percent of maximum ± SEM for groups stimulated at 1.0Hz for 30 minutes with and without the presence of DMSO and a group stimulated at 1.0Hz for 25minutes which had the MLCP inhibitor OA added following 15 minutes of 1.0Hz stimulation. Developed tension as a percent of maximum stays the same between the two groups before the addition of OA. OA in this case caused a dramatic increase in the level of developed tension at 25 minutes. The asterisk represents a statistically significant difference in developed tension between the groups.

*Contraction Time-OA*

Figure 8 shows the contraction time as a percent of maximum (end of equilibration) both with and without the addition of OA. A two-way (time, group) repeated measures (time) ANOVA revealed no significant interaction ($p=0.15$) and no significant group effect ($p=0.08$). There was however a significant time effect ($p=0.03$) and the Scheffe test revealed that the increase in contraction time from 2 minutes to 20 minutes was significant ($p=0.03$) but the contraction time at 25 minutes was not significantly different from that at 2 minutes ($p=0.29$) or 20 minutes ($p=0.15$).

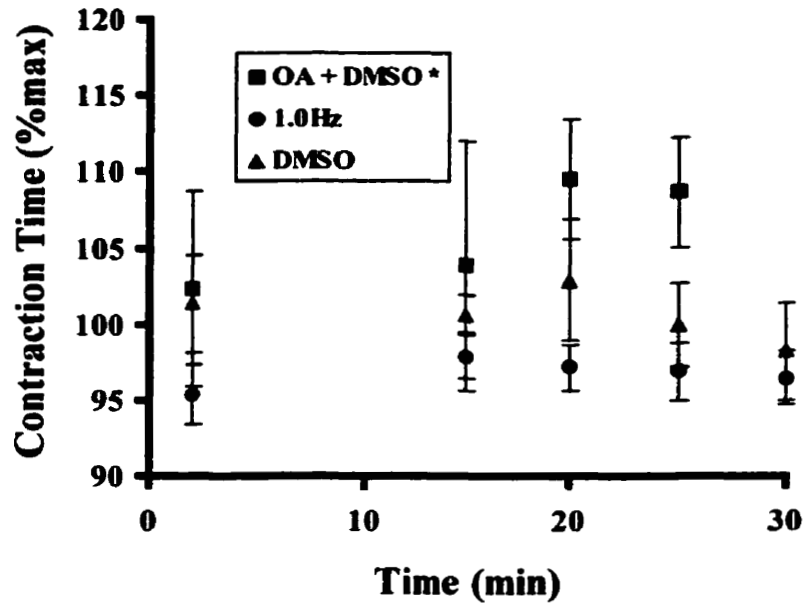


**Figure 8.** The average contraction time as a percent of maximum ± SEM for the1.0Hz, DMSO controls, and DMSO + OA groups. OA was added to the tissue bath following 15 minutes of stimulation at 1.0Hz. As observed in this graph there was a small but significant increase in the average contraction times for the OA group as compared to the average contraction times for the 1.0Hz group.

*Average Rate of Force Development/Peak Rate of Relaxation-OA*

In Figure 9a, the average rate of force development as a percent of maximum (end of equilibration) is plotted against time (min). This graph displays the same pattern as developed tension versus time (Figure 7). A two-way (group, time) repeated measures (time) ANOVA revealed a significant interaction between frequency and time ($p<0.001$). A one-way ANOVA (time) looking for simple main effects revealed that there were no significant time effects for either the 1.0Hz data ($p>0.3$) or the DMSO controls ($p>0.1$) but that there was a significant time effect for the OA group ($p<0.04$). The Scheffe test on the OA group then revealed that the 25-minute data were significantly different from the 2-minute data ($p<0.05$) but that 2 minutes and 25 minutes were not different from 20 minutes.

Figure 9b shows the peak rate of relaxation as a percent of maximum (end of equilibration) versus time (min) for the 1.0Hz, DMSO, and DMSO + OA groups. A two-way (group, time) repeated measures (time) ANOVA showed a significant interaction ($p<0.03$). A one-way ANOVA checking for simple main effects revealed no significant change across time for the 1.0Hz data and no significant difference for the DMSO group ($p=0.4$). In the OA group, there was a significant difference between 20 minutes and 25 minutes ($p<0.05$).

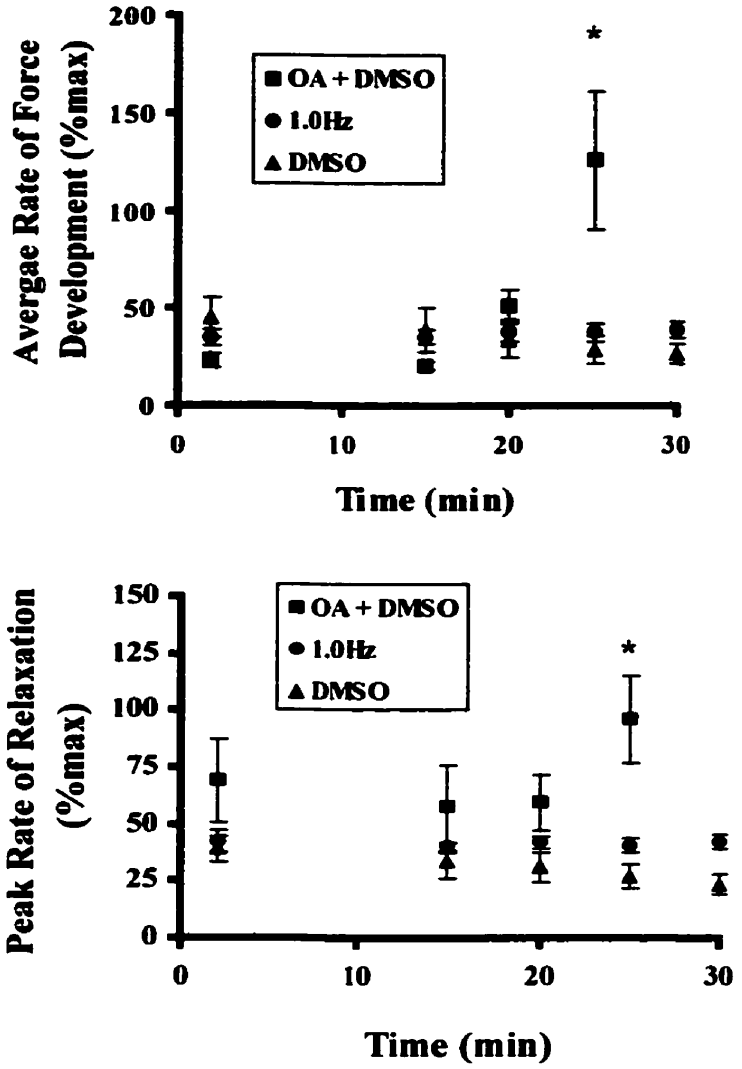


**Figure 9. a)** Mean values representing the average rate of force development as a percent of maximum ± SEM for the 1.0Hz, DMSO controls, and DMSO + OA groups. OA was added to the tissue bath following 15 minutes at 1.0Hz. The addition of OA to the tissue bath caused the average rate of force development as a percent of maximum in the OA group to rise. Following 25 minutes the average rate of force development as a percent of maximum for the OA group was significantly different from both the 1.0Hz group and DMSO group (*). **b)** Represents the peak rate of relaxation as a percent of maximum for the OA + DMSO, 1.0Hz, and DMSO groups. Following 25 minutes the peak rate of relaxation, as a percent of maximum was significantly different from 20 minutes in the OA group (*).

*RLC Phosphorylation-OA*

Figure 10 shows examples of the 1-minute rest, 30 minutes rest, and the DMSO + OA (from left to right respectively) isoelectric focussing slab gels. Biochemical analysis of these atria for RLC phosphorylation revealed that there were statistically significant differences between the atria samples that received no stimulation for 30 minutes as compared to either the DMSO controls or the 1.0Hz samples ($p<0.01$). The samples that received OA for 10 minutes also contained statistically higher levels of RLC phosphorylation as compared to either the DMSO controls or the 1.0Hz experiments ($p<0.01$). There was also no difference between the percent phosphorylation of the DMSO controls ($27.09 \pm 4.3$) versus the percent phosphorylation following 1.0Hz stimulation ($29.40 \pm 0.01$) ($p=0.7$). The data for each group are presented in Table 2 as the percent phosphorylation ± the SEM. Table 2 also shows the regulatory light chains as a percent of the total light chains ± SEM.

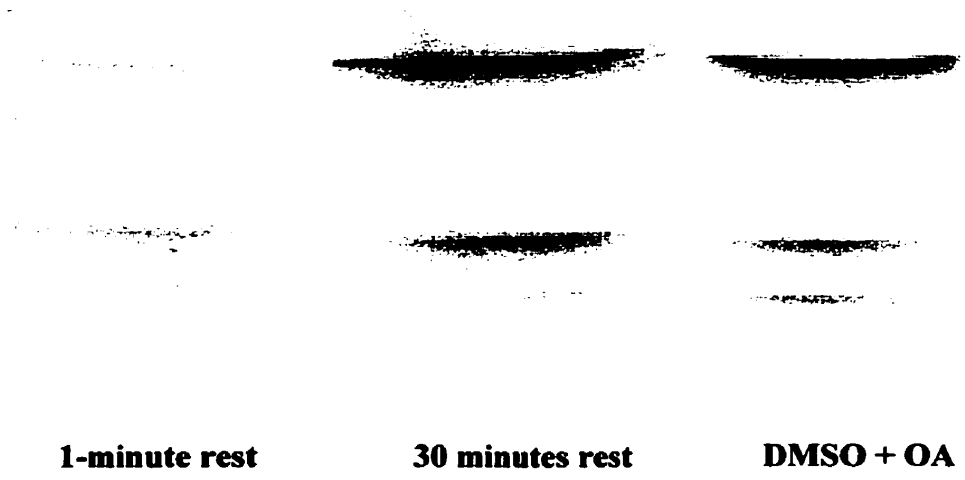


**Figure 10**. Representative samples of gels from experiments in which the muscle received 1-minute of rest, 30 minutes of rest, and finally DMSO + OA from left to right respectively. The top bands are the essential myosin light chains the second bands are the non-phosphorylated myosin RLC, and the third bands are phosphorylated myosin RLC. In the 1-minute rest sample there is also the appearance of the fourth band that represents diphosphorylated myosin RLC.

**Table 2.** Myosin light chain phosphorylation levels for the 1.0Hz as compared to the group which received 30minutes of no stimulation or 1 minute of no stimulation, 1.0Hz plus DMSO, and 1.0Hz plus OA ± SEM. The bar represents no significant differences between the data. The average values represent the percent of RLC phosphorylation as determined by vertical lane analysis using the program Un-Scan-it.

| | n | % RLC Phosphorylation | | RLC as a % of all LC | |
|---|---|---|---|---|---|
| 30-min no stimulation | 5 | 12.98 | ±0.92 | 34.08 | ±1.18 |
| 1.0Hz DMSO control | 5 | 27.09 | ±4.26 | 40.99 | ±1.70 |
| 1.0Hz;flow | 9 | 29.35 | ±3.01 | 44.89 | ±2.21 |
| 1-min no stimulation | 9 | 32.75 | ±3.64 | 45.81 | ±2.97 |
| 1.0Hz DMSO + OA | 5 | 44.56 | ±1.98 | 35.29 | ±2.97 |

## Discussion

### *Conditions for RLC Phosphorylation*

The major finding of this study was that conditions were established for which different levels of myosin RLC phosphorylation could be expected in rat atrial cardiac muscle. To obtain a low level (12%) of myosin RLC phosphorylation the muscle must be stimulated for 30 minutes followed by 30 minutes of no stimulation. For a moderate level (30%) of myosin RLC phosphorylation, cardiac muscle can be stimulated in the range of 1.0 to 4.0Hz for 30 minutes whereas the addition of OA for 10 minutes produced high levels (45%) of myosin RLC phosphorylation.

The results from these experiments show that 30 minutes of stimulation at 1.0Hz and 2.0Hz resulted in equal levels of myosin RLC phosphorylation (Table 1). Though the myosin RLC phosphorylation levels at 1.0Hz were not different from those at 2.0Hz the amount of developed tension produced at 1.0Hz was significantly higher than the developed tension produced at 2.0Hz (Figure 3). The significant difference between the developed tension recorded at 1.0Hz as compared to at 2.0Hz and with no change in terms of myosin RLC phosphorylation is probably the result of different calcium transients at the different frequencies.

Increasing the frequency of contraction increases in the number of calcium transients, which then augments MLCK activation. The increase in number of calcium transients with increased stimulation frequency also results in shorter periods of time between each calcium transient resulting in a reduction in the time for MLCK

deactivation (Stull et al. 1990). The 1.0Hz experiments had a significantly higher developed tension, probably as the result of larger calcium transients, as compared to the 2.0Hz results where the lower force was most likely the result of smaller calcium transients. Similar myosin RLC phosphorylation levels between the 1.0 and 2.0Hz frequencies would then be the result of a combination of more frequent small calcium transients and less frequent large calcium transients.

The results of the $4.0Hz_{short}$ and 4.0Hz data are interesting in that there was a significant initial rise in the level of myosin RLC phosphorylation (39% at 15 minutes) followed by a decline in the myosin RLC phosphorylation after 30 minutes (32%) back to near equilibration values (27%). Though the myosin RLC phosphorylation levels were increased after 15 minutes of 4.0Hz stimulation, there was no change in the developed tension (Figure 3). The mechanism that produced the rise and subsequent fall in myosin RLC phosphorylation with no changes to developed tension is unclear. Changing the stimulation frequency following equilibration to 4.0Hz causes the calcium transients to become smaller but more frequent, giving an unpredictable effect on MLCK activity. Since higher levels of RLC phosphorylation were observed, it can be deduced that MLCK activity was increased. The subsequent decline in RLC phosphorylation could not have been due to smaller calcium transients since DT did not decrease. Either regulation of MLCP or inhibition of MLCK could perhaps explain the rise and fall in myosin RLC phosphorylation levels with no change to the developed tension. If calcium transients were constant from 2 minutes to 30 minutes at 4.0Hz stimulation then

phosphorylation of the myosin RLC in cardiac muscle has no effect on the contractile response in intact atria.

*Frequency Dependence of Myosin RLC Phosphorylation*

A study by Silver et al. (1986) showed that myosin RLC phosphorylation is frequency dependent. Using a rabbit ventricular preparation paced between 0 and 2Hz the myosin RLC phosphorylation increased linearly with increasing stimulation frequency. The results of the present study indicate that myosin RLC phosphorylation is not frequency dependent in a rat cardiac preparation paced between 1.0 and 4.0Hz over 15 or 30 minutes.

This difference in results is most likely a consequence of the type of preparation utilized (rat versus rabbit). There are fundamental differences in the action potentials of rat and rabbit cardiac muscle (Yuan et al. 1996). In rat cardiac muscle, the action potential is of very short duration with a small plateau phase where the L-type calcium channels are usually open. This small plateau region only allows small amounts of calcium to enter the cell and therefore rat cardiac muscle is more dependent on the sarcoplasmic reticulum for calcium to activate the contractile response. In comparison, the action potentials of rabbit cardiac muscle have a long-duration plateau. The long duration plateau means the L-type calcium channel is open longer and plays a greater role in increasing intracellular calcium concentrations (Yuan et al. 1996). As a result of this difference in calcium supply for rat

(sarcoplasmic reticulum) as compared to the rabbit (membrane permeability to calcium) the force frequency curves are different as well.

In a rabbit ventricular preparation there is a linear increase in force production with an increase in frequency of stimulation (positive force frequency relationship) whereas in a rat preparation there is a negative force frequency relationship (decreased force with increased frequency of stimulation) (Koch-Weser & Blinks, 1963). This positive force frequency relationship in the rabbit can be linked to the linear relationship between myosin RLC phosphorylation and stimulation frequency through calcium transients. Increased calcium transients (size and number) with increased stimulation frequency in the rabbit allows for more calcium to be present in the cytosol (long action potentials) resulting in an increased MLCK activation (Yuan et al. 1996). The increase in MLCK activation would then result in augmented myosin RLC phosphorylation.

Apparently for 1.0Hz and 2.0Hz the negative force frequency relationship that is observed in rat cardiac muscle would produce the opposite effects as that observed in rabbit cardiac muscle. Higher stimulation frequencies would result in smaller but more frequent calcium transients. The force would remain constant with small calcium transients but MLCK activation would not increase resulting in no change to the myosin RLC phosphorylation levels.

*OA Function*

Addition of the potent phosphatase inhibitor OA can alter many functions within the muscle. A study of the effect of OA on protein phosphatases determined that OA has a high specificity for type 2A, type 1, and polycation-modulated phosphatases. The activities of type 2C, phosphotyrosyl, inositol trisphosphate phosphatase, acid phosphatases, and alkaline phosphatases are not compromised by the addition of OA (Bialojan & Takai, 1988).

Other studies using isolated guinea-pig cardiac myocytes have demonstrated that the addition of OA causes both an increase in action potential duration (Kodama et al. 1986) and an increase in L-type calcium currents (Hescheler et al. 1988). Lee et al. (1991) have also shown that the addition of OA to a ferret papillary muscle increases the magnitude of calcium transients. All of these observations led Takai (1988) to the conclusion that addition of OA must suppress the dephosphorylation mechanism resulting in elevated phosphorylation of calcium channel proteins and a subsequent increase in calcium channel opening probability.

The results of this study show that the addition of OA corresponds with an increase in the phosphorylation levels of the myosin RLC (Table 2). The anticipated mechanism through which OA produced the increase in myosin RLC phosphorylation is inhibition of MLCP similar to what has been observed in smooth muscle (Bialojan et al. 1988). Addition of OA to the tissue bath also produced a significant increase in developed tension (Figure 7). The developed tension could be enhanced either as the result of higher levels of myosin RLC phosphorylation or perhaps increased calcium

within the cytosol as a result of calcium channel phosphorylation (increased magnitude of calcium transients).

Increasing the calcium transients as the result of addition of OA could also contribute to the activation of MLCK in the muscle. Increased MLCK activity together with decreased MLCP activity would then result in enhanced myosin RLC phosphorylation. The preliminary experiments performed with OA showed a subsequent decline in developed tension following 10 minutes exposure. This decline could be the result of OA affecting another protein phosphatases within the muscle thus producing a detrimental effect that overrides the positive effect of myosin RLC phosphorylation and/or increased calcium transients. For example, phosphorylation of troponin I results in decreased calcium sensitivity (Robertson et al. 1982).

*MLCK and MLCP Activity*

Changes to the level of RLC phosphorylation in cardiac muscle is much slower than what is observed in skeletal muscle. This is perhaps the result of lower MLCK activity in the cardiac cell. Cardiac muscle of rodents contains approximately 9 times to 20 times less MLCK than either rat slow twitch soleus or fast twitch extensor digitorum longus muscles respectively (Fitzsimons et al. 1989). Since cardiac muscle contains less MLCK it would be expected that a longer period of time is required for augmentation of myosin RLC phosphorylation. It would also be expected that the relationship between the rate of MLCK activation and inactivation,

which is dependent on the kinetics of both calcium release and sequestration, would influence the amount of cardiac myosin RLC phosphorylation observed.

Results from this study show that stimulation of the muscle for 30 minutes at 1.0Hz followed by 30 minutes of no stimulation led to a significant reduction in the level of myosin RLC phosphorylation (Table 2). This significant reduction in myosin RLC following a long rest occurred most likely as the result of MLCK inactivation and maintenance of MLCP activity at a low level. Stimulation of the atria for 30 minutes at 1.0Hz followed by 1-minute with no stimulation did not significantly change the level of observed myosin RLC phosphorylation (Table 2). Low activity of the MLCP in cardiac tissue is the most likely reason the 1-minute rest was not accompanied by significant reductions in myosin RLC phosphorylation. Therefore the results of this study are consistent with the known low activity of both MLCK and MLCP in cardiac muscle.

*Myosin RLC Diphosphorylation*

A very unique result of this study was the appearance of a fourth band directly below the phosphorylated myosin RLC band (Figure 6). This fourth band most likely represents diphosphorylation of the atrial myosin RLC but it was not consistently seen in all of the samples. Only one other study has shown that atrial muscle can be diphosphorylated (Morano et al. 1989). In this study Morano et al. (1989) determined that ventricular myosin had two different myosin RLC forms which could be

monophosphorylated but that atrial myosin contains only one RLC form which may be both mono- or diphosphorylated.

Phosphorylation of myosin RLC increases the calcium dependent force of contraction in skinned cardiac muscles (Morano et al. 1985, 1986, 1988 and Morano & Ruegg, 1986). Diphosphorylation of atrial muscle may play a significant physiological role. Addition of OA to the tissue bath also resulted in no diphosphorylation of the atrial RLC (Figure 10). This may be the result of many factors such as the addition of either DMSO or OA or both, therefore further research needs to be conducted in this area to determine the factors associated with diphosphorylation of the myosin RLC in cardiac muscle.

*RLC as a Percent of Total LC*

The myosin RLC as a percent of the total light chains should be approximately 50% and as seen in Table 2 the calculated values are lower than 50%. This is most likely the case because the essential light chains on the isoelectric focussing gels were very dark and distinct whereas the RLC bands were neither as dark nor distinct (Figures 6 and 10). As a result of the essential light chain bands being very dark they had a very steep transition from background to band as compared to the very gradual transition for the regulatory light chains which were not as dark. There were also very obvious changes to the degree of background shading (Figures 6 and 10) that altered the ability to detect the edge of the bands. The combination of a gradual transition and altered background shading most likely resulted in an underestimation of the

density for the regulatory light chains and therefore of the percent of RLC as a total of all the light chains. The possibility that the fourth band was another essential light chain instead of a diphosphorylated RLC is quite unlikely, as the values for the percent of total light chains would then be even lower than they are.

# Chapter 4

## Summary and Future Directions

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### Summary

The experiments conducted have allowed for the determination of conditions in which known levels of myosin RLC phosphorylation can be observed. Addition of the toxin OA, a potent phosphatase inhibitor, did enhance both the amount of force produced by the atria and the level of myosin RLC phosphorylation obtained. The appearance of a diphosphorylated myosin RLC band is unique and may play a yet unknown physiological role. Calcium levels were not measured in this set of experiments. This would be necessary to determine if calcium sensitivity, the calcium transients or both were altered with the addition of OA, as this may be a potential mechanism aiding in the modulation of cardiac contractility.

**Future Directions**

The results of the study presented here have provided conditions under which known levels of myosin RLC phosphorylation can be obtained. With this information it is now possible to look at functional correlates such as work, force, and power in an intact cardiac preparation. It may also be worth while to repeat the experiments conducted in this thesis with a ventricular preparation so that the functional correlates can be measured in a ventricular preparation as well. Another area that would provide important information in the study of myosin RLC phosphorylation in cardiac muscle would be the measurement of calcium transients and calcium sensitivity with various myosin RLC phosphorylation levels.

# References

Allen, D.G. & Kurihara S. (1980). Calcium transients in mammalian ventricular muscle. **European Heart Journal.** 1(Supplement A): 5-15.

Bialojan, C., Ruegg, J.C., & Takai, A. (1988). Effects of okadaic acid on isometric tension and myosin phosphorylation of chemically skinned guinea-pig taenia coli. **Journal of Physiology.** 398: 81-95.

Bialojan, C., & Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. **Biochemical Journal.** 256: 283-290.

Bryg, R.J., Williams, G.A., & Labovitz, A.J. (1987). Effect of aging on left ventricular diastolic filling in normal subjects. **American Journal of Cardiology.** 59: 971-974.

Ebashi, S. (1991). Excitation-contraction coupling and the mechanism of muscle contraction. **Annual Reviews of Physiology.** 53: 1-16.

Fabiato, A. (1982). Calcium release in skinned cardiac cells: variations with species, tissues, and development. **Federation Proceedings.** 41: 2238-2244.

Fitzsimons, D.P., Bodell, P.W., & Baldwin, K.M. (1989). Phosphorylation of rodent cardiac myosin light chain 2: effects of exercise. **Journal of Applied Physiology.** 67: 2447-2453.

Fitzsimons, D.P., Bodell, P.W., & Baldwin, K.M. (1990). Myocardial functional correlates of cardiac myosin light chain 2 phosphorylation. **Journal of Applied Physiology.** 68: 2426-2433.

Grange, R.W., Vandenboom, C.R., & Houston, M.E. (1995). Myosin phosphorylation augments force-displacement and force-velocity relationships of mouse fast muscle. **American Journal of Physiology.** 269: C713-C724.

Giles, W.R. (1989). Intracellular electrical activity in the heart. In **Textbook of Physiology.** H.D. Patton, A.F. Fuchs, B. Hille, A.M. Scher, & R. Steiner, eds., pp782-795, W.D. Saunders Company.

Hescheler, J., Míeskes, G., Ruegg, J.C., Takai, A., & Trautwein, W. (1988). Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. **Pflügers Archiv.** 412: 248-252.

High, C.W., & Stull, J.T. (1980). Phosphorylation of myosin in perfused rabbit and rat hearts. **American Journal of Physiology.** 239: H756-H764.

Jeacocke, S.A., & England, P.J. (1980). Phosphorylation of myosin light chains in perfused rat heart. **Biochemistry Journal.** 188: 763-768.

Kennelly, P.J., Starovasnik, M., & Krebs, E.G. (1989). Activation of rabbit skeletal muscle myosin light chain kinase by calmodulin: a mechanistic overview. In: H. Hidaka, E. Carafoli, A.R. Means, & T. Tanaka (Eds.), **Calcium Protein Signalling.** (pp. 155-164). Plenum Publishing Corporation. New York.

Koch-Weser, J., & Blinks, J.R. (1963). The influence of the interval between beats on myocardial contractility. **Pharmacological Reviews.** 15: 601-652.

Kodama, I., Kondo, N., & Shibata, S. (1986). Electromechanical effects of okadaic acid isolated from black sponge in guinea-pig ventricular muscles. **Journal of Physiology.** 378: 359-373.

Kopp, S.J., & Barany, M. (1979). Phosphorylation of the 19,000-dalton light chain of myosin in perfused rat heart under the influence of negative and positive inotropic agents. **Journal of Biological Chemistry.** 254:12007-12012.

Lee. J.A., Takai, A., & Allen, D.G. (1991). Okadaic acid, a protein phosphatase inhibitor, increases the calcium transients in isolated ferret ventricular muscle. **Experimental Physiology.** 76: 281-284.

Manning, D.R., & Stull, J.T. (1979). Myosin light chain phosphorylation and phosphorylase $\alpha$ activity in rat extensor digitorum longus muscle. **Biochemistry and Biophysics Research Communications.** 90:164-170.

Manning, D.R., & Stull, J.T. (1982). Myosin light chain phosphorylation-dephosphorylation in mammalian skeletal muscle. **American Journal of Physiology.** 242: C234-C241.

Metzger, J.M., Greaser, M.L., & Moss, R.L. (1989). Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibres. **Journal of General Physiology.** 93: 855-883.

Moore, R.L., Houston, M.E., Iwamoto, G.A., & Stull, J.T. (1985). Phosphorylation of rabbit skeletal muscle myosin in situ. **Journal of Cellular Physiology.** 125: 301-305.

Moore, R.L., Musch, T.I., & Cheung, J.Y. (1991). Modulation of cardiac contractility by myosin light chain phosphorylation. **Medicine and Science in Sports and Exercise.** 23: 1163-1169.

Moore, R.L., & Stull, J.T. (1984). Myosin light chain phosphorylation in fast and slow skeletal muscles in situ. **American Journal of Physiology.** 247: C462-C471.

Morano, I., Hofmann, F., Zimmer, M., & Ruegg, J.C. (1985). The influence of P-light chain kinase on the calcium sensitivity of chemically skinned heart fibres. **FEBS Letters.** 189: 221-224.

Morano, I., Arndt, C., Bachle-Stolz, C., & Ruegg, J.C. (1986). Further studies on the effects of myosin P-light chain phosphorylation on contractile properties of skinned cardiac fibres. **Basic Research in Cardiology.** 81: 611-619.

Morano, I., Bachle-Stolz, C., Katus, A., & Ruegg, J.C. (1988). Increased calcium sensitivity of chemically skinned human atria by myosin light chain kinase. **Basic Research in Cardiology.** 83: 350-359.

Morano, I., Wankerl, M., Bohm, M., Erdmann, E., & Ruegg, J.C. (1989). Myosin P-light chain isoenzymes in the human heart: evidence for diphosphorylation of the atrial P-LC form. **Basic Research in Cardiology.** 84: 298-305.

Morano, I., & Ruegg, J.C. (1986). Calcium sensitivity of myofilaments in cardiac muscle – effect of myosin phosphorylation. **Basic Research in Cardiology.** 81 (supplement 1): 17-23.

Morgan, M., Perry, S.V., & Ottaway, J. (1976). Myosin light chain phosphatase. **Biochemical Journal.** 157: 687-697.

Palmer, B.M., & Moore, R.L. (1989). Myosin light chain phosphorylation and tension potentiation in mouse skeletal muscle. **American Journal of Physiology.** 257: C1012-C1019.

Persechini, A., Stull, J.T., & Cooke, R. (1985). The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibres. **Journal of Biological Chemistry.** 260: 7951-7954.

Pires, E., Perry, S.V., & Thomas, M.A.W. (1974). Myosin light chain kinase, a new enzyme from striated muscle. **FEBS Letters.** 41: 292-296.

Rankin, L.L., Enoka, R.M., Volz, K.A., & Stuart, D.G. (1988). Coexistence of twitch potentiation and tetanic force decline in rat hindlimb muscle. **Journal of Applied Physiology.** 65: 2687-2695.

Robertson, S.P., Johnson, J.D., Holroyde, M.J., Kranias, E.G., Potter, J.D., & Solaro, R.J. (1982). The effect of troponin I phosphorylation on the $Ca^{2+}$-binding properties of the $Ca^{2+}$-regulatory site of bovine cardiac troponin. **Journal of Biological Chemistry.** 257: 260-263.

Sayers, S.T., & Barany, K. (1983). Myosin light chain phosphorylation during contraction of turtle heart. **FEBS Letters.** 154: 305-309.

Shabb, J.B., & Corbin, J.D. (1992). Protein phosphorylation in the heart. In: Fozzard, H.A., Jennings, R.B., Haber, E., Katz, AM, & Morgan, H.E. (Eds.), **The Heart and Cardiovascular System.** (pp. 1539-1555) Raven Press. Ltd. New York.

Siegman, M.J., Butler, T.M., & Mooers, S.U. (1989). Effects of okadaic acid on the relationships among force, myosin light chain phosphorylation and ATPase activity in skinned portal vein. In: Paul, R.J., Elzinga, G., & Yamada, K. (Eds.), **Muscle Energetics.** (pp. 305-316). Alan R. Liss Inc. New York.

Silver, P.J., Buja, L.M., & Stull, J.T. (1986). Frequency dependent myosin light chain phosphorylation in isolated myocardium. **Journal of Molecular and Cellular Cardiology.** 18: 31-37.

Stuart, D.S., Lingley, M.D., Grange, R.W., & Houston, M.E. (1987). Myosin light chain phosphorylation and contractile performance of human skeletal muscle. **Canadian Journal of Physiology and Pharmacology.** 66: 49-54.

Stull, J.T., Blumenthal, D.K., Millar, J.R., & DiSalva, J. (1982). Regulation of myosin phosphorylation. **Journal of Molecular and Cellular Cardiology.** 14: 105-110.

Stull, J.T., Nunnally, M.H., Moore, R.L., & Blumenthal, D.K. (1985). Myosin light chain kinases and myosin phosphorylation in skeletal muscle. **Advances in Enzyme Regulation.** 23: 123-140.

Stull, J.T., Bowman, B.F., Gallagher, P.J., Herring, B.P., Hsu, L., Kamm, K.E., Kubota, Y., Leachman, S.A., Sweeney, H.L., & Tansey, M.G. (1990). Myosin phosphorylation in smooth and skeletal muscles: regulation and function. **Progress in Clinical and Biological Research.** 327: 107-126.

Sutko, J.L., & Willerson, J.T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium. Comparison with dog, cat, and rabbit ventricular tissues. **Circulation Research.** 46: 333-343.

Sweeney, H.L., & Kushmerick, M.J. (1985). Myosin phosphorylation in permeabilized rabbit psoas fibres. **American Journal of Physiology.** 249: C362-C365.

Sweeney, H.L., & Stull, J.T. (1986). Phosphorylation of myosin in permeabilized mammalian cardiac and skeletal muscle cells. **American Journal of Physiology.** 250: C657-C660.

Sweeney, H.L., & Stull, J.T. (1990). Alteration of cross-bridge kinetics by myosin light chain phosphorylation in rabbit skeletal muscle: Implications for regulation of actin-myosin interaction. **Proceedings of the National Academy of Science.** 87: 414-418.

Takai, A. (1988). Okadaic acid, protein phosphatase inhibition and muscle contractile effects. **Journal of Muscle Research and Cell Motility.** 9: 563-565.

Tubman, L.A. (1996). Myosin light chain phosphorylation and twitch potentiation in disuse atrophied skeletal muscle. **Doctoral Dissertation.** pp. 19.

Wolf H., & Hofmann, F. (1980). Purification of myosin light chain kinase from bovine cardiac muscle. **Proceedings of the National Academy of Science.** 77: 5852-5855.

Yuan, W., Ginsberg, K.S., Bers, D.M. (1996). Comparison of sarcolemmal calcium channel current in rabbit and rat ventricular myocytes. **Journal of Physiology.** 493: 733-746.

# Appendix A

## Methods for All Experimental Protocols

The left atrium was dissected away from the heart of an anaesthetized female Sprague-Dawley rat (220- 320g) and horizontally mounted in a tissue bath, superfused with Tyrodes solution. The composition of the Tyrodes solution was 137 mM NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 23.3 mM $NaHCO_3$, 1.78 mM $NaH_2PO_4$, 0.5 mM $MgCl_2$, and 5.6 mM dextrose. This solution was equilibrated with 95% $O_2$ and 5% $CO_2$ to give an extracellular pH of 7.40. The atrio-ventricular ring end of the atrium was attached to the force transducer with a silk ligature (size 4-0) and the free end was fastened under a spring-loaded clip. The temperature of the bath was monitored throughout the experiment by a probe inserted in the muscle bath and maintained at a constant temperature of 32.5°C ± 0.5°C. This preparation is illustrated in Figure 11.

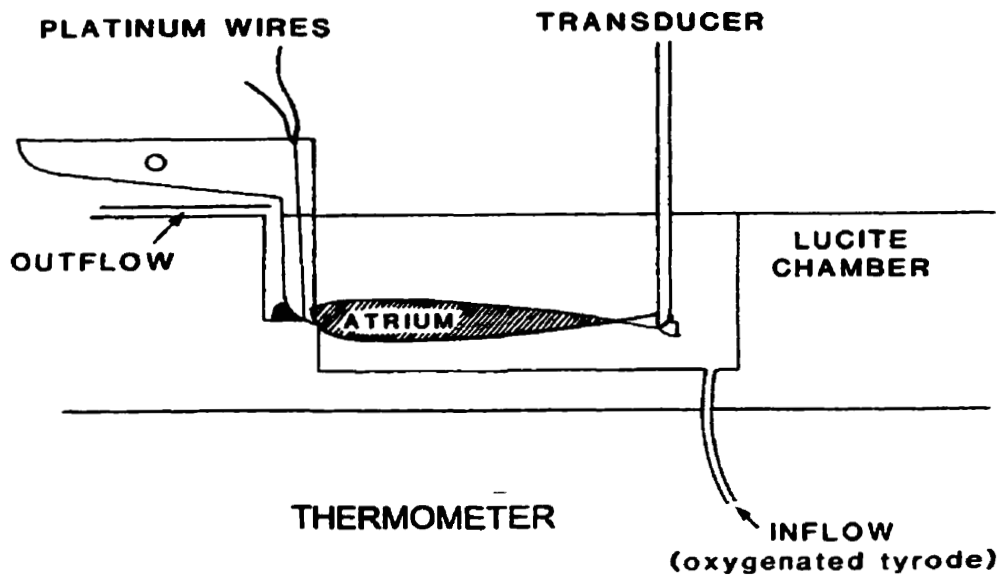


**Figure 11.** A diagram of the muscle tissue bath used for the atria preparations. The muscle is suspended in oxygenated Tyrodes solution by clipping one end under a spring loaded clip and fastening the atrio-ventricular ring end with silk to the force transducer. Platinum wires connected to the stimulator allow the muscle to be stimulated at set frequencies. The temperature thermistor recorded the temperature of Tyrodes solution within the muscle bath.

*Muscle Stimulation Patterns*

The length of the atrium was adjusted in the muscle bath by moving the force transducer forward or backward to the position where maximal isometric force was obtained. This was considered to be the muscle's optimal length. The atrium was stimulated with suprathreshold square pulses of 0.5ms duration (Grass model S88, Grass Instruments, Quincy Mass. USA). Following surgery the atrium underwent a 45-minute equilibration period during which it was continuously stimulated at 0.2Hz. Following the 45-minute equilibration period the stimulation frequency was set at: 1.0Hz, 2.0Hz, or 4.0Hz. A muscle twitch was recorded at the end of the equilibration period and at 2, 15, and 30 minutes post equilibration. At the end of either 15 (only for 4.0Hz stimulation) or 30 minutes the Tyrodes solution was removed from the muscle bath and the atria were frozen with isopentane pre-chilled with liquid nitrogen then stored in foil packets in the $-70^{\circ}C$ freezer for further analysis. The recorded twitches were then analyzed using a macro programmed into Microsoft Excel for the following contractile properties: contraction time ($C_t$), half relaxation time (1/2 $R_t$), developed tension (DT), average rate of tension development ($DT \bullet C_t^{-1}$), and peak rate of relaxation ($dP \bullet dt^{-1}_{min}$) (Figure 12). These contractile properties are important because with RLC phosphorylation it would be expected that $C_t$ does not change but the rates $DT \bullet C_t^{-1}$ and $dP \bullet dt^{-1}_{min}$ would become faster.

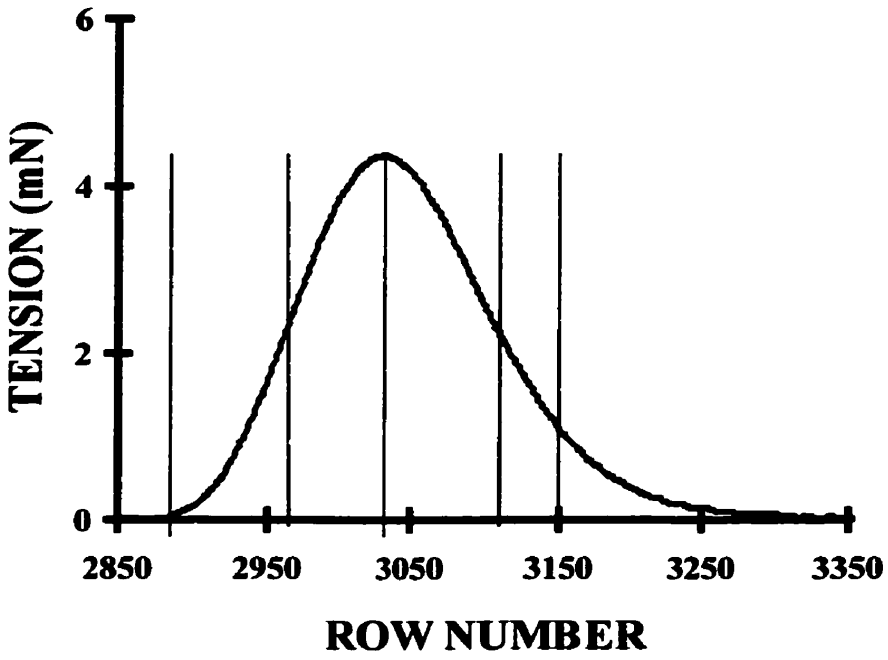


**Figure 12.** An example of a twitch (June6a00) recorded following the equilibration period. The point where the lines crosses the curve represents the value determined by the macro for the initial rise in tension, peak rate of force development (maximum slope), peak of contraction ($C_t$ = peak of contraction – initial rise in force), ½ relaxation time, and 75% relaxation time from left to right respectively.

*One-Minute Rest Protocol*

In another series of experiments the atrium was removed from the rat in the same manner as described previously and was again stimulated at 0.2Hz for a 45-minute equilibration period. After the equilibration period a sample twitch was collected at 0.2Hz before the muscle stimulation pattern was changed to 4.0Hz for 30 minutes. During the 30 minutes at 4.0Hz a sample twitch was collected at 2, 15, 20, 25, and 30 minutes. Following the 30-minute stimulation period the muscle was allowed to rest with no stimulation for a period of one minute. Subsequent to the 1-minute rest period the atrium was frozen immediately.

*30-Minute Rest Protocol*

Using the same dissection technique as described above another set of experiments was completed. Following equilibration (45 minutes at 0.2Hz) the atria were stimulated for 30 minutes at 1.0Hz and then were left in the flowing Tyrodes solution for another 30 minutes with no stimulation before they were frozen. The purpose of these of experiments was to determine if a substantial period of time (30 minutes) would significantly reduce the myosin RLC phosphorylation levels.

*Okadaic Acid Protocol*

The left atrium was removed from the rat and mounted in the tissue bath as described previously. Following the 45-minute equilibration period a sample twitch was recorded before the stimulation pattern was changed to 1.0Hz and the flow of Tyrodes solution was stopped. The Tyrodes solution remaining in the bath (2mL) was oxygenated by a tube attached to the muscle bath which supplied 95% $O_2$ and 5% $CO_2$ for the duration of the experiment. The muscle was left contracting in the Tyrodes solution at 1.0Hz for the first 15 minutes of the experiment with sample contractions taken at the 2 and 15-minute mark. After the 15-minute contraction was recorded the 2-mL of Tyrodes in the bath was removed and replaced by 2-mL of fresh Tyrodes solution. The fresh Tyrodes solution contained a) 0.25% Dimethylsulfoxide (DMSO) or b) DMSO plus Okadaic Acid (OA) with a final concentration of 62μm. Once the Tyrodes solution containing either DMSO or DMSO + OA was added to the bath the muscle was stimulated for an additional 10 minutes with contractions recorded at the 20 and 25 minute marks. In total each atrium in this protocol was stimulated for 25 minutes at 1.0Hz with contractions recorded at 2, 15, 20, and 25min.

The addition of either DMSO or DMSO + OA was done at the 15 minute mark and the experiment lasted for only 10 minutes more. This was because in preliminary experiments it was observed that the maximal level of force enhancement had been reached following 10 minutes of exposure to OA. In these preliminary experiments the developed tension tended to decrease when the muscle was exposed to OA for a period longer than 10 minutes.

The solutions containing DMSO and DMSO + OA were added after 15 minutes and not directly following the equilibration period so that data collection could be done at the 2 and 15 minute marks and compared to control values of muscles stimulated at 1.0Hz for 30 minutes. These twitches were analyzed for the same contractile properties as described above. Following the 25 minutes of stimulation the Tyrodes solution was again removed from the bath and the atria were quick-frozen with chilled isopentane and stored at –70°C for further analysis.

*Muscle Tissue Preparation*

In order to determine the levels of myosin RLC phosphorylation in the muscle it was necessary for each sample to be frozen for further analysis. Therefore following the 30 minutes of stimulation the Tyrodes solution was quickly removed from the tissue bath and the atrium was quick frozen to -60°C by pipetting pre-chilled isopentane into the muscle bath. The frozen muscle sample was then removed from the tissue bath and placed in a labeled tin foil wrap, then dropped into liquid nitrogen before being transferred to the –70°C freezer.

Once a sufficient number of samples had been collected, the frozen muscle samples were retrieved from the freezer. Each muscle was individually removed from its foil wrap and placed into a mortar and crushed into a fine powder with a pestle. Both the mortar and pestle had been pre-chilled with liquid nitrogen. The muscle powder was then transferred with a cold metal spatula into a cooled Eppendorf tube. Each tube was labeled with the experiment number then immediately placed back into

the liquid nitrogen until the homogenizing process could begin. During this procedure it was important that all the items being used were kept very cold and that the muscle sample never had the opportunity to thaw. The muscle was kept frozen because increased phosphorylation of the myosin light chains may occur as a result of muscle thawing.

To begin the homogenizing process the homogenizing solution (Appendix B) was pipetted into the appropriate glass vials and allowed to freeze, once frozen the vial was placed on a cooled weigh scale and 8 – 10 mg of the frozen crushed muscle tissue was placed inside. The vial was then warmed slightly to thaw the homogenizing solution and the muscle sample was homogenized with a kinematic Polytron (Brinkmann Instruments, Rexdale Ont. Canada) for 3-4 seconds. The homogenizing solution was then poured out of the vial into a labeled eppendorf tube and diluting solution (Appendix B) was added to the vial, which was then homogenized again for 3-4 seconds with the polytron. The diluting solution was homogenized with the polytron so that any pieces of the muscle remaining on the polytron head would be removed. The diluting solution was then poured out of the vial and into the same eppendorf tube containing the homogenizing solution. Following the homogenizing process each sample was centrifuged at 7500 rpm and 0°C for 15 minutes after which the supernatant was poured off into a new set of chilled labeled eppendorf tubes and then returned to liquid nitrogen to freeze the sample.

*Pyrophosphate Polyacrylamide Gel Electrophoresis: (PAGE)*

Before preparing the pyrophosphate polyacrylamide gel solutions (Appendix B) the bottom of the glass tubes was sealed with a square of parafilm and placed upright in a test tube stand. The gel solution was then prepared with the addition of both TEMED and ammonium persulfate iast. Once this step was completed the solution was pipetted into the glass tubes taking care to keep the levels of the gel in the tubes identical at about 0.5cm from the top. Once all the tubes were filled they were allowed to polymerize for 5 minutes before being topped off with deionized water and then covered with plastic wrap for an additional 45 minutes, this allowed for complete polymerization to occur. After preparing the running buffer (Appendix B) for the gel electrophoresis unit (Pharmacia GE-2/4, Baie d'Urfe, Quebec, Canada) it was poured in and the refrigeration unit (Haake D3-G, SaddleBrook NJ, USA) was started to cool the buffer to 0°C.

After the tubes had polymerized the top layer of water was removed and the tubes were placed into the electrophoresis unit. The pump was then turned on so that the empty portion of each tube filled with running buffer. The samples were then removed from the freezer and placed on ice. To load the gel tubes each sample was individually thawed then shaken to stir up the supernatant after which 50µl of the supernatant was pipetted into the tubes on top of the gel in a circular pattern. Once each sample was used it was returned to the liquid nitrogen to be re-frozen. After all the samples had been loaded the pump was restarted and the lid was placed on the

electrophoresis unit and the power leads were connected. The gels were then run at 90V for 3.0-3.5 hours.

To remove the gels from the glass tubes a syringe with water flowing through it was used to rim the top and bottom of each tube. The gel would then slide from the glass tube into your gloved hand after which it was immediately placed into a glass culture tube. Commassie Blue stain (Appendix B) was added to each culture tube after which they were capped and placed on a tube rocker (American Tube Rocker R4185-10). After five minutes in the staining solution the Commassie Blue was drained from the tube and the gels were rinsed with deionized water prior to the addition of the destain solution (Appendix B). The destain solution was left in each tube for approximately 5 minutes before being drained and the gel removed from the tube. The gel was placed on a light table that allowed the observation of the myosin band within the gel. After identification of the myosin band it was cut from the gel with a razor blade and the slice of gel was placed into a labeled eppendorf tube and stored in the –70°C freezer until the next step (Isoelectric Focussing Slab Gel) could be performed.

*Isoelectric Focussing Slab Gels (IEF)*

Once the myosin band was removed from the tube gel and placed in the eppendorf tube 90µl of IEF denaturant (Appendix B) was added and the gel was broken up into small pieces and left for 45 minutes. During this 45-minute period the IEF gel solution (Appendix B) was prepared and pipetted between the two glass plates

that had been cammed together with a 1mm spacer in between. Enough of the gel solution was added so that the placement of combs in between the two glass plates allowed wells to form. Once the combs were in place the gel was left undisturbed for 45 minutes to allow polymerization to occur.

To load the gels the combs were removed and the wells were rinsed with 0.2N NaOH (Appendix B) taking care not to allow the wells to dry out. Each sample was then loaded into a corresponding well with a Drummond pipette. Once all the samples were loaded into the wells the plates were detached from the casting stand and clipped into the electrophoresis unit (Idea Scientific, Mini Slab, Minneapolis MN, USA). The inner buffer chamber was filled with 0.2N NaOH and the outer buffer chamber was filled with the $H_3PO_4$ buffer (Appendix B). The entire electrophoresis unit was then placed in a bucket partially filled with ice and water. Once surrounded by the ice and water the lid was attached to the electrophoresis unit and the power leads were connected. The samples were then left to migrate through the gel for 3.0-3.5 hours at a constant power of 5 watts and a voltage that did not exceed 575V.

To stain the IEF gels the gel was removed from between the two glass plates and put into a covered glass container that contained a trichloroacetic acid (TCA) fixative (Appendix B) and was agitated for 30 minutes. When the fixative was removed from the plate a methanol/acetic acid solution (Appendix B) was added. This was left for 30 minutes before the solution was removed and this step was repeated leaving the gels in the methanol/acetic acid solution for an additional 2 hours. Once this step was completed the methanol/acetic acid was decanted and an ethanol solution (Appendix B) was added to the gel plate. After 30 minutes, the

ethanol solution was removed and the gel was placed into a glutaraldehyde solution (Appendix B) for another 30 minutes. Once this 30-minute stage was completed the gels had to be thoroughly washed with deionized water for at least 2 hours, making sure to change the water in the plate every 10 minutes. After removing the final water wash the silver stain solution (Appendix B) was added and the gels are agitated in the stain for 17 minutes. After 17 minutes in staining solution the gels are transferred to another dish containing deionized water and washed three times before half of the developing solution (Appendix B) was added. The first half of the developing solution was left mixing with the gels until the myosin bands began to appear. Once the bands were visible the first half of the developing solution was decanted off and the remainder was added to the dish. Once optimal band appearance was achieved the gels were removed from the developing solution and placed into an acetic acid solution (Appendix B) to stop the developing process. The gels were then stored at 4°C in small plastic bags with a minimal amount of deionized water.

To determine the level of phosphorylation of the myosin light chains the gels were scanned using a program called UN-ScanIt-Gel version 5.1 (Silk Scientific Corporation, Orem, Utah). This program analyzed each sample individually with a vertical lane analysis. Vertical lane analysis identified all four of the bands found in the lane of each sample and then determined the density of each band before plotting the densities in terms of area. A comparison of all four bands (essential light chain, regulatory light chain, phosphorylated regulatory light chain, and diphosphorylated regulatory light chain) can then be done because the total area of the four bands is equivalent to 100% and each band makes up a portion of that 100%. The essential

light chain was included in this analysis because it was a good determinant of how well the gels were done. It was expected that each sample had approximately 50% of its density in the band that corresponds to the essential light chain since there should be a 1:1 stoichiometry between essential and regulatory light chains.

*Statistical Analysis*

There were three sets of experiments conducted for this thesis. The first and third set of experiments where the atrial preparation was stimulated over a period of 30 minutes at one of three stimulation frequencies required that a two-way analysis of variance (time, frequency) with repeated measures (time) be performed. If there was a significant interaction then a one-way analysis of variance was performed to check the simple main effects. If this one-way analysis of variance had a significant result then it was followed with post hoc analysis using the Scheffe test. If there was no significant interaction on the two-way analysis of variance but there was either a significant group or time effect then the Scheffe test was utilized to determine the difference.

The RLC phosphorylation data was analyzed using a one-way analysis of variance between groups. Post hoc analysis of this data was done with the Scheffe test. All statistics were performed on Statistica and a significance level of $p<0.05$ was used for all of the data.

# Appendix B

**Solutions Utilized In Gel Preparations**

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The following list of solutions is mentioned in the Methods section (Appendix A). These solutions were utilized from the homogenization of the frozen muscle samples through to the polyacrylamide gel electrophoresis and the isoelectric focussing section of the Methods. Preparation of these solutions was done with care for exact measurement.

## 1.1 Homogenizing and Diluting Solutions

| | |
|---|---|
| Homogenizing or Diluting Stock Solution | 10ml |
| PMSF | 100µl |
| Trasylol | 100µl |
| Leupeptin | 100µl |
| β-Mercaptoethanol | 20µl |

## 1.2 Tube Gel Solution

| | |
|---|---|
| Monomer Stock | 6.3ml |
| Ppi Gel Buffer | 15ml |
| EDTA | 0.75ml |
| NaF | 8.0ml |
| TEMED | 63µl |
| 10% Ammonium Persulfate | 0.125g |

### 1.3 Running Buffer

| | |
|---|---|
| $Na_4P_2O_7*10H_2O$ | 71.0 g |
| NaF | 8.4 g |
| Glycerol | 400 ml |
| $H_2O$ fill to | 4 L |

### 1.4 O.15% Coomassie Stain Stock

| | |
|---|---|
| Coomassie Blue R-250 | 0.75 g |
| Methanol | 250 ml |
| Acetic acid | 35 ml |
| $H_2O$ fill to | 500 ml |

### 1.5 Destain Solution

| | |
|---|---|
| Methanol | 50 ml |
| Acetic acid | 25 ml |
| $H_2O$ fill to | 500 ml |

### 1.6 IEF Denaturant

| | |
|---|---|
| Urea | 11.5 g |
| Ampholines 4-5 | 0.8 ml |
| Ampholines 5-6 | 0.8 ml |
| β-Mercaptoethanol | 26 µl |
| $H_2O$ fill to | 24 ml |

### 1.7 IEF Gel Solution

| | |
|---|---|
| Monomer Stock | 4.4ml |
| Urea | 11.4g |
| 10% Triton X-100 | 4.4ml |
| H2O | 3.1ml |
| TEMED | 44µl |

10% Ammonium Persulfate 0.0065g

### 1.8 IEF Running Buffers

0.02 N NaOH

| | |
|---|---|
| NaOH | 0.8 g |
| $H_2O$ | 1000 ml |

0.026 M $H_3PO_3$

| | |
|---|---|
| $H_3PO_4$ | 0.875 ml |
| $H_2O$ fill to | 500 ml |

**1.9 15% TCA**

| | |
|---|---|
| 100% TCA | 37.5 g |
| $H_2O$ fill to | 250 ml |

**1.10 50% Methanol: 10% Acetic Acid**

| | |
|---|---|
| Methanol | 200 ml |
| Acetic Acid | 40 ml |
| $H_2O$ fill to | 400 ml |

**1.11 10% Ethanol**

| | |
|---|---|
| Ethanol | 30 ml |
| $H_2O$ fill to | 300 ml |

**1.12 10% Glutaraldehyde**

| | |
|---|---|
| 50% glutaraldehyde | 50 ml |
| $H_2O$ fill to | 250 ml |

**1.13 Silver Staining Solution**

| | |
|---|---|
| 0.36% NaOH | 83 ml |
| $NH_4OH$ | 1.5 ml |
| 19.4% $AgNO_3$ | 1.6 ml |
| $H_2O$ fill to | 150 ml |

**1.14 Developing Solution**

| | |
|---|---|
| Citric Acid | 0.02 g |
| $H_2O$ | 400 ml |
| 37% Formaldehyde | 216 µl |

**1.15 10% Acetic Acid**

| | |
|---|---|
| Acetic Acid | 20 ml |
| $H_2O$ fill to | 200 ml |