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Myosin Light Chain Phosphorylation and Twitch Potentiation in Disuse Atrophied Skeletal Muscle

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Lynda Aaron Tubman

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a dissertation entitled "Myosin Light Chain Phosphorylation and Twitch Potentiation in Disuse Atrophied Skeletal Muscle" submitted by Lynda Aaron Tubman in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Supervisor, Dr. B. R. MacIntosh
Department of Medical Science

Dr. W. Herzog
Department of Medical Science

Dr. H. ter Keurs
Department of Medical Physiology

Dr. M. Walsh
Department of Medical Biochemistry

External Examiner, Dr. P. Gardiner
Departement d'education, Universite de Montreal

July 15, 1996

Abstract

The mechanism for twitch potentiation in fast-twitch skeletal muscle has not been fully elucidated. The most widely accepted theory is that phosphorylation of the regulatory light chains (R-LC) of myosin is primarily responsible for the enhanced developed tension observed during low frequency repetitive stimulation (staircase potentiation) and following a tetanic stimulation (posttetanic potentiation). This hypothesis is based on experimental evidence demonstrating a strong positive correlation between R-LC phosphorylation and twitch potentiation. Twitch potentiation is significantly attenuated in atrophied skeletal muscle, but the causes of this attenuation are not clear. If it is assumed that R-LC phosphorylation is the primary mechanism of twitch potentiation, then it would be expected that phosphorylation would be diminished concomitantly with potentiation in atrophied muscles. Two models of disuse atrophy were used in this dissertation. In the initial study, application of tetrodotoxin (TTX) was used to induce atrophy in rat gastrocnemius muscle. R-LC phosphorylation and contractile characteristics were measured *in situ* during low frequency repetitive stimulation (10 Hz for 10 s) in TTX-treated, sham-treated and control muscles. In the

subsequent study, a spinal hemisection was completed to elicit disuse atrophy and then similarly assess R-LC phosphorylation and contractile properties *in situ* following a tetanic stimulation (2 s of 200 Hz). Staircase potentiation was severely attenuated in atrophied muscles, and this was accompanied by a virtual absence of R-LC phosphorylation. Alternatively, posttetanic potentiation was depressed but not abolished in atrophied muscle, and this event was associated with a moderate incorporation of phosphate in the R-LC. It appears that the potentiation-phosphorylation relationship is maintained in disuse atrophy. It is also evident that there is an impairment of the phosphorylation process in atrophied muscles. Since R-LC phosphorylation is dependent upon the Ca^{2+}-calmodulin activation of myosin light chain kinase (MLCK), a study was completed to determine if calmodulin content was reduced in atrophied muscle. However, calmodulin cellular content was similar for atrophied and non-atrophied muscles. Regardless of the mechanism for impairment of phosphorylation, the major finding in this dissertation was that attenuation of potentiation is associated with decreased R-LC phosphorylation in atrophied muscles. This supports the hypothesis that R-LC phosphorylation is the primary mechanism for twitch potentiation in skeletal muscle.

Preface

The chapters 3 and 5 of this dissertation are based on the following two manuscripts:

Tubman, L.A., Rassier, D.E. and MacIntosh B.R. Absence of Myosin Light chain Phosphorylation and Twitch Potentiation in Atrophied Skeletal Muscle. (Accepted) *Can. J. Physiol. Pharmac.*, 1996.

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Dedication

This dissertation is dedicated to:

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List of Abbreviations

AP	action potential
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate ($C_8H_4BrClNNa_2O_4P$)
Ca^{2+}	calcium
$[Ca^{2+}]$	calcium concentration
$[Ca^{2+}_i]$	intracellular calcium concentration
CaM	calmodulin
C_t	contraction time
$dP \cdot dt^{-1}_{max}$	peak rate of force development
$dP \cdot dt^{-1}_{min}$	peak rate of relaxation
DT	developed tension
$DT \cdot C_t^{-1}$	average rate of tension development
EDL	extensor digitorum longus
EDTA	ethylenediaminetetraacetic acid

FG	fast glycolytic muscle fiber type
FOG	fast oxidative glycolytic muscle fiber type
FT	fast-twitch fiber type
$\frac{1}{2}R_t$	half-relaxation time
IEF	isoelectric focussing
L_o	optimal muscle length
MG	medial gastrocnemius
MLCK	myosin light chain kinase
NBT	p-nitro blue tetrazolium chloride ($C_{40}H_{30}Cl_2N_{10}O_6$)
PTP	posttetanic potentiation
R-LC	regulatory or phosphorylatable light chains of myosin, also known as light chain 2
R_t	relaxation time
SR	sarcoplasmic reticulum
ST	slow-twitch muscle fiber type
T12	twelfth thoracic vertebra
TEMED	tetramethylethylenediamine
TTX	tetrodotoxin

Chapter 1

Introduction

The transient rise and fall of isometric force occurring as a consequence of a single pulse of stimulation in skeletal muscle is a twitch contraction. In mammalian fast-twitch skeletal muscle, enhancement of twitch amplitude can occur as a result of activity-dependent potentiation. Twitch potentiation has been consistently observed during low frequency repetitive stimulation, staircase potentiation, and following tetanic stimulation, posttetanic potentiation (Close and Hoh, 1968; Desmedt and Hainaut, 1968; Krarup, 1981a), yet the underlying mechanism has not been fully elucidated. Mechanisms for modulation of twitch force are complex and still require extensive investigation.

There are currently two theories which attempt to explain activity-dependent potentiation in skeletal muscle: i) potentiation results from phosphorylation of the regulatory light chains of myosin (Moore and Stull, 1984; Manning and Stull, 1979; Manning and Stull, 1982), or ii) potentiation results from increased availability of calcium during activation (Close, 1972). Although it is possible that both mechanisms contribute

to twitch potentiation, there is strong evidence that R-LC phosphorylation is the primary mechanism (Stull et al., 1985). For this reason the main objective of this dissertation was to further investigate the relationship between R-LC phosphorylation and twitch potentiation.

The observation that twitch potentiation is attenuated with certain types of disuse atrophy, suggests that an atrophy model could be used to gain insight into the underlying mechanisms of potentiation. Disuse atrophy which results from a period of inactivity has several effects on the contractile response of muscle. One of these effects is a change in the time-course of a twitch as a result of a prolonged contraction and relaxation time (St-Pierre and Gardiner, 1985). Also, compared with non-atrophied muscles, the amplitude of a tetanic contraction is reduced and twitch:tetanus ratio is elevated (St-Pierre and Gardiner, 1985). While the absolute amplitude of a twitch is preserved in disuse atrophied muscles, twitch potentiation is attenuated (MacIntosh et al., 1988).

Disuse atrophy associated with denervation has been shown to result in a depression of posttetanic potentiation (Dulhunty, 1985; Matsushita et al., 1988), and tetrodotoxin-induced atrophy results in an absence of staircase potentiation (MacIntosh et al., 1988). The reason for diminished potentiation in atrophied muscles is not known; however, by assessing the extent of R-LC phosphorylation and the degree of twitch potentiation simultaneously, further evidence could be revealed. If R-LC phosphorylation is the primary mechanism for potentiation, then it would be expected that R-LC phosphorylation would also be attenuated in atrophied muscles. However, it should be recognized that the presence of R-LC phosphorylation in atrophied muscles would not

disprove the theory that this mechanism is normally the primary mechanism of twitch potentiation.

In this research project two models of disuse atrophy were used: application of tetrodotoxin to the sciatic nerve and a spinal hemisection at the level of the twelfth thoracic vertebra (T12). Tetrodotoxin (TTX) is a sodium channel blocker that prevents the activation of the muscle fibers by the motoneurons (Lavoie et al., 1977), therefore if TTX application to the nerve is successful it is assumed that muscle activation would be totally blocked. Alternatively, a successful spinal hemisection results in a complete block of voluntary motor function of the affected muscle groups. In the hemisection model there is a possibility of some muscle activation as a result of intact spinal reflexes. Regardless of this difference in the disuse models, both interventions result in significant atrophy of the targeted muscle group.

The rat gastrocnemius muscle was chosen for these studies because it is predominantly composed of fast-twitch muscle fibers (28-38% FOG, and 58-65% FG) (Ariano et al., 1973; Armstrong and Phelps, 1984). An *in situ* preparation was used to measure twitch and tetanic contractile characteristics in the rat gastrocnemius muscle. This preparation has been well documented in the literature (MacIntosh et al., 1993; MacIntosh and Gardiner, 1987; Gardiner et al., 1992), with the advantage that the muscle can be studied at physiological temperature while blood flow is maintained. Additionally, previous work specifically examining contractile properties of atrophied muscles has commonly used the rat gastrocnemius muscle (Gardiner et al., 1992; MacIntosh et al., 1988; St-Pierre and Gardiner, 1985), thus allowing relevant comparisons with our results.

The initial *in situ* study of this research project examined the consequences of low frequency repetitive stimulation on contractile characteristics and R-LC phosphorylation in tetrodotoxin-disuse atrophied muscles (chapter 3). The notable observation was that the magnitude of R-LC phosphorylation was significantly attenuated in atrophied muscle, and this discovery lead to an additional investigation into the mechanism responsible for this impairment. Since R-LC phosphorylation is dependent upon the Ca^{2+}-calmodulin dependent activation of myosin light chain kinase, it was relevant to determine if the content of calmodulin was altered in atrophied muscles. These experimental data are presented in chapter 4 of this dissertation. The subsequent *in situ* study examined the relationship between posttetanic potentiation and R-LC phosphorylation. The effects of a 2 s tetanic stimulation on twitch contractile properties and R-LC phosphorylation was evaluated in muscles atrophied via spinal hemisection (chapter 5).

In summary, the motivation for conducting this research project was the question: What is the primary mechanism for twitch potentiation? This question was addressed by completing these specific objectives: (1) to confirm that potentiation is attenuated in atrophied muscles, (2) to assess the extent of R-LC phosphorylation during repetitive stimulation and following a tetanic contraction in atrophied muscles, (3) to examine the potentiation-phosphorylation relationship under two different stimulation schemes (10 Hz for 10 s, and 200 Hz for 2 s), and (4) to explore the possibility that impaired R-LC phosphorylation in atrophied muscles is due to a decreased calmodulin concentration. If the primary mechanism for twitch potentiation is R-LC phosphorylation, then the potentiation-phosphorylation relationship should be preserved even under conditions of

muscle atrophy.

The following section is a review of the literature and is intended to develop some background information relevant for the understanding of this research proposal. In this review, several underlying principles will be discussed in detail including: twitch contractions, twitch potentiation, myosin light chain phosphorylation, disuse atrophy and the diminution of potentiation in atrophied muscle.

Chapter 2

Review of Literature

Contractile Properties of Twitch Contractions

A twitch contraction is the response of skeletal muscle to a single activation and lasts for a fraction of a second. The contraction-relaxation cycle is primarily dependent upon the Ca^{2+} exchange between the sarcoplasmic reticulum (SR) and the myofibrils (Celio and Heizmann, 1982). Stimulation of skeletal muscle results in a rapid increase in the intracellular Ca^{2+} concentration from less than 10^{-7} M to ~ 10^{-5} M, due to the arrival of the action potential at the transverse tubules triggering Ca^{2+} release from the SR (Klug and Tibbits, 1988). In skeletal muscle, contraction is regulated by the thin filament system, and it is the binding of Ca^{2+} to troponin-C that initiates muscle contraction. This event is followed by Ca^{2+} sequestration by the SR which permits relaxation of the muscle (Desmedt and Hainaut, 1968).

The amount of force developed during a single twitch may be modulated by many

factors such as muscle length, temperature, and previous muscle activity. Desmedt and Hainaut (1968) have shown that complete activation of the contractile filaments of mammalian (human) muscle *in vivo* is not achieved with a single pulse of stimulation. Since a single activation results in incomplete activation, a change in the amplitude of contraction could result from variations in the level of activation. The primary mechanisms underlying twitch force modulation include changes in intracellular free Ca^{2+} concentration and/or alterations in the Ca^{2+} sensitivity of the myofilaments. When the consequences of these events result in an enhanced contractile response this is referred to as twitch potentiation.

Measurement of some twitch kinetics can provide important indirect insight into the Ca^{2+} handling characteristics of the SR. For example the time course of a twitch which is reflected by C_t (contraction time) or time to peak tension, has been associated with the rate at which Ca^{2+} is accumulated by the SR (Brody, 1976). Brody (1976) measured the twitch C_t of two different ST muscles (crureus and soleus muscles), and although the C_t was shorter in the crureus muscle the myosin ATPase activity was similar for both muscles. The difference in C_t was attributed to the rate of SR Ca^{2+} uptake, which was observed to be significantly greater in the crureus muscle (Brody, 1976).

Theoretically, however, these observations do not rule out the possibility that an increased C_t is a reflection of a transient increase in sarcoplasmic free Ca^{2+} concentration. This situation could arise as a result of an increased amount of Ca^{2+} released by the SR or residual free Ca^{2+} from previous muscle activation. For example, an enhanced release of Ca^{2+} by the SR would be expected to prolong C_t, and this idea is consistent with

twitch contractile measurements following exposure to caffeine (Sandow and Brust, 1966). Alternatively, it is possible that during repetitive stimulation the non-contractile binding sites for Ca^{2+} become saturated. The possible consequence of this Ca^{2+} buffering would be that a subsequent pulse of stimulation may provide a greater free sarcoplasmic [Ca^{2+}]. This in turn would be expected to enhance the amplitude of the twitch response, but the potentiation would be expected to be associated with a prolongation of C_t.

Furthermore, Ca^{2+} handling properties are also associated with half-relaxation time ($½R_t$) and peak rate of relaxation ($dP \cdot dt^{-1}_{min}$) which are used to describe relaxation characteristics of a twitch. Specifically, lengthening of the twitch $½R_t$ may be due to an increased free [Ca^{2+}] in the sarcoplasm as a result of a greater SR Ca^{2+} release or a slowing of the Ca^{2+} uptake by the SR. Alternatively, greater Ca^{2+} binding to non-contractile proteins can serve to accelerate relaxation. For example FT muscle has a significant concentration of parvalbumin (Celio and Heizmann, 1982), thus parvalbumin could potentially assist in the removal of free sarcoplasmic Ca^{2+} and decrease $½R_t$. On the other hand, if repetitive stimulation results in a relative saturation of the non-contractile Ca^{2+} binding sites, then subsequent release of Ca^{2+} from the SR could result in a higher peak free [Ca^{2+}]. If Ca^{2+} buffering sites are occupied, then removal of Ca^{2+} from the sarcoplasm would be slowed and $½R_t$ would be prolonged.

The positive slope of tension during a twitch contraction can be measured as peak rate of tension development ($dP \cdot dt^{-1}_{max}$) or average rate of force development ($DT \cdot C_t^{-1}$), and these parameters are thought to reflect the intensity of activation of the muscle (Desmedt and Hainaut, 1968). Because of their association with Ca^{2+} handling in the

sarcoplasm, all of these contractile characteristics provide insight into the possible mechanisms for twitch potentiation.

Twitch Potentiation

In mammalian muscle, potentiation or enhanced twitch tension development is prominent in fast-twitch fibers (Close and Hoh, 1968; Krarup, 1981b; MacIntosh and Gardiner, 1987). Chemical potentiation of twitch contractions can be produced with the administration of zinc, nitrate and caffeine (Desmedt and Hainaut, 1968). During chemical potentiation, the enhancement of tension development is accompanied by increased C_t, $\frac{1}{2}R_t$ and $dP \cdot dt^{-1}{}_{max}$ (Desmedt and Hainaut, 1968). Therefore these potentiators increase the length of activation time, as well as the intensity of activation.

Enhancement of twitch DT can also occur with activity-dependent potentiation, as a result of sustained or repetitive muscle stimulation. Staircase is one form of activity-dependent potentiation where there is a progressive enhancement of twitch DT during the initial phase of repetitive low frequency stimulation (MacIntosh, 1991). Posttetanic potentiation (PTP) is characterized by the augmentation of twitch force following a tetanic contraction (Krarup, 1981a). Both staircase and posttetanic potentiation exhibit some typical characteristics as discussed below.

Staircase

Peak force potentiation during staircase in rat gastrocnemius muscle has been observed to range from 75% to 85% during 10 Hz stimulation (MacIntosh and Kupsh, 1987; MacIntosh et al., 1993). Specifically the staircase response has been observed to peak at approximately 10 s during 10 Hz stimulation, followed by a slow decline in DT (MacIntosh and Kupsh, 1987; Moore and Stull, 1984). Figure 1 illustrates the classic staircase response of rat gastrocnemius muscle in superimposed tracings of twitch tension transients, emphasizing the changes in amplitude and time course of contractions at 0, 5, 10 and 15 s of 10 Hz stimulation. The increased amplitude of the twitch contraction is accompanied by an increase in $dP \cdot dt^{-1}_{max}$ with no significant change in C_t. These observations support the hypothesis that increased intensity of activation rather than a prolongation of activation is the mechanism by which staircase potentiation is elicited in mammalian fast-twitch skeletal muscle at 37°C.

Posttetanic Potentiation

Twitch magnitude is increased following tetanic stimulation, and this enhanced contractile response is called posttetanic potentiation. For example, in rat EDL muscle a peak PTP of 132% has been observed after a tetanic stimulation (125 Hz for 1.5 s) (Krarup, 1981a), while in rat gastrocnemius muscle twitch potentiation of 50% following a 1 s tetanic contraction (200 Hz) has been demonstrated (MacIntosh and Gardiner, 1987).

Figure 2 illustrates an increased twitch amplitude from pretetanic to posttetanic conditions. Similar to the staircase response, analysis of the contractile features of the posttetanic twitch demonstrates that potentiation is most often accomplished by an increase in the $dP \cdot dt^{-1}_{max}$ rather than a prolonged C_t (MacIntosh and Gardiner, 1987).

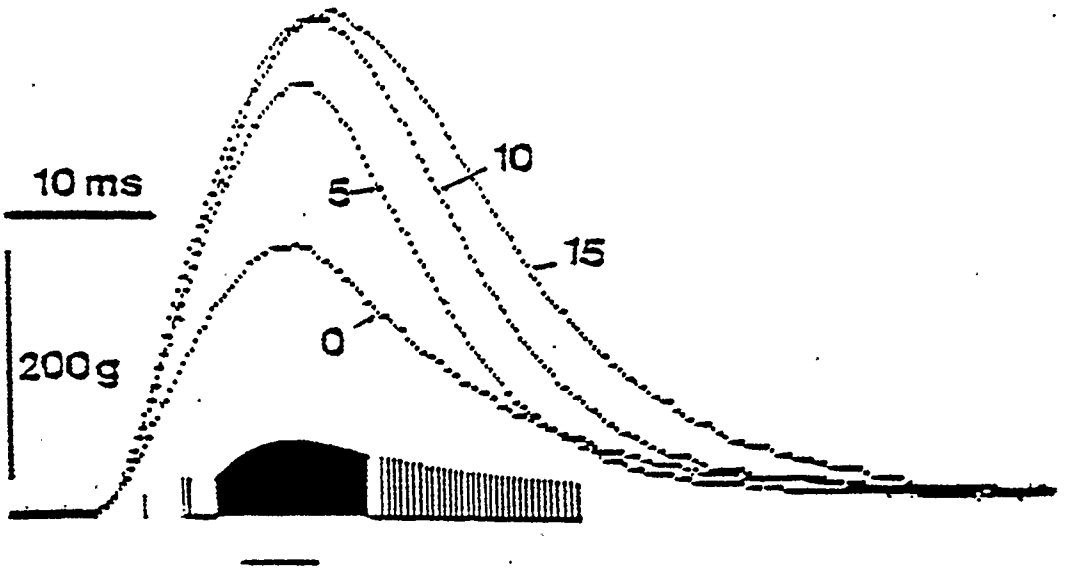

Figure 1. Staircase response in rat gastrocnemius-muscle showing twitches at 0, 5, 10, and 15 s of 10 Hz stimulation. Vertical calibration bars for the digitized contractions (outer) and slow tracings (inner) represent 200 g. Horizontal bars represent 10 milliseconds or 15 seconds, respectively (modified from MacIntosh, 1991).

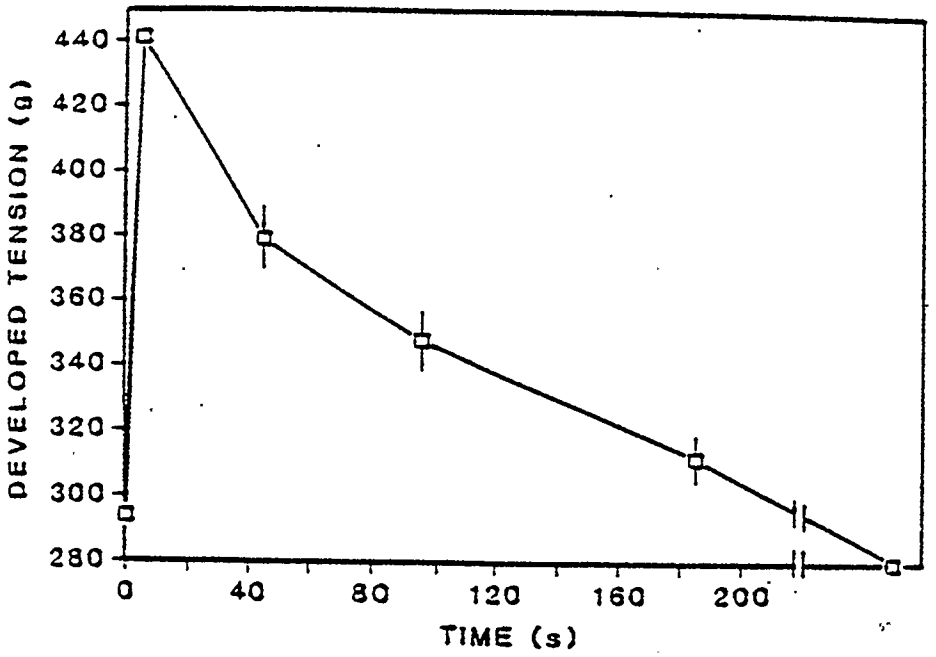

Figure 2. Twitch DT is plotted with time. The first contraction (zero time) is the twitch measured prior to the tetanic contraction (200 Hz for 1 s). This is followed by measurements for the 1st, 5th, 10th and 19th (184 s after the tetanic contraction) posttetanic twitches (MacIntosh and Gardiner, 1987).

Mechanisms for Twitch Potentiation

Initially from studies using chemical potentiators it was speculated that enhanced twitch force occurred due to a prolongation of the active state (Sandow, 1964). However this may not be true for activity-dependent potentiation. It has been established in mammalian muscle that potentiation is primarily achieved by an increased intensity of activation, as reflected by measurements of twitch contraction time and relaxation time (Desmedt and Hainaut, 1968; Krarup, 1981b; MacIntosh and Gardiner, 1987). Although the mechanism for twitch potentiation has not been fully elucidated, there are two main theories to account for this increased intensity of activation: i) a greater concentration of Ca^{2+} in the sarcoplasm, or ii) an increased Ca^{2+} sensitivity of the myofilaments via regulatory light chain phosphorylation. The proposed study will focus on theory (ii), but this review will provide evidence supporting both possible mechanisms.

Increased Ca^{2+} Concentration in the Sarcoplasm

It has been proposed that repetitive stimulation increases the Ca^{2+} concentration in the sarcoplasm and, therefore, more myofibrils are activated and potentiation occurs. Close and Hoh (1968) measured twitch characteristics following prolonged stimulation in rat EDL muscle *in vitro* (35°C), to try to elucidate the mechanism for twitch potentiation. Since an increase in peak tension was accompanied by a prolonged C_t, these authors (Close and Hoh, 1968) suggested that the observed potentiation was largely due to an

increase in the duration of activation.

A prolongation of activation could result from an increased free Ca^{2+} concentration within the cell. It is possible that a higher sarcoplasmic free [Ca^{2+}] could occur via an increased Ca^{2+} release per action potential, similar to that which appears to occur with caffeine intervention. However, it appears that the mechanism for potentiation is different in caffeine-induced potentiation as compared to posttetanic potentiation (MacIntosh and Gardiner, 1987). It has been well established that caffeine enhances the release of Ca^{2+} from the SR (Poledna and Morad, 1983), suggesting that during caffeine potentiation an enhanced Ca^{2+} availability is the principal mechanism by which the twitch is potentiated. MacIntosh and Gardiner (1987) showed that while PTP is accompanied by an increase in $dP \cdot dt^{-1}_{max}$ (without a change in C_t) caffeine potentiation is primarily achieved by an increased C_t.

Furthermore, it has been argued by Blinks and coworkers (1978) that following tetanic stimulation of frog muscle there is not a greater release of "activator", since they observed a reduced aequorin response. Together these observations suggest that Ca^{2+} release by the SR is not enhanced following tetanic stimulation. However, a higher free [Ca^{2+}] could still be attained with less Ca^{2+} release if the tetanus left residual Ca^{2+} in the sarcoplasm. If during a tetanic stimulation the Ca^{2+} binding sites become saturated, then a subsequent pulse of stimulation may result in a transient increase in free sarcoplasmic [Ca^{2+}]. This suggests that a higher sarcoplasmic [Ca^{2+}] is unlikely to result from a greater release of Ca^{2+} per action potential, but may arise due to the high buffering capacity of the cell and slow Ca^{2+} exchange kinetics of the cell buffers (Blinks et al., 1978).

During repetitive or prolonged stimulation there is potential for a cumulative effect of Ca^{2+} release. For example, incomplete removal of Ca^{2+} from the sarcoplasm prior to a subsequent activation could occur as a result of Ca^{2+} binding to non-contractile proteins (e.g., parvalbumin). In this case, the Ca^{2+} buffering capacity of the muscle cell would become saturated allowing for a greater free Ca^{2+} concentration with a subsequent SR Ca^{2+} release.

If twitch potentiation is achieved due to an enhanced Ca^{2+} availability, it could be argued that there would be a concomitant increase in contraction time during potentiation. Although twitch C_t has been observed to be increased following prolonged tetanic stimulation (Olson and Swett, 1971; Close and Hoh, 1968), in most potentiation studies increased twitch tension is associated with an increase in $dP \cdot dt^{-1}_{max}$ without significant prolongation of C_t (Krarup, 1981a; Klug et al., 1982; Yagi et al., 1993). Measurements of twitch kinetics during potentiation indirectly support the theory that Ca^{2+} availability is not the primary mechanism responsible for twitch potentiation.

There is little evidence presently to support the theory that residual Ca^{2+} plays an essential role in twitch potentiation. In amphibian muscle, the prolongation of contraction/relaxation time has been correlated with twitch tension potentiation (Sandow, 1964) whereas these changes do not necessarily occur in mammalian muscle. This, coupled with the fact that mammalian FT skeletal muscle has a large potential for intracellular Ca^{2+} removal by the SR, suggests that an alternative theory (e.g. R-LC phosphorylation) has a primary role in potentiation. However, further research is required to more precisely determine residual Ca^{2+} distribution in mammalian skeletal muscle

following tetanic or repetitive contractions.

Myosin Light Chain Phosphorylation

Phosphorylation of the regulatory light chains of myosin may be the primary mechanism for twitch potentiation. This theory originates from observations that a strong positive correlation exists between the extent of R-LC phosphorylation and the magnitude of twitch potentiation (Klug et al., 1982; Manning and Stull, 1979; Manning and Stull, 1982; Stull et al., 1985). Furthermore, Moore and Stull (1984) have demonstrated that during muscle stimulation, phosphorylation and potentiation occur in a time and frequency dependent manner.

To better understand the potential role of R-LC phosphorylation in potentiation it is important to examine the biochemical process associated with the incorporation of phosphate into the light chains. Phosphorylation of the 18,500 dalton R-LC occurs via the enzyme myosin light chain kinase (MLCK), which is actively regulated by Ca^{2+}-bound calmodulin (Kennelly et al., 1989). The increased intracellular Ca^{2+} concentration caused by muscle stimulation results in the formation of a Ca^{2+}-calmodulin complex which in turn binds to and activates MLCK. Active MLCK catalyzes the transfer of the terminal phosphoryl group of ATP to the light chain. During muscle relaxation intracellular Ca^{2+} levels fall, causing the dissociation of the Ca^{2+}-calmodulin complex and inactivation of MLCK (Stull et al., 1990). Dephosphorylation of R-LC is catalyzed at a slow rate via myosin light chain phosphatase, an enzyme which does not appear to be actively regulated

(Morgan et al., 1976). This process is illustrated in Figure 3.

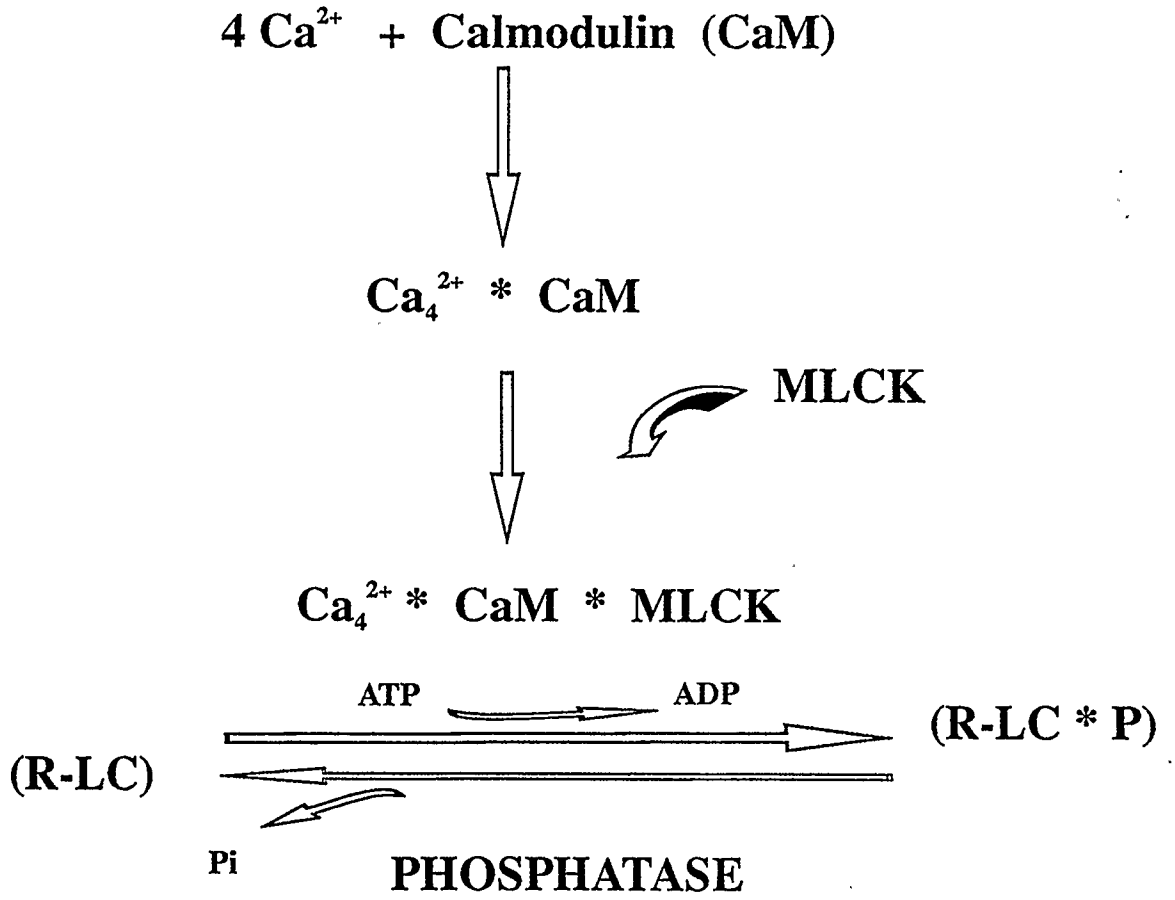

Figure 3. Phosphorylation of the regulatory light chains of myosin (modified from Stull et al., 1990).

The hypothesis that R-LC phosphorylation may enhance force development has been supported by studies using skinned fibers, which demonstrate an increased myofilament sensitivity to Ca^{2+} due to phosphorylation (Persechini et al., 1985; Stephenson and Stephenson, 1993). Persechini and coworkers (1985) examined the effect of R-LC phosphorylation on isometric force in skinned rabbit skeletal muscle fibers at low (0.6 μM) and high (10 μM) Ca^{2+} concentrations. At high Ca^{2+} concentrations, force was maximal and phosphorylation had no effect, whereas at low Ca^{2+} concentrations phosphorylation (induced by addition of MLCK and calmodulin) resulted in a 50% increase in isometric force. Similar results showing an increased force response due to R-LC phosphorylation have been reported using an endogenous MLCK phosphorylating system (Stephenson and Stephenson, 1993).

In another study, Sweeney and Stull (1986) examined the effect of R-LC phosphorylation over a wide range of Ca^{2+} concentrations in permeabilized rabbit skeletal muscle fibers. A leftward shift in the pCa-tension relationship was observed and this was associated with a decrease in the slope of the pCa-tension relationship. This observation is consistent with the idea that although R-LC phosphorylation does not enhance isometric force at saturating levels of Ca^{2+}, it does affect actin-myosin interactions at submaximal Ca^{2+} levels. Together, these data suggest that R-LC phosphorylation elicits potentiation of isometric twitch contractions due to an increased Ca^{2+} sensitivity, and the specific mechanism may be related to alterations in the crossbridge kinetics which will be discussed next.

Metzger and Moss (1992) using protein extraction techniques demonstrated that

removal of the R-LC resulted in an increased Ca^{2+} sensitivity of the myofilaments similar to that observed when the R-LC are phosphorylated. Partial extraction of the R-LC, at submaximal Ca^{2+} concentrations, resulted in an enhanced rate of force redevelopment in skinned skeletal muscle fibers and reconstitution of the R-LC fully restored the original sensitivity to Ca^{2+} (Metzger and Moss, 1992). Therefore R-LC may act in an inhibitory fashion, limiting the availability of myosin crossbridges for binding to actin, and subsequently, phosphorylation of the R-LC may act to remove this inhibition. Metzger and Moss (1992) further speculated that R-LC represses weak to strong transitions of crossbridges and that this repression can be eliminated by extracting the R-LC.

Recent experimental evidence suggests that R-LC phosphorylation manifests its potentiating effect on isometric force production by increasing the rate at which crossbridges enter the force-producing state. Sweeney and Stull (1990) utilized a two-state crossbridge model (Brenner, 1988) to examine the effect of R-LC phosphorylation on isometric ATPase activity and crossbridge properties. In this model, the transition from non-force-generating states to force-generating states is represented by the rate constant, f_{app}, whereas the reverse rate constant is g_{app}. To determine if the enhanced force development due to phosphorylation is a result of an increase in the number of cycling crossbridges or by an increase in the fraction of crossbridges in the force-generating state, measurements of stiffness, isometric force, rate of force redevelopment (following shortening and restretch) and ATPase activity were made over a range of submaximal Ca^{2+} concentrations, with both phosphorylated and unphosphorylated R-LC.

Increases in force were proportional to increases in fiber stiffness, during both

phosphorylated and unphosphorylated conditions. Further, increments in force were also proportional to increases in ATPase activity under both conditions. In other words R-LC phosphorylation did not change the ratio between these variables. For example, if R-LC phosphorylation had resulted in an increased force for any given level of stiffness (shifting the stiffness-force relationship to the right) this would suggest that phosphorylation executes its potentiating effects via a greater force per crossbridge. The observation that the relationship between ATPase activity and force was not changed in the phosphorylated condition, suggests that the rate at which the crossbridges leave the force-generating state (g_{app}) is also unchanged as a result of R-LC phosphorylation.

These constant ratios suggest that g_{app} is constant over all levels of Ca^{2+} activation whether or not myosin is phosphorylated and thus increments in the rate of force redevelopment (K_{redev}) must be due to an increase in f_{app} (since $K_{redev} = f_{app} + g_{app}$) (Sweeney and Stull, 1990). In this case, an increase in f_{app} would result in the crossbridges spending a greater fraction of time (during the whole cycle) in the force-generating state. In conclusion, at low Ca^{2+} concentrations the mechanism by which R-LC phosphorylation may produce twitch potentiation is by increasing the probability of crossbridges entering the force-producing state.

The culmination of the above studies provides convincing evidence to support the hypothesis that R-LC phosphorylation has a primary role in twitch potentiation.

Disuse Atrophy

Muscle atrophy results from inactivity which can be induced by various methods in order that contractile, biochemical or histochemical changes can be evaluated. Hindlimb suspension, denervation, limb immobilization, tenotomy and tetrodotoxin application to the motor nerve are only a few of the interventions that have been used extensively. The contractile changes in the muscle are a result of the absence of activity, or are due to the loss of some neurotrophic influence (Davis, 1985).

To elicit disuse atrophy in the present studies both TTX and a spinal hemisection were used. Application of the sodium channel blocker, TTX, allows for the maintenance of the neurotrophic influence while the muscle activation is totally or almost completely impaired (Lavoie et al., 1977; Lapointe and Gardiner, 1984; St-Pierre and Gardiner, 1985). TTX completely blocks motoneuron action potential propagation while not interfering with fast axoplasmic transport (Lavoie et al., 1977). Hemisection of the spinal cord at the level of the twelfth thoracic vertebra in the rat completely impairs voluntary motor function of the hindlimb. Some minimal activation of the muscle may occur as a result of intact spinal reflexes. The successful execution of both interventions results in significant atrophy of the affected muscle groups.

Many studies have reported that TTX-blockade results in significant atrophy of skeletal muscle and also produces some histochemical changes. TTX-disuse has resulted in a reduction in gastrocnemius muscle weight of approximately 40-50% compared to sham-treated animals (Gardiner et al., 1992; St-Pierre et al., 1987). While this weight loss

was accompanied by a decrease in fiber cross-sectional area of approximately 68% for FT fibers and 48% for ST fibers in one study (Gardiner et al., 1992), in similar research this weight loss was associated with no preferential atrophy of the FT fibers and a reduction of ~ 59% in both FT and ST fibers (St-Pierre and Gardiner, 1985).

Many skeletal muscle contractile changes have been demonstrated following TTX-induced atrophy. In terms of tension generating capacity, TTX studies have demonstrated that absolute tetanic tension is lower in atrophied rat gastrocnemius muscle (Gardiner et al., 1992; Lapointe and Gardiner, 1984; St-Pierre et al., 1987). This can be explained in part by decreases in muscle fiber size (Gardiner et al., 1992). A decrease in the cross-sectional area of myofibrils could lead to a reduction in the number of available crossbridges during maximal activation and would explain the attenuated tetanic DT observed in atrophied muscles.

Conversely, studies have consistently reported no change in absolute twitch tension of TTX-atrophied versus control muscles (Gardiner et al., 1992; Lapointe and Gardiner, 1984; MacIntosh et al., 1988; St-Pierre and Gardiner, 1985). The observed decrease in tetanic DT with no change in twitch DT in atrophied skeletal muscle results in an increased twitch:tetanic contraction ratio which has been confirmed by several investigators (Gardiner et al., 1992; Lapointe and Gardiner, 1984; St-Pierre and Gardiner, 1985).

In the rat gastrocnemius muscle the twitch amplitude is preserved following disuse; however, this is accomplished by a prolongation of the C_t and an increase in $\frac{1}{2}R_t$, while $dP \cdot dt^{-1}_{max}$ remains relatively unchanged (Lapointe and Gardiner, 1984; St-Pierre and

Gardiner, 1985). These measurements of twitch kinetics suggest that twitch magnitude is maintained in disuse atrophied muscle by a lengthening of activation time rather than by an increased intensity of activation and this would be consistent with an enhanced Ca^{2+} release and/or slowed reuptake of Ca^{2+}.

Engel and Stonnington (1974) reported that the proportion of SR compared to other myoplasmic protein in rat muscle fibers increases in disuse atrophy. It has also been suggested that atrophied muscles have an increased Ca^{2+} release per activating pulse relative to the quantity of contractile protein (Finol et al., 1981). An enhanced SR Ca^{2+} release (per gram of muscle) would explain the observations that the twitch amplitude is preserved and that the twitch:tetanus ratio is elevated in atrophied muscles.

Diminished Potentiation in Atrophied Muscle

An important observation for the present study is the marked attenuation of the staircase response in skeletal muscle following TTX-induced atrophy (MacIntosh et al., 1988; St-Pierre and Gardiner, 1985), and the depression of PTP in atrophied muscle following denervation (Dulhunty, 1985; Matsushita et al., 1988). Presently it is not clear why twitch potentiation is attenuated in atrophied muscle.

MacIntosh and coworkers (1988) demonstrated an absence of staircase potentiation in atrophied muscles. It has been proposed that the attenuation of the staircase response may be due to the disproportionate amount of Ca^{2+} released in the initial twitch compared to successive twitches (MacIntosh et al., 1988). If this were true, in atrophied muscle the

initial twitch would produce activation of a greater proportion of crossbridges compared to non-atrophied muscle. As a result, in subsequent twitches hypothetically there would be less additional force development since the first twitch already produced a greater degree of Ca^{2+} saturation in the atrophied muscle.

However, if the Ca^{2+} release is less with each pulse of repetitive stimulation then one would expect that the twitch kinetics would reflect this change. It has been suggested that prolongation of C_t is associated with an increased intracellular free [Ca^{2+}] (Sandow and Brust, 1966). If this hypothesis were true, then since C_t is increased during repetitive stimulation in atrophied muscles (MacIntosh et al., 1988), this suggests that decreased Ca^{2+} availability is not responsible for attenuated potentiation. This theory is also supported by the observation that $½R_t$ is also prolonged in atrophied muscles during repetitive stimulation (MacIntosh et al., 1988). An increase in $½R_t$ suggests that there is an accumulation of Ca^{2+} in the sarcoplasm rather than a depletion.

Matsushita and coworkers (1988) demonstrated that PTP was virtually abolished in rat EDL muscle fibers (35°C) 7 days after denervation. This depressed PTP was associated with a proportional decrease in the extent of R-LC phosphorylation. The strong correlation (r=0.96) between the rates of reduction for PTP and R-LC phosphorylation provides some evidence that these two events may be causally linked.

Summary

As stated earlier there are two main theories explaining the mechanism for twitch potentiation: i) a greater concentration of Ca^{2+} in the sarcoplasm, or ii) an increased Ca^{2+} sensitivity of the myofilaments via myosin light chain phosphorylation. This research project focussed on theory (ii) and attempts to elucidate the role of R-LC phosphorylation in twitch potentiation, in both staircase and posttetanic potentiation, by using a disuse atrophy model. Since it has been established that potentiation is significantly reduced in atrophied muscle, then the next logical step is to determine if this event is accompanied by attenuated R-LC phosphorylation. This was the main purpose of this research project.

HYPOTHESES

Since it has been postulated that both staircase and posttetanic potentiation occur via the same mechanism, the hypotheses tested in this dissertation were:

Hypothesis #1: *It is expected that staircase potentiation will be absent in TTX-disuse atrophied muscle and this observation will be accompanied by an absence of R-LC phosphorylation.*

Hypothesis #2: *It is hypothesized that posttetanic potentiation will be depressed in muscles atrophied via a spinal hemisection and this event will be associated with an attenuation of R-LC phosphorylation.*

Chapter 3

The Absence of Myosin Light Chain Phosphorylation and Staircase Potentiation in Atrophied Muscle

INTRODUCTION

Repetitive activation of fast-twitch skeletal muscle normally results in a progressive but transient increase in twitch developed tension referred to as staircase potentiation. There are currently two theories which attempt to explain this potentiation in non-atrophied skeletal muscle: i) phosphorylation of the regulatory light chains (R-LC) of myosin (Klug et al., 1982), and ii) increased availability of intracellular calcium (Ca^{2+}) during activation in the potentiated state (Close, 1972). R-LC phosphorylation is thought to produce twitch potentiation by increasing the tension development for any given submaximal intracellular Ca^{2+} level (Persechini et al., 1985). More specifically, it has been proposed that phosphorylation of R-LC increases the probability that crossbridges will enter the force-producing state, resulting in a higher proportion of active crossbridges at any given time during the twitch (Sweeney and Stull, 1990).

Studies examining changes in contractile properties associated with potentiation have provided evidence to support theory (i). MacIntosh and coworkers (1988) demonstrated that, in non-atrophied rat gastrocnemius muscle, staircase potentiation during 10 Hz stimulation was associated with an increase in rate of force development with no prolongation of contraction time. Alternatively, caffeine-induced potentiation, resulting from an enhanced sarcoplasmic reticulum Ca^{2+} release, is associated with a prolonged contraction time (MacIntosh and Gardiner, 1987). If modulation of intracellular Ca^{2+} were the primary mechanism for potentiation it would be expected that twitch contraction time would be lengthened. Measurement of R-LC phosphorylation during a brief period of repetitive stimulation confirms that there is a strong relationship between potentiation and R-LC phosphorylation (Klug et al., 1982; MacIntosh et al., 1993; Moore and Stull, 1984).

If R-LC phosphorylation is the primary mechanism for twitch potentiation, then it would be anticipated that R-LC phosphorylation would be reduced or absent when potentiation is severely attenuated. It has been reported that twitch potentiation is absent in tetrodotoxin (TTX)-induced disuse atrophy (MacIntosh et al., 1988; St-Pierre and Gardiner, 1985). Further, Matsushita and coworkers (1987) have demonstrated that there is a complete absence of the phosphorylated form of R-LC in rested muscle following denervation. Since staircase potentiation is absent in TTX-atrophied muscle, it would be of value to determine the corresponding levels of R-LC phosphorylation. Thus the present study addressed the question: Is R-LC phosphorylation absent in disuse atrophied muscle, under conditions when twitch potentiation is abolished? If absence of twitch potentiation

is associated with attenuated light chain phosphorylation, this would support the theory that R-LC phosphorylation is the primary mechanism for potentiation.

METHODS

Disuse Atrophy

Female Sprague-Dawley rats weighing approximately 200 grams were utilized for this study. Care and treatment of these animals was according to the Canadian Council on Animal Care and all procedures were approved by a committee for the ethical use of animals for research. The rats were randomly assigned to one of 5 groups: a) TTX-treated, stimulated (n=7); b) Sham-treated, stimulated (n=5); c) Control-untreated, stimulated (n=5); d) TTX-treated, rested (n=5); or e) Control-untreated, rested (n=4).

Disuse atrophy of the left hindlimb muscles was induced with the chronic application of TTX to the left sciatic nerve for 14 days. TTX is a sodium channel blocker, and therefore prevents motoneuron activation of the hindlimb muscles (Narahashi, 1974; St-Pierre and Gardiner, 1985). An osmotic pump (Alzet mini-osmotic pumps, model 2002) loaded with TTX (0.30 $mg \cdot ml^{-1}$) in a penicillin solution was surgically implanted subcutaneously in the upper back region of the anesthetized animals (St-Pierre and Gardiner, 1985). A Silastic cuff was placed around the left sciatic nerve and this cuff was connected to the pump via polyethylene tubing to deliver TTX to the nerve. The sham-treated group underwent the same surgery as the TTX group, but the pump was

loaded with sterile physiological saline only. After 14 days, the osmotic pumps were removed and contractile measurements were done 24 hours later.

Muscle Preparation

The rats were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 $mg \cdot kg^{-1}$). The left gastrocnemius muscle was isolated *in situ* (MacIntosh et al., 1988) to examine the isometric contractile properties. The gastrocnemius muscle was attached to a force transducer (Grass FT.10) via the Achilles tendon by a stainless steel wire. The soleus and plantaris muscles were cut at their insertion to the Achilles tendon to remove them from the preparation.

The sciatic nerve was cleared of connective tissue and severed close to the spine. The distal stump of the severed nerve was placed across a pair of stainless steel electrodes for indirect stimulation of the gastrocnemius muscle. Stimulation (Grass Model S88) was obtained with supramaximal (1-10 V) square pulses, 50 μs in duration. The loosened skin from the hindlimb was stretched and anchored to form a pocket for warmed mineral oil. Rectal and mineral oil temperatures were monitored and regulated at approximately 37°C throughout the experiment using radiant heat.

Contractile Properties

Initially the optimal muscle length (L_o) was determined using double pulse

stimulation with a 5 ms delay. Subsequent to setting the muscle at L_o, four twitch contractions were elicited at 20 s intervals to ensure consistent twitch developed tension (DT) in the gastrocnemius muscle. Subsequently, contractile responses were recorded during 10 s of 10 Hz stimulation. Immediately following this repetitive stimulation, the muscle was rapidly freeze-clamped with pre-chilled tongs and placed in liquid nitrogen. For the "rested" groups the muscle was freeze-clamped after a period of quiescence lasting at least five minutes to obtain resting values for R-LC phosphorylation. A microcomputer was used to monitor the output from the force transducer and the contractions were displayed visually throughout the experimental procedures. The contractions were sampled at 0, 5 and 10 s during the repetitive stimulation. The twitch contractions were analyzed for developed tension (DT), contraction time (C_t), average rate of tension development ($DT \cdot C_t^{-1}$) calculated as developed tension divided by contraction time, half-relaxation time ($\frac{1}{2}R_t$), and peak rate of relaxation ($dP \cdot dt^{-1}_{min}$).

Biochemical Analysis

The frozen muscle samples were analyzed to determine the proportion of phosphorylated P-light chains. The procedure utilized was that described by Silver and Stull (1982) and modified by Moore and Stull (1984) and Stuart et al. (1987). The muscle was pulverized and then homogenized at 0°C with a Polytron. The homogenate was centrifuged (5°C) and the supernatant was loaded onto a pyrophosphate polyacrylamide tubular gel and electrophoresed at 90 V for 3 hours at 5°C to isolate the

myosin. The tubular gels were stained with 0.15% Coomassie Blue (R-250, Sigma), 50% methanol, 10% acetic acid solution to identify the myosin band which was then excised.

Subsequently the isolated myosin was denatured and loaded onto an isoelectric focussing (IEF) polyacrylamide gel with a pH gradient of 4.5 to 6 to separate the phosphorylated from the nonphosphorylated form of R-LC. Electrophoresis was carried out at a constant power of 5 W and a temperature of 5^0C for 3 hours. Silver staining was used to distinguish the 4 bands representing the light chains. The stained protein bands corresponding to the phosphorylated and nonphosphorylated forms of R-LC were quantified using a laser densitometer (LKB 2202 Ultroscan). The densities of the two bands identified as phosphorylated and nonphosphorylated R-LC were used to calculate the proportion of phosphorylated R-LC: moles of phosphorylated R-LC per mole of total R-LC = density of phosphorylated R-LC band/density of phosphorylated R-LC band + density of nonphosphorylated R-LC band.

Statistical Analyses

A two-way analysis of variance (group, time) with repeated measures (time) was used to determine differences in each of the contractile variables (DT, C_t, $DT \cdot C_t^{-1}$, $\frac{1}{2}R_t$, and $dP \cdot dt^{-1}_{min}$) in all of the stimulated groups. For the R-LC phosphorylation values, because of the unbalanced groups (no resting values for the sham-treated group) a one-way ANOVA was used to detect differences between groups (control, TTX-, and sham-treated). Also, a one-way ANOVA was performed to determine differences between

conditions (rested or stimulated) for the control and for the TTX-treated group. The Student Newman-Keuls test was utilized for post-hoc analysis. A significance level of $p<0.05$ was used in all analyses.

RESULTS

Developed Tension

As shown in Figure 4 twitch DT progressively increased for both the control and sham-treated groups during 10 s of 10 Hz stimulation, demonstrating a classic positive staircase response. A significant group by time interaction was observed for developed tension. The values for DT at 5 and 10 s, for the control and sham-treated groups, were significantly higher than the DT for first pulse of stimulation at 0 s. In contrast to the non-atrophied groups, the DT for the TTX-treated group did not rise above the DT of the initial contraction at 0 s. The DT was significantly lower for the TTX-treated group compared to the other groups at 5 and 10 s of 10 Hz stimulation. Note: in atrophied muscle a single twitch contraction may already be "potentiated" compared to non-atrophied muscles, thus the potential for further relative potentiation is much less in these muscles.

Contraction Time and Average Rate of Tension Development

The changes in contraction time with repetitive stimulation at 10 Hz are shown in Figure 5. There was no significant group by time interaction, and no time effect, for C_t. However a significant group effect was observed. The TTX-treated group had a significantly longer C_t than the control group.

There was a significant group by time interaction for average rate of tension development. The $DT \cdot C_t^{-1}$ increased during low frequency repetitive stimulation for the control and sham-treated groups, but decreased for the TTX-treated group. From 0 to 5 s of stimulation the non-atrophied muscles demonstrated a marked increase in $DT \cdot C_t^{-1}$, and there was no further change from 5 to 10 s. The $DT \cdot C_t^{-1}$ at 5 and 10 s of repetitive stimulation in the TTX-treated muscles did not change significantly from the 0 s value. The TTX-treated group demonstrated a significantly lower average rate of tension development at all time points compared to the control group, and at 5 and 10 s compared to the sham-treated group (Figure 6).

Half-Relaxation Time and Peak Rate of Relaxation

A significant group by time interaction was observed for $\frac{1}{2}R_t$. Figure 7 demonstrates that $\frac{1}{2}R_t$ increased progressively from 0 to 10 s of stimulation for the TTX-treated group, whereas it did not change for the two non-atrophied groups. Half-relaxation time was significantly greater for the TTX-treated group at 5 and 10 s of 10

Hz stimulation compared to the other groups.

A significant group by time interaction was observed for $dP \cdot dt^{-1}_{min}$. The $dP \cdot dt^{-1}_{min}$ remained relatively constant for the TTX-treated group during repetitive stimulation, whereas this rate increased greatly from 0 to 5 s of 10 Hz stimulation and then stabilized for the other two groups. Figure 8 shows peak rate of relaxation was significantly lower for the TTX-treated group at 5 and 10 s of 10 Hz stimulation compared to other groups.

R-LC Phosphorylation

The degree of light chain phosphorylation was assessed in muscle which was frozen at rest or immediately following 10 s of 10 Hz stimulation, since this point is thought to correspond with peak twitch potentiation. The first observation from the biochemical analysis of the muscles was the presence of the phosphorylated form of the light chains in atrophied muscle. The band representing the phosphorylated R-LC was identified by the downward shift of its isoelectric point to a more acidic region (Silver and Stull, 1982). As anticipated, the control muscles demonstrated a significantly elevated phosphorylation level at 10 s of 10 Hz stimulation compared to rested conditions. In contrast the TTX-treated muscles demonstrated a relatively constant level of phosphorylation under both rested and stimulated conditions. R-LC phosphorylation was significantly diminished in TTX-atrophied muscle compared to both the control and sham-treated muscles which were not different (Table 1).

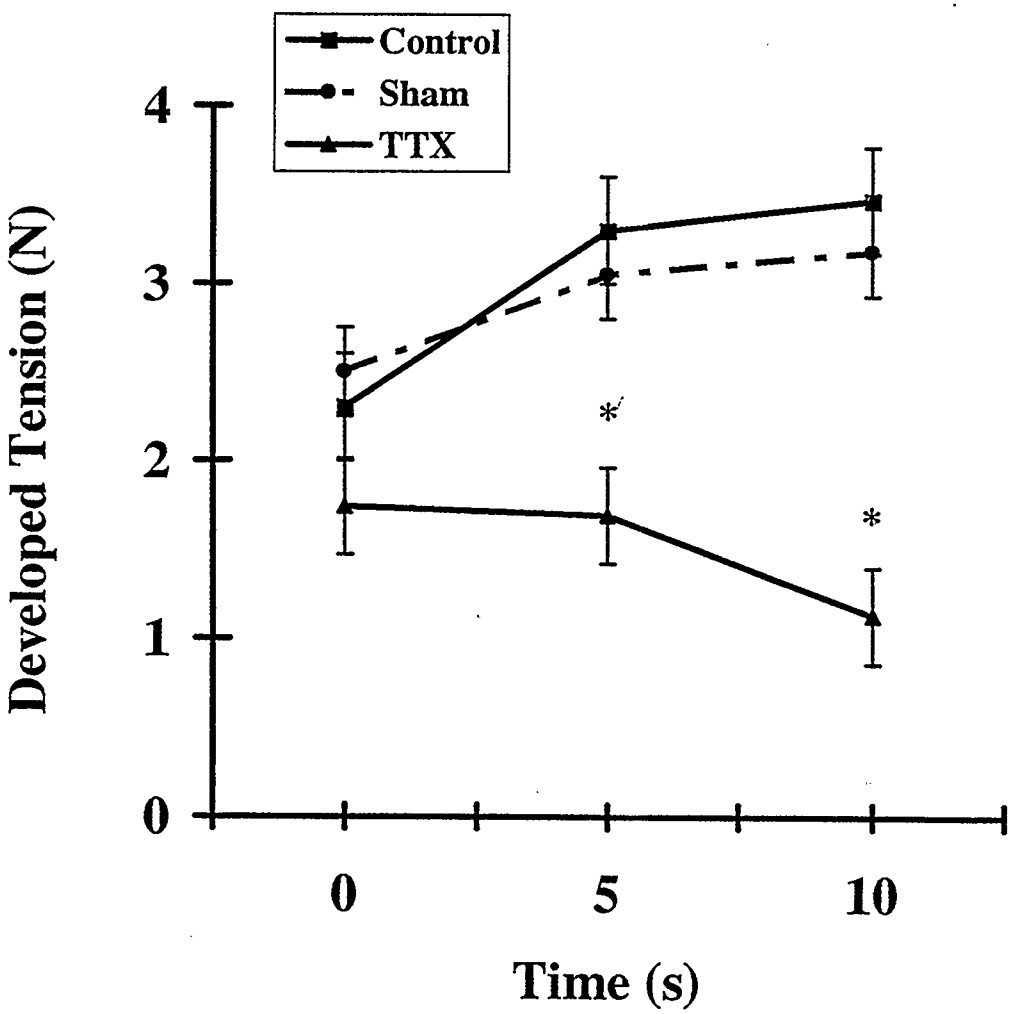

Figure 4. Mean twitch developed tension versus time during 10 Hz stimulation for control, sham- and TTX-treated groups. Asterisks represent significant differences between the TTX-treated group and the other groups at 5 and 10 s of repetitive stimulation. Vertical bars represent ± SE.

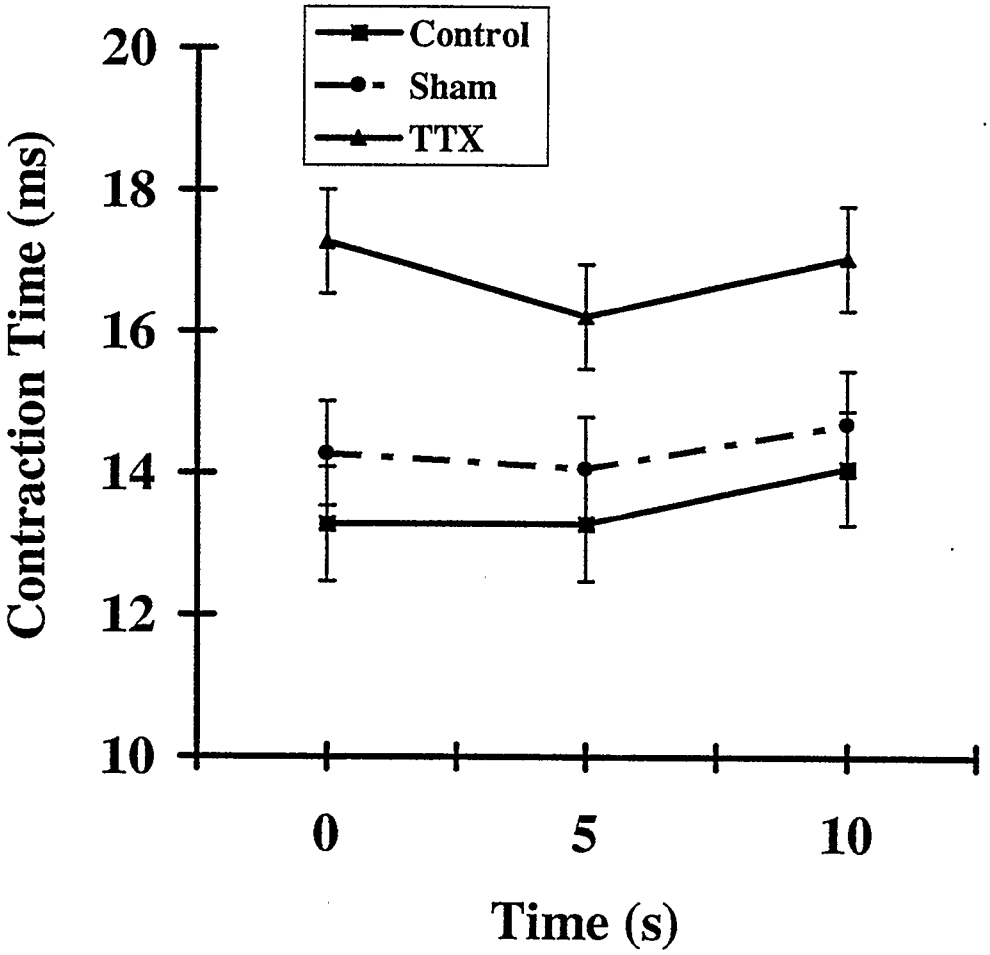

Figure 5. Mean twitch contraction time versus time during 10 Hz stimulation for control, sham- and TTX-treated groups. A significant group effect was observed. The TTX-treated group had a significantly prolonged C_t compared to the other groups. Vertical bars represent $\pm$ SE.

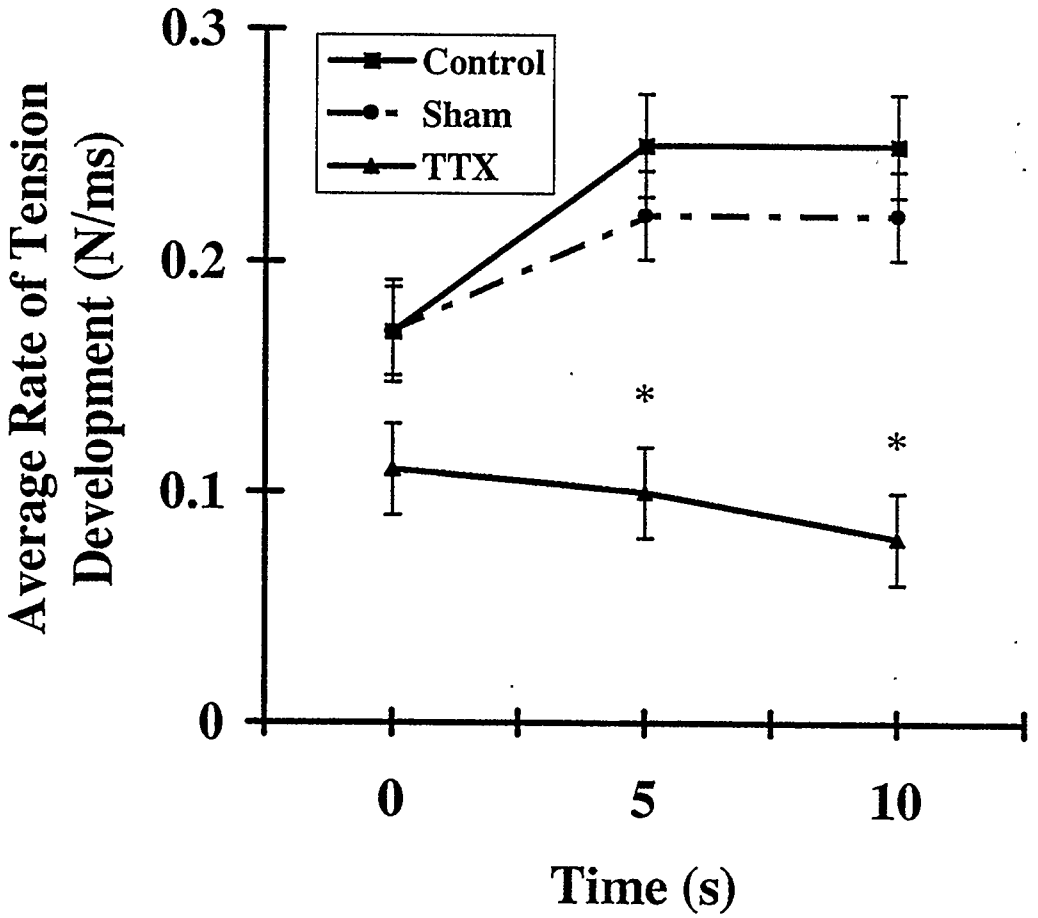

Figure 6. The average rate of force development (mean values) for twitches sampled at 0, 5 and 10 s of 10 Hz stimulation in three groups. Asterisks represent significant differences between the TTX-treated group and the other two groups at 5 and 10 s of repetitive stimulation. Vertical bars represent $\pm$ SE.

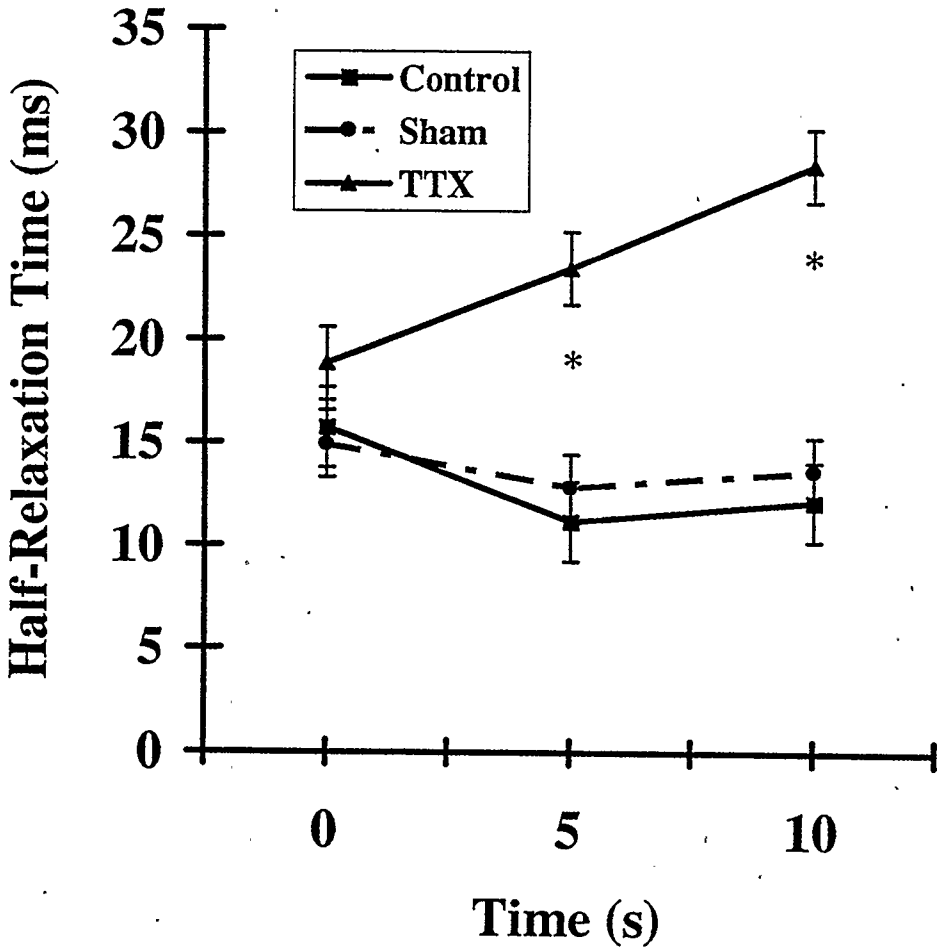

Figure 7. Mean values for half-relaxation time for twitches sampled at 0, 5 and 10 s of 10 Hz stimulation. Asterisks represent significant differences between the TTX-treated group and the other two groups at 5 and 10 s of repetitive stimulation. Vertical bars represent $\pm$ SE.

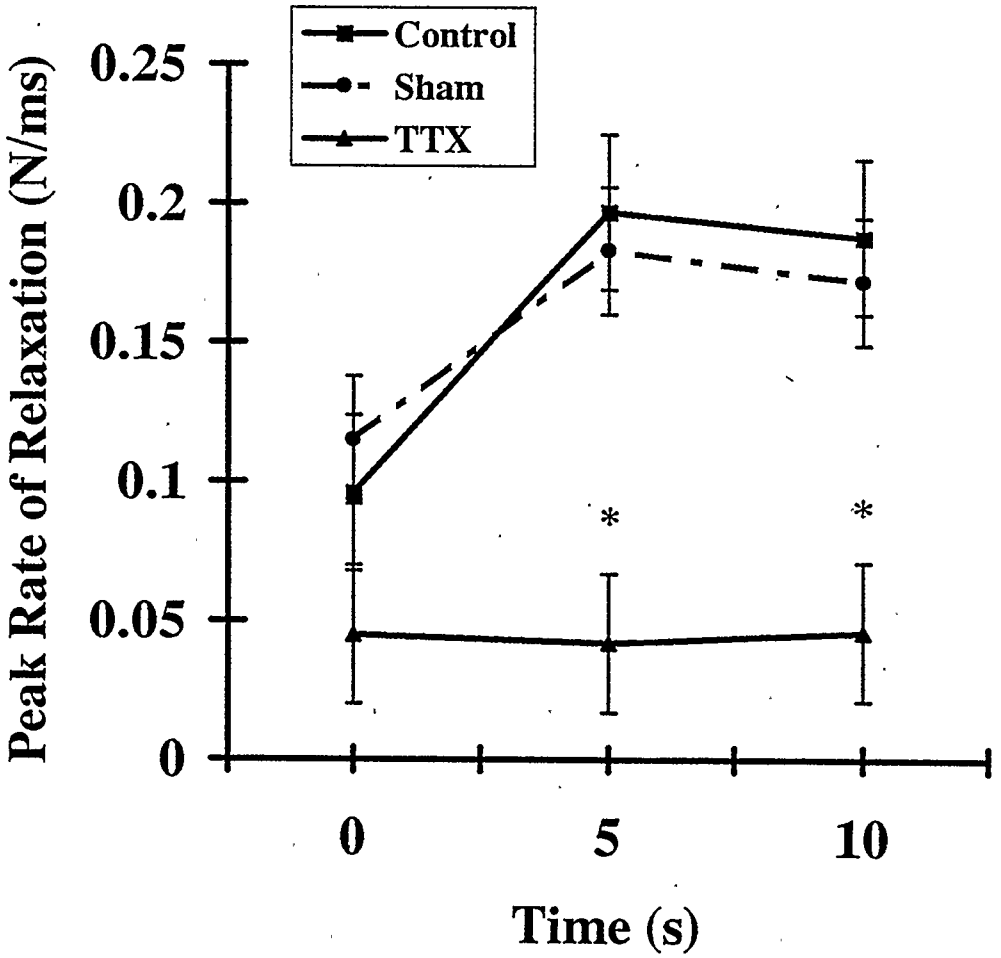

Figure 8. Mean values for peak rate of relaxation for twitches sampled at 0, 5 and 10 s of 10 Hz stimulation. Asterisks represent significant differences between the TTX-treated group and the other two groups at 5 and 10 s of repetitive stimulation. Vertical bars represent $\pm$ SE.

Table 1. R-LC phosphorylation values (moles of phosphate/mole of R-LC) for 3 groups (control, sham-treated and TTX-treated) and for two conditions (rested and stimulated).

	Rested	n	Stimulated	n
Control	0.21 ± 0.04	4	0.57 ± 0.03 *	5
Sham-Treated	-	-	0.46 ± 0.06	5
TTX-Treated	0.05 ± 0.03 #	5	0.09 ± 0.03 #	7

* Significantly different from rested condition for the control group ($p<0.05$).
# Significantly different from the corresponding control and sham-treated group values ($p<0.05$).

DISCUSSION

The major finding in the present study was that the absence of twitch potentiation in atrophied skeletal muscle is accompanied by little or no myosin light chain phosphorylation. The observation that staircase potentiation is absent in atrophied muscle supports previous studies (MacIntosh et al., 1988; St-Pierre and Gardiner, 1985). The correspondingly low levels of R-LC phosphorylation during repetitive stimulation in atrophied muscle have not been reported previously, and this observation is consistent with the hypothesis that R-LC phosphorylation is the primary mechanism for twitch potentiation.

In the present study, the control and sham-treated groups demonstrated a progressive increase in twitch DT with 10 Hz stimulation from 0 to 10 s. In contrast, this classic staircase response was not seen in the TTX-treated group. The DT measured at 5 and 10 s of repetitive stimulation did not rise above the value observed at 0 s in the atrophied muscles. During repetitive stimulation of disuse atrophied muscle, the changes in the contractile properties of the twitch were not the same as for the non-atrophied muscle. The most apparent contractile changes due to TTX-induced disuse were a significantly increased twitch contraction time even for a single twitch, and prolongation of half-relaxation time during repetitive stimulation. This prolonged twitch time course is consistent with previous studies examining contractile changes in atrophied muscle (Gardiner et al., 1992; MacIntosh et al., 1988; St-Pierre and Gardiner, 1985).

It is expected that a prolonged C_t, with no change in the rate of force development,

would be associated with an enhanced DT. Considering that C_t is prolonged in the atrophied muscle even before repetitive stimulation suggests that a single twitch of atrophied muscle represents an enhanced twitch. This is further confirmed by the large twitch:double pulse contraction ratio (MacIntosh et al., 1988) and the large twitch:tetanus ratio (St-Pierre and Gardiner, 1985) observed in atrophied muscle. The fact that $½R_t$ was prolonged during repetitive stimulation suggests that either crossbridge kinetics are slowed or Ca^{2+} uptake is progressively attenuated. Either of these processes would be expected to enhance not diminish twitch DT. Therefore, it seems reasonable to postulate that coincident with these changes which prolong the twitch, some other factor(s) counteract the tendency for an enhanced twitch, possibly early fatigue.

The enhanced DT observed during staircase potentiation in non-atrophied muscle has been reported to be associated with an increased peak rate of force development rather than an increased C_t (Krarup, 1981a; MacIntosh, 1991). The results of the present study are consistent with this observation. In contrast, it was found that repetitive stimulation of atrophied muscle resulted in no twitch potentiation, and this corresponded with no change in average rate of force development. These observations are consistent with the notion that R-LC phosphorylation may be responsible for potentiation by enhancing the force development at a submaximal $[Ca^{2+}]$ in the myoplasm (Grange et al., 1993). R-LC phosphorylation has been shown to result in an increase in peak rate of tension development during tension redevelopment over a range of Ca^{2+} concentrations in permeable skeletal muscle fibers (Metzger et al., 1989). The absence of an increase in the rate of force development observed in atrophied muscle may be due to the absence

of R-LC phosphorylation.

The increase in phosphorylation levels observed for control and sham-treated muscles following 10 s of repetitive stimulation in the present study, are comparable to previous studies using the rat gastrocnemius muscle (MacIntosh et al., 1993; Moore and Stull, 1984). In contrast, atrophied muscle was observed to have only 0.09 moles of phosphate/mole of R-LC after 10 Hz stimulation and in several muscles the phosphorylated band was completely absent. The conservation of the light chains and attenuation of phosphorylation in rested atrophied muscle has been previously demonstrated by Matsushita and coworkers (1987) who examined the effect of denervation on the myosin light chains in rested rat extensor digitorum longus (EDL) muscle. Prior to denervation the mean resting phosphorylation level was 21%. Following 2 days of denervation the resting level of R-LC phosphorylation was half that of the control muscles, and after 3 days there was a total absence of R-LC phosphorylation in the rested muscle. The low level of R-LC phosphorylation (~ 9%) observed in the present study is even more notable since the muscles were evaluated following stimulation.

The observation that the light chains are present but not phosphorylated in atrophied muscle suggests that the process of phosphorylation is somehow impaired. The fact that some phosphorylation was observed in these atrophied muscles, in contrast with the study by Matsushita et al. (1987), suggests that an intact motor neuron, or in our case possibly contractile activity during the 24 hour recovery period, is required to preserve the mechanism of R-LC phosphorylation. Phosphorylation of the 18,500-dalton light chain is catalyzed by myosin light chain kinase (MLCK). In a non-atrophied muscle, the

activation of MLCK first requires the binding of calcium to calmodulin, and then the binding of the Ca^{2+}-calmodulin complex to the inactive form of MLCK to initiate the kinase activity (Klug et al., 1982). During repetitive stimulation, with each pulse of activation intracellular Ca^{2+} concentration rises and falls transiently. Thus the first pulse of stimulation would only activate a small fraction of the MLCK, but subsequent pulses would result in additional activation of the MLCK. As repetitive stimulation continues, and more MLCK is activated, the rate of R-LC phosphorylation will increase, and progressively more R-LC will be phosphorylated.

From this brief review of the known mechanism for R-LC phosphorylation, it is clear that impaired phosphorylation of R-LC in atrophied muscle could be due to any of several factors: i) low intracellular Ca^{2+} concentration, ii) reduced calmodulin, iii) impaired binding of the Ca^{2+}-calmodulin complex to MLCK, and, iv) decreased activity of MLCK. One or more of these factors may result from disuse atrophy.

There is some evidence that Ca^{2+} availability is not the limiting factor in the phosphorylation process of atrophied muscle. It has been hypothesized that the intracellular Ca^{2+} concentration during stimulation may be enhanced in atrophied skeletal muscle since there is an increase in the volume fraction of sarcoplasmic reticulum (SR) in these muscles (Freilich et al., 1995). Also, Zorzato and coworkers (1989) have demonstrated that following denervation there is an increase in the number of Ca^{2+} release channels of junctional SR. Further, it has been demonstrated that disuse atrophy results in an elevated twitch:tetanus ratio (St-Pierre and Gardiner, 1985). The observation that twitch amplitude is preserved while tetanic tension is diminished, may indicate that Ca^{2+}

availability (per activating pulse) is enhanced in atrophied muscles. These studies support the idea that reduced Ca^{2+} availability is not likely the basis for impaired myosin light chain phosphorylation in atrophied muscle. However, it is not known whether or not the apparently enhanced Ca^{2+} release is sustained with each activating pulse during repetitive stimulation.

In the present study the activity levels of the MLCK and the corresponding phosphatase were not measured, therefore, we can only speculate as to their potential role in the observed impairment of R-LC phosphorylation in atrophied muscles. An attenuation of R-LC phosphorylation may be due to either an impairment of the Ca^{2+}-calmodulin-MLCK system, or due to an accelerated dephosphorylation via the myosin light chain phosphatase. Some of the changes in fast-twitch fibers with disuse atrophy are indicative of a change from fast-twitch (FT) to slow-twitch (ST) contractile characteristics (ie. longer contraction time and slower relaxation). It has been observed that phosphatase activity is greater in slow-twitch muscle than in fast-twitch muscle (Moore and Stull, 1984). This observation gives credence to the possibility that absence or attenuation of R-LC phosphorylation could be due to increased phosphatase activity.

Alternatively, impairment of R-LC phosphorylation may be due to a reduction in MLCK activity since it has been demonstrated that this enzyme's action is influenced by contractile activity. Klug and coworkers (1986; 1992) showed that chronic stimulation of FT muscles resulted in a significant decrease in MLCK activity. Their hypothesis that this alteration occurs due to a fast-to-slow conversion in muscle fiber-type characteristics is consistent with the observation that MLCK activity is 3 times greater in FT versus ST

muscle (Moore and Stull, 1984). Thus the attenuated phosphorylation observed in the present study could be due to a depressed MLCK activity because TTX-disuse results in greater loss of FT versus ST fibers (68% versus 48%, respectively) in rat gastrocnemius muscle (Gardiner et al., 1992) and a slowing of speed-related twitch contractile properties (Gardiner et al., 1992; St-Pierre and Gardiner, 1985).

Regardless of the mechanism responsible for the evident impairment of myosin light chain phosphorylation in disuse atrophied muscle, the association between the loss of potentiation and corresponding absence of phosphorylation does provide indirect evidence supporting R-LC phosphorylation as the primary mechanism for potentiation. Alternatively, these results do not exclude other mechanisms such as enhanced Ca^{2+} availability playing a modulatory role in potentiation of twitch developed tension in non-atrophied muscle.

SUMMARY

Staircase potentiation is absent in atrophied rat gastrocnemius muscle. Since twitch potentiation has been strongly correlated with myosin light chain phosphorylation, our purpose was to determine if R-LC phosphorylation is also diminished in atrophied gastrocnemius muscle with repetitive stimulation. Following 2 weeks of disuse, induced by delivery of TTX to the sciatic nerve, phosphorylation and twitch developed tension were measured at 10 s of 10 Hz stimulation *in situ* in control, sham and TTX-treated rats. Phosphorylation was significantly diminished in TTX-treated animals (9.3 $\pm$ 3.7%),

compared to control (57.0 $\pm$ 3.4%) and sham-treated (45.5 $\pm$ 5.6%) animals. Concomitantly, at 10 s of 10 Hz stimulation potentiation was absent in atrophied muscles (74.4 $\pm$ 9.4% of initial twitch) while control (188.4 $\pm$ 10.3%) and sham-treated (151.7 $\pm$ 10.3%) muscles demonstrated a high degree of potentiation. The twitch contraction of the atrophied muscles had a decreased rate of tension development and peak rate of relaxation, and an increased contraction time and half-relaxation time. The observation that the light chains are present, but not phosphorylated, suggests that the process of phosphorylation is impaired. The association of diminished phosphorylation with the absence of staircase supports the theory that R-LC phosphorylation may be the principal mechanism for twitch potentiation, but does not rule out other contributing factors.

Chapter 4

The Quantification of Calmodulin in Disuse Atrophied Skeletal Muscle

INTRODUCTION

In skeletal muscle the 18,500 dalton regulatory light chains of myosin (R-LC) are phosphorylated by the Ca^{2+}-calmodulin dependent enzyme, myosin light chain kinase (MLCK). This phosphorylation process is initiated by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) from resting levels of ~ 0.2 μM to 10 μM or greater, as a result of Ca^{2+} release from the sarcoplasmic reticulum (SR) during muscle stimulation (Stull et al., 1985). This event allows Ca^{2+} to bind to calmodulin (CaM) and this Ca^{2+}-calmodulin complex, in turn, binds to MLCK converting this enzyme from an inactive to active form (Sweeney et al., 1993). The activated form of MLCK phosphorylates a specific serine residue in the amino-terminal portion of the R-LC (Sweeney et al., 1993). During relaxation Ca^{2+} is taken up into the SR, and the concentration of intracellular free Ca^{2+}

decreases resulting in the dissociation of Ca^{2+} from the Ca^{2+}-calmodulin-MLCK complex. This in turn, causes inactivation of the kinase and a decreased rate of R-LC phosphorylation (Burger et al., 1983). Dephosphorylation of the R-LC occurs via a protein phosphatase, the activity of which does not appear to be regulated (Morgan et al., 1976). The extent of R-LC phosphorylation is dependent upon the relative activities of these two enzymes, MLCK and the phosphatase, and therefore regulation of R-LC phosphorylation is dependent on the Ca^{2+}-CaM system.

Contrary to observations with non-atrophied skeletal muscle, R-LC phosphorylation is virtually absent in atrophied muscles following 10 s of 10 Hz stimulation (Tubman et al., 1996). This may be the main reason for an absence of staircase potentiation demonstrated in atrophied skeletal muscle (MacIntosh et al., 1988; St-Pierre and Gardiner, 1985), since there is a strong correlation between R-LC phosphorylation and twitch potentiation (Houston et al., 1985; Klug et al., 1982; Manning and Stull, 1979; Manning and Stull, 1982; Moore and Stull, 1984). However, it is not clear what mechanisms underlie the impairment of R-LC phosphorylation in atrophied muscle. The possible sites of impairment in the phosphorylation process of the regulatory light chains include: i) low intracellular free Ca^{2+} concentration, ii) reduced calmodulin content, iii) decreased activity of MLCK, and iv) increased phosphatase activity.

Calmodulin has an essential role in the process of R-LC phosphorylation, since the Ca^{2+}- calmodulin complex is the primary regulator of MLCK (Sweeney et al., 1993). Calmodulin is a 16,800 dalton protein whose crystal structure shows a dumbbell-shaped molecule in which a central helix connects two lobes which are each composed of a pair

of Ca^{2+}-binding domains (Persechini et al., 1994). The activation of MLCK requires interaction with these two separate sites on CaM (Klee et al., 1980). In the absence of CaM, the CaM-binding site of MLCK, which is located at the extreme carboxyl terminus of the enzyme (Blumenthal et al., 1985), binds to the MLCK active site causing inhibition (Stull et al., 1990). Binding of the Ca^{2+}-CaM complex to the CaM-binding domain reverses this inhibitory state by removing this domain from the active site (Stull et al., 1990). This model of MLCK activation is the most widely accepted and is referred to as the autoinhibitor or pseudosubstrate hypothesis (Kennelly et al., 1989).

In vitro studies have demonstrated that the quantity of calmodulin relative to the kinase in skeletal muscle is important for the effectiveness of MLCK activation (Blumenthal and Stull, 1980). Calmodulin (CaM) appears to be effective in activating MLCK up to 80-90% when present in amounts equimolar with respect to MLCK (Nairn and Perry, 1979). In this study we tested the hypothesis that the quantity of calmodulin, relative to the protein concentration in the muscle extract, is diminished in atrophied muscle. This would result in the impairment of the Ca^{2+}-calmodulin-MLCK system and, subsequently, an attenuation of R-LC phosphorylation.

METHODS

Muscle Samples

Muscle samples analyzed in the present study were derived from the staircase

potentiation study previously described in Chapter 3. Rat gastrocnemius muscle from each of the treatment groups (control, n=9; sham-treated, n=7; and TTX-treated, n=11) were assessed for the quantity of CaM relative to the protein content of the muscle extract. These muscle samples were obtained from both the resting and stimulated (10 s of 10 Hz) conditions.

Gel Electrophoresis and Western Immunoblot

The frozen crude muscle samples were first pulverized in liquid nitrogen. The frozen muscle powder was then homogenized (Polytron) in a buffer consisting of 0.5 M Tris (Tris(hydroxymethyl) aminomethane)-HCl, pH 6.8, 1% sodium dodecylsulfate (SDS), 2% 2-mercaptoethanol, 0.01% bromophenol blue and 30% glycerol at a ratio of 10 ml SDS buffer:1 g of muscle tissue. The homogenate was transferred to Eppendorf tubes, and underwent heat-treatment (10 min) and centrifugation (15 min). The insoluble material was removed and the supernatant preserved. For each 100 µl sample (supernatant), 8 µl of EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) was added resulting in a final concentration of 10 mM EGTA. These samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Supernatants (108 µl) were loaded on SDS-PAGE gels to separate and concentrate the CaM. Also, four samples containing known concentrations of pure skeletal muscle CaM (1.3 $mg \cdot ml^{-1}$) were loaded on the gels (appropriate volumes were loaded to result in final concentrations of 0.5, 1.0, 2.0 and 3.0 µg of CaM). The resolving gel (15-20%

acrylamide gradient) consisted of 0.375 M Tris-HCl, pH 8.8, 0.1% SDS and a 1.3 - 2.6% glycerol gradient. The upper stacking gel was composed of 0.125 M Tris-HCl, 0.1% SDS and 5% acrylamide. Electrophoresis was carried out in a running buffer comprised of 25 mM trizma base, 192 mM glycine, and 0.1% SDS. Electrophoresis was conducted for 16 hours at 7-8 mA per gel.

Following resolution of the sample by gel electrophoresis, the proteins were transferred to nitrocellulose (0.1 μm) at 40 volts and 5°C for 14 hours in a potassium phosphate transfer buffer: 0.025 M $KHPO_4$, pH 7.0. Upon removal from the transfer-blot unit, the gels were fixed and stained with Coomassie blue for 4 hours and then destained for 4 hours to monitor the efficiency of transfer. The nitrocellulose was then placed in a tray containing blocking buffer: TBST (Tris-buffered saline; 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 with 0.05% Tween-20) and 10% Blotto. Membranes were blocked for 1 hour which efficiently blocked most nonspecific binding sites for immunoglobulins. Subsequently, the nitrocellulose was washed with TBST and TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). The specific monoclonal CaM antibody (Upstate Biotechnology Incorporated) was dissolved in TBST containing 1% bovine serum albumin (BSA). The membranes were then incubated in the presence of the primary antibody solution for 2 hours at room temperature on a rotating platform. After several washes with TBST/TBS, the nitrocellulose was incubated for 1 hour with a secondary antibody (anti-mouse IgG - Alkaline phosphatase conjugate, Sigma Immuno Chemicals A-5153) dissolved in a blocking solution. Following extensive washing with TBST followed by TBS, the immunoreactive bands were visualized using the alkaline phosphatase substrates NBT (p-

nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) in a buffer consisting of 100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 9.5.

The amount of CaM in each sample was determined by quantitative densitometry using a two-dimensional scanning system (Pharmacia LKB-ImageMaster) of Western blots using purified skeletal muscle CaM as the standard. The concentration of CaM was expressed relative to the protein content of the muscle homogenate (supernatant). The total protein content of each muscle sample was determined using a Bradford protein assay system (Bio-Rad) with BSA standards. Absorbance was measured at 595 nm. The amount of protein in each of the samples was calculated using their absorbance measurements in a linear regression equation derived from the standard curve.

Statistical Analyses

A one-way analysis of variance was used to detect differences in the relative quantity of CaM between groups. A significance level of $p<0.05$ was used.

RESULTS

Figure 9 shows a typical Western blot from the present study. A calibration curve was calculated from the densitometric scanning of lanes on each blot containing known amounts of purified CaM. The quantity of CaM present in the experimental muscle samples was interpolated from this calibration curve (or in some cases extrapolated). In

this figure, one example from each of the treatment groups: TTX-Treated (T5), Sham-Treated (S1) and Control (C5) is shown. The quantities of CaM for these individual samples were determined to be 2.70, 3.04 and 2.42 μg, respectively.

Figure 10 illustrates the protein assay standard curve for BSA. The protein concentration for each of the homogenized muscle samples was then calculated from their individual absorbance measurements. Subsequently the quantity of CaM in each sample, as determined from the Western blots, was presented relative to the total protein concentration of the homogenized muscle sample.

The relative quantity of CaM found in the homogenized muscle samples was the same in atrophied versus non-atrophied muscles. Table 2 illustrates the individual values for each group as well as some descriptive statistics. There was no difference in CaM concentration among the three treatment groups.

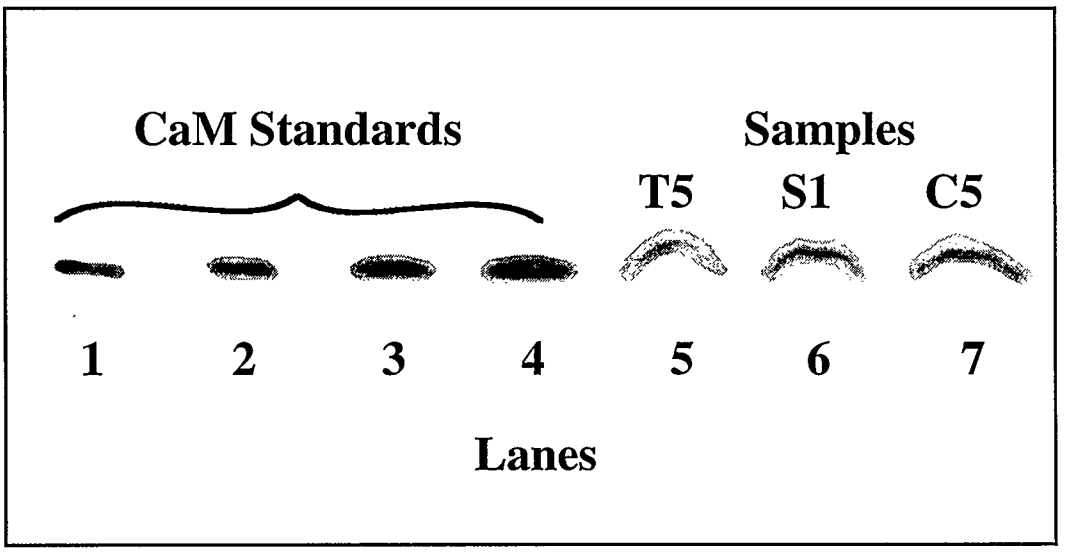

Figure. 9. Western immunoblot of skeletal muscle calmodulin, quantified using a two-dimensional laser densitometer scanning system (Pharmacia LKB ImageMaster). The first four lanes represent the calmodulin standards and the final three lanes represent the blots of the crude samples from rat gastrocnemius muscles. Lane 1, 0.5 μg CaM; Lane 2, 1.0 μg CaM; Lane 3, 2.0 μg CaM; Lane 4, 3.0 μg CaM; Lane 5, TTX-treated/atrophied muscle (sample #T5) with 2.70 μg CaM; Lane 6, sham-treated/non-atrophied muscle (sample #S1) with 3.04 μg CaM; Lane 7, control/non-atrophied muscle (#C5) with 2.42 μg CaM.

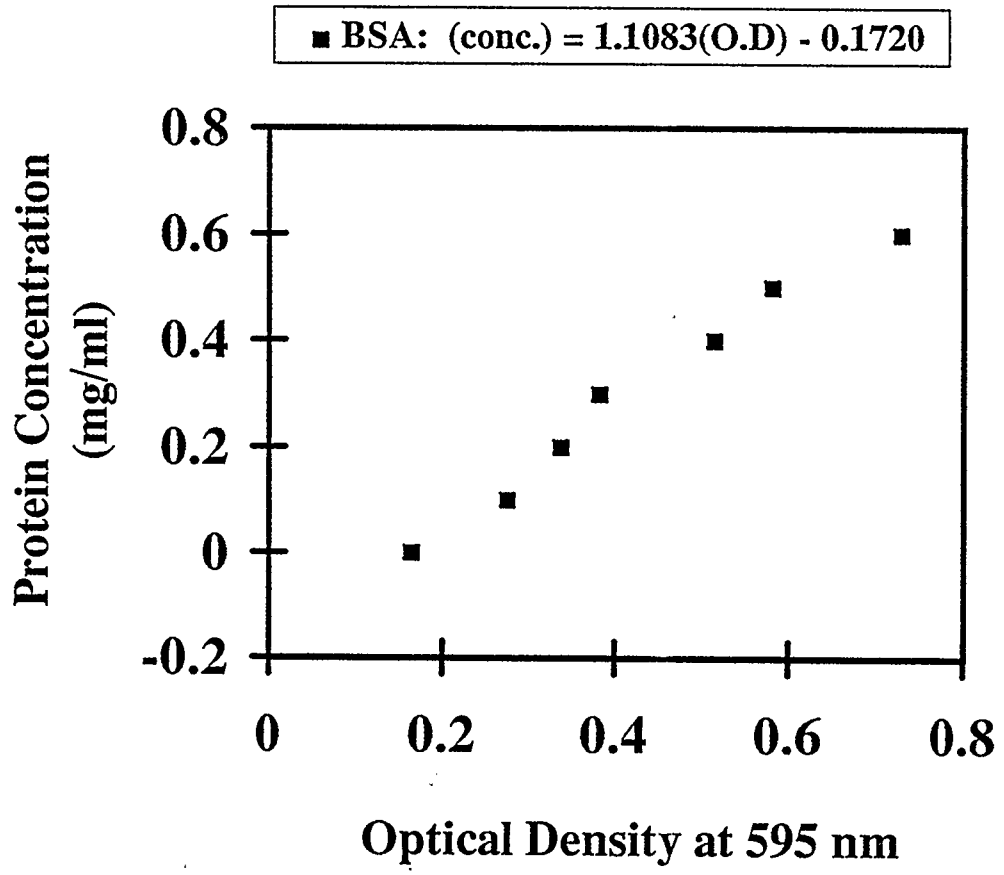

Figure. 10. The standard curve for the Bio-Rad protein assay procedure using bovine serum albumin (BSA: Standard II). Absorbance (optical density) was read at 595 nm. Linear regression was used to estimate the protein concentration of the muscle homogenate.

Table 2. The content of calmodulin for each individual rat gastrocnemius muscle, expressed in μg of CaM per mg of total protein ($\mu g \cdot mg^{-1}$). Mean values and SEM for the groups are also presented.

Group	**Control**	**Sham-Treated**	**TTX-Treated**
	0.27	**0.39**	**0.33**
	0.61	**0.50**	**0.34**
	0.30	**0.14**	**0.43**
	0.31	**0.36**	**0.29**
	0.24	**0.20**	**0.32**
	0.29	**0.14**	**0.27**
	0.13	**0.28**	**0.29**
	0.38		**0.44**
	0.14		**0.14**
			0.32
			0.40
Mean	**0.30**	**0.29**	**0.33**
SEM	**0.05**	**0.05**	**0.03**

DISCUSSION

In the introduction of this chapter, it was suggested that the impairment of R-LC phosphorylation in atrophied muscles could be due to a reduced CaM content. The major finding in the present study was that the relative calmodulin content in atrophied muscles is no different than in non-atrophied muscles and thus is not responsible for the observed impairment of myosin light chain phosphorylation.

Since these results rule out changes in the cellular content of CaM as being a major factor in the impairment of R-LC phosphorylation during repetitive stimulation (10 Hz for 10 s) in atrophied muscles, other possible explanations for this phenomenon need to be considered. These would include a low $[Ca^{2+}]_i$, a reduction in MLCK or an increased phosphatase activity. Evidence of an augmented volume fraction of sarcoplasmic reticulum (SR) (Engel and Stonnington, 1974), and an increased number (Zorzato et al., 1989) and mean open time (Delbono and Chu, 1995) of Ca^{2+}-release channels in the junctional SR of denervated muscles, supports the theory that Ca^{2+} availability is not the limiting factor in R-LC phosphorylation. In fact, in atrophied muscle an enhanced Ca^{2+} transient is probably attained during muscle stimulation. This is consistent with the increase in the twitch:tetanus ratio (Gardiner et al., 1992; St-Pierre et al., 1987) and twitch:double pulse contraction ratio (MacIntosh et al., 1988) in atrophied muscles when compared with non-atrophied muscles. The preservation of the twitch force in atrophied muscles concomitant with attenuation of the tetanic force suggests that Ca^{2+} availability during a single twitch contraction is not different than in

non-atrophied muscles.

Even with no difference in the content of CaM, it is possible that there is an impairment in the activation of the Ca^{2+}-calmodulin-MLCK system in atrophied muscles. Under normal circumstances, the maximal rate of R-LC phosphorylation exceeds the maximal rate of dephosphorylation by 60-fold in mammalian striated muscle (Stull et al., 1990). Therefore, considering the high activity level of MLCK in fast-twitch muscle (Moore and Stull, 1984), an alteration in the activity of this kinase could potentially result in a significant decrease in the amount of phosphate incorporated into the R-LC. On the other hand, a proportional increase in the phosphatase activity would not as significantly alter the final extent of R-LC phosphorylation.

It has been demonstrated that the activation of MLCK can be modulated under certain conditions. Klug and coworkers (1992) demonstrated that chronic stimulation of rabbit fast-twitch muscle produced a 36% decrement in MLCK activation, suggesting that this enzyme may be altered by changes in the activity pattern of the muscle. Thus it could be speculated that MLCK activity may be reduced with TTX-treatment, since this intervention also changes the activity pattern of the muscle. Conversely, in the same study (Klug et al., 1992), the phosphatase activity remained unchanged as a result of chronic stimulation suggesting that the phosphatase is not affected by changes in muscle activity. A decrease in the activation of MLCK may be due to a reduced quantity of MLCK in atrophied muscles rather than a limited Ca^{2+} availability.

The present study provides evidence that the CaM content is not changed as a result of TTX-induced atrophy, and supports the theory that CaM is not the cause of

impaired R-LC phosphorylation. Further research involving the quantification of MLCK, or measurement of intracellular free Ca^{2+} concentration, is required to determine the exact mechanism responsible for an attenuated R-LC phosphorylation in atrophied muscles.

Chapter 5

Attenuation of Myosin Light Chain Phosphorylation and Posttetanic Potentiation in Atrophied Skeletal Muscle

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INTRODUCTION

Marked twitch potentiation has been observed in rat fast-twitch muscle following tetanic stimulation (Krarup, 1981a; MacIntosh and Gardiner, 1987), and this has been referred to as posttetanic potentiation (PTP). The mechanism(s) for this apparent modulation of twitch amplitude has not been fully elucidated. The observation that PTP in rat fast-twitch muscle is achieved with an increased rate of force development without a significant change in contraction time (Desmedt and Hainaut, 1968; Krarup, 1981b; MacIntosh and Gardiner, 1987) suggests that potentiation results from a greater intensity of activation rather than a greater duration of activation.

It is possible that phosphorylation of the regulatory light chains (R-LC) of myosin may provide a greater intensity of activation during a posttetanic twitch. It has been

repeatedly demonstrated that PTP is strongly correlated with the extent of R-LC phosphorylation in fast-twitch skeletal muscle (Manning and Stull, 1979; Manning and Stull, 1982; Moore and Stull, 1984). This is consistent with the observation in skinned fibers that R-LC phosphorylation enhances force development for any given submaximal intracellular free Ca^{2+} concentration, via a leftward shift in the force-pCa relationship (Moore and Stull, 1984; Persechini et al., 1985; Sweeney and Stull, 1986). Furthermore, it has been proposed that R-LC phosphorylation increases the probability that crossbridges will enter the force-producing state, resulting in a higher proportion of active crossbridges at any given time during the twitch (Sweeney and Stull, 1990). This may occur due to a movement of the myosin crossbridges away from the thick filament (Sweeney et al., 1993).

Staircase potentiation is absent in tetrodotoxin (TTX)-induced disuse atrophy (MacIntosh et al., 1988; St-Pierre and Gardiner, 1985) and recent experimental data show that this is accompanied by an apparent attenuation of R-LC phosphorylation (Tubman et al., 1996). This observation is consistent with the theory that R-LC phosphorylation is the mechanism for staircase potentiation. At present it is known that PTP is depressed in atrophied muscle following denervation (Dulhunty, 1985; Matsushita et al., 1988) but the cause for this attenuation is not known. However if R-LC phosphorylation is the primary mechanism responsible for PTP, then the diminution of potentiation in atrophied muscle should be accompanied by proportional attenuation of R-LC phosphorylation.

R-LC phosphorylation has not yet been measured in rat gastrocnemius muscles paralyzed by spinal cord hemisection following a tetanic stimulation. Because tetanic

stimulation, in contrast to repetitive (10 Hz) stimulation, may provide a higher sustained intracellular Ca^{2+} concentration during activation, the consequences for PTP and R-LC phosphorylation may be quite different. It was the purpose in this study to determine if significant potentiation and/or R-LC phosphorylation occurs in atrophied muscle as a result of tetanic stimulation. If PTP is absent and is associated with an absence of R-LC phosphorylation, these results would indirectly provide further support for the theory that R-LC phosphorylation is the primary mechanism for PTP.

METHODS

Female Sprague-Dawley rats weighing 160 - 220 grams were utilized for this study. Care and treatment of these animals were according to the Canadian Council on Animal Care and all procedures were approved by a committee for the ethical use of animals for research. The rats were randomly assigned to one of three primary treatment groups: hemisected (n=15), sham-treated (n=17) and control-untreated (n=13). For the purposes of this chapter the term hemisected group will be interchangeable with the term atrophied group.

Disuse Atrophy

To induce disuse atrophy a spinal hemisection was performed at the level of the twelfth thoracic vertebra (T12) under sterile surgical conditions. A laminectomy of T12

was completed to expose the dorsal aspect of the spinal cord. Subsequently, the left side of the spinal cord was severed from the midline laterally. The paraspinal muscles, superficial muscle layer and skin were subsequently sutured. These animals received a subcutaneous injection of streptomycin (1 cc.) to prevent infection and to replace fluid loss incurred during surgery. Regular monitoring of the toe-spread reflex in conjunction with a toe-pinch pain test was done to ensure that complete paralysis was present over the 2 week period. The sham-treated animals underwent the same surgery as the atrophy group excluding the hemisection of the spinal cord, such that the T12 laminectomy was the extent of the surgical intervention. These animals were also monitored for 2 weeks to ensure an intact toe-spread reflex following surgery. After 2 weeks, acute measurement of contractile responses was performed on all groups.

Muscle Preparation

The rats were deeply anaesthetized with an intramuscular injection of ketamine:xylazine (ketamine 100 $mg \cdot ml^{-1}$, xylazine 100 $mg \cdot ml^{-1}$; mixed 85:15 IM; dosage 0.22 ml). The left gastrocnemius muscle was isolated *in situ* (MacIntosh et al., 1988) to examine the isometric contractile properties. The gastrocnemius muscle was attached to a force transducer (Grass FT.10) via the Achilles tendon by a stainless steel wire. The soleus and plantaris muscles were cut at their insertions to the Achilles tendon to remove them from the preparation.

The sciatic nerve was cleared of connective tissue and severed close to the spine.

The distal stump of the severed nerve was placed across a pair of stainless steel electrodes for indirect stimulation of the gastrocnemius muscle. Stimulation was performed with supramaximal (1-5 V) square pulses, 50 μs in duration (Grass Model S88). The loosened skin from the hindlimb was stretched and anchored to form a pocket which was filled with warmed mineral oil. Rectal and mineral oil temperatures were monitored and regulated at approximately 37°C throughout the experiment using radiant heat.

Contractile Properties

Initially the optimal muscle length (L_o) was determined utilizing double pulse stimulations with a 5 ms delay. Subsequent to setting the muscle at L_o, a conditioning tetanic contraction was elicited to ensure that the experimental setup was stable and the muscle contractions were isometric. Next, four control twitch contractions and a double pulse contraction were obtained at 20 s intervals to ensure consistent twitch developed tension (DT). Up to this point in time the treatment was identical for all groups.

Subsequently, animals from the three primary treatment groups (hemisected, sham-treated and control) were subdivided into either resting or stimulated conditions. Following pre-tetanic twitch contractions, animals in the resting condition were allowed 10 min of inactivity before freezing the muscle for assessment of resting R-LC phosphorylation. Animals designated to the stimulated condition completed pre-tetanic twitch contractions, and then were given 2 s of 200 Hz stimulation, and 20 s later a single twitch contraction was elicited. Immediately following this posttetanic twitch, the muscle

was rapidly freeze-clamped with tongs precooled in liquid nitrogen and the frozen muscle sample was placed in liquid nitrogen. These muscles were subsequently assessed for R-LC phosphorylation levels.

A microcomputer was used to monitor the output from the force transducer and the contractions were displayed visually throughout the experimental procedures. Digitized (4000 Hz) contractions were analyzed for the following parameters: developed tension (DT), the amplitude difference between the rest (initial) tension and the peak of the contraction; contraction time (C_t), the time from the first detectable increase in tension to the peak of the contraction; and average rate of tension development ($DT \cdot C_t^{-1}$), the developed tension divided by contraction time.

Biochemical Analysis

The frozen muscle samples were analyzed to determine the proportion of phosphorylated R-LC. The procedure utilized was that described by Silver and Stull (1982) and modified by Moore and Stull (1984) and Stuart et al. (1987). The muscle was pulverized and then homogenized at 0°C with a Polytron. After 15 min of centrifugation at 5°C, the supernatant was loaded onto a pyrophosphate polyacrylamide tubular gel and underwent electrophoresis at 90 V for 3 hours at 0°C to isolate the myosin. The tubular gels were stained with 0.15% Coomassie Blue (R-250, Sigma), 50% methanol, 10% acetic acid solution to identify the myosin band which was then excised.

The isolated myosin was subsequently denatured and loaded onto an isoelectric

focussing (IEF) polyacrylamide gel with a pH gradient of 4.5 to 6 to separate the phosphorylated from the nonphosphorylated form of R-LC. Electrophoresis was carried out at a constant power of 5 W and a temperature of 5°C for 3 hours. Silver staining was used to distinguish the 4 bands representing the light chains. The stained protein bands corresponding to the phosphorylated and nonphosphorylated forms of R-LC were quantified using a laser densitometer (LKB 2202 Ultroscan). Phosphate content was expressed as moles of phosphate per mole of total R-LC.

Statistical Analyses

A one-way analysis of variance was used to determine differences in twitch contraction parameters, and twitch:double pulse contraction ratios between hemisected/atrophied, sham-treated and control primary groups. Differences in twitch:tetanus ratio and tetanic developed tension between the stimulated subgroups were also determined with a one-way analysis of variance. The posttetanic twitch contractile variables: DT, C_t and $DT \cdot C_t^{-1}$ were expressed relative to the pre-tetanic twitch (100%) and a one-way analysis of variance was used to detect differences between subgroups for the posttetanic twitch. For the R-LC phosphorylation values, a two-way analysis of variance was used to detect differences between groups (hemisected, sham-treated and control) and conditions (rested, stimulated). The Student Newman-Keuls test was utilized for post-hoc analysis determined a priori. A significance level of $p<0.05$ was used in all analyses.

RESULTS

It was not possible to measure the weight of individual muscles which were studied in these experiments to confirm the presence of atrophy. However, visual inspection revealed that there was obvious atrophy present in the left gastrocnemius muscle of hemisected animals, and the differences in the contractile characteristics when compared to the control and sham-treated groups confirmed that spinal cord hemisection resulted in muscle atrophy.

Tetanic Developed Tension

The tetanic contractions were analyzed for absolute developed tension in each of the stimulated subgroups. All groups were significantly different from each other. The atrophied muscles demonstrated the lowest absolute tetanic DT. The fact that the sham-treated group demonstrated a higher absolute tetanic DT than the control group (Table 3) was most likely due to a greater mean body weight of the sham-treated animals (205.4 $\pm$ 3.5 g) compared to the control animals (176.8 $\pm$ 6.6 g). Table 3 illustrates that when tetanic DT was normalized for body weight ($mN \cdot g^{-1}$) there was no difference between control and sham-treated animals, but these values were significantly greater than for the hemisected animals (mean body weight = 195.0 $\pm$ 4.0 g).

Twitch Developed Tension

Figure 11 illustrates the results from two typical experiments with a sham-treated (A) and an atrophied (B) muscle, respectively. The superimposed traces represent twitch contractions recorded before (the smaller of each pair) and after a tetanus. It is obvious that the degree of posttetanic potentiation is greater in the sham-treated animal than in the hemisected animal. Further, it appears that the rate of force development is greater during the twitch in the sham-treated muscle compared to the atrophied muscle following tetanic stimulation. These apparent differences were confirmed by statistical comparisons.

The absolute DT of the pre-tetanic twitches was not different between treatment groups, indicating that twitch force was preserved in the atrophied muscles (Table 3). When the posttetanic twitch DT is presented relative to the pre-tetanic value (100%), it is clear that tetanic stimulation (2 s at 200 Hz) potentiated the subsequent twitch contraction in all groups demonstrating classic posttetanic potentiation. However, PTP was significantly less in the atrophied group (128.7 $\pm$ 2.6%) than in sham-treated (149.9 $\pm$ 2.4%) and control (142.9 $\pm$ 2.7%) groups.

Twitch:Double Pulse and Twitch:Tetanus Ratios

Analysis of the developed tension during paired pulse contractions shows that the atrophied muscles had a significantly higher twitch:double pulse ratio than the non-atrophied muscles (Table 3). Also, Table 3 illustrates that, as anticipated, the

twitch:tetanus ratio for the atrophied group was significantly greater than for the control and sham-treated groups. The higher twitch:tetanus ratio observed was due to the preservation of twitch amplitude in the atrophied muscles while tetanic tension was greatly attenuated in these muscles.

Posttetanic Twitch Contractile Characteristics

The contractile characteristics C_t and $DT \cdot C_t^{-1}$ were analyzed to determine if there was a difference between groups in the change (%) from pre-tetanic to posttetanic conditions. Twitch C_t increased significantly from pre- to posttetanic twitch for all groups. The twitch C_t following a 2 s tetanic stimulation expressed relative to the pre-tetanic contraction was not significantly different between sham-treated (117.3 $\pm$ 2.3%), control (112.0 $\pm$ 2.5%) and atrophied (123.9 $\pm$ 2.4%) muscles. The change in the average rate of tension development of the posttetanic twitch relative to the pre-tetanic twitch was significantly greater for the sham-treated (128.3 $\pm$ 3.9%) and control (132.5 $\pm$ 5.6%) groups compared to the atrophied group (105.2 $\pm$ 5.2%). Thus, the greater posttetanic potentiation observed in non-atrophied muscle was accompanied by a greater increase in $DT \cdot C_t^{-1}$ from the pre- to post-condition, compared to atrophied muscles.

R-LC Phosphorylation

Figures 12A and 12B illustrate examples of individual lanes of polyacrylamide

isoelectric focussing gels completed on the three experimental groups. The gel analysis of the muscles during rest conditions (Figure 12A) indicates that the proportion of phosphorylated R-LC (R-LC*) is similar for control and sham-treated muscles but appears to be less in the atrophied muscles. The enhancement of R-LC phosphorylation following a tetanic stimulation (Figure 12B) is evident by the increased density of the phosphorylated band (R-LC*) in all groups, but is much more apparent in the non-atrophied muscles. These observations were confirmed with statistical analysis.

The degree of light chain phosphorylation was assessed in muscles frozen 20 s following a tetanic contraction (2 s at 200 Hz), since this point is thought to correspond with peak posttetanic potentiation (Manning and Stull, 1979; Moore and Stull, 1984). A significant interaction was observed for group (hemisected, sham-treated and control) and condition (rested, stimulated). Tetanic stimulation produces a significant increase in the extent of R-LC phosphorylation compared to the rested condition in all groups, including atrophied muscles. However, there was significantly less R-LC phosphorylation in the atrophied muscles both at rest and following tetanic stimulation (Figure 13).

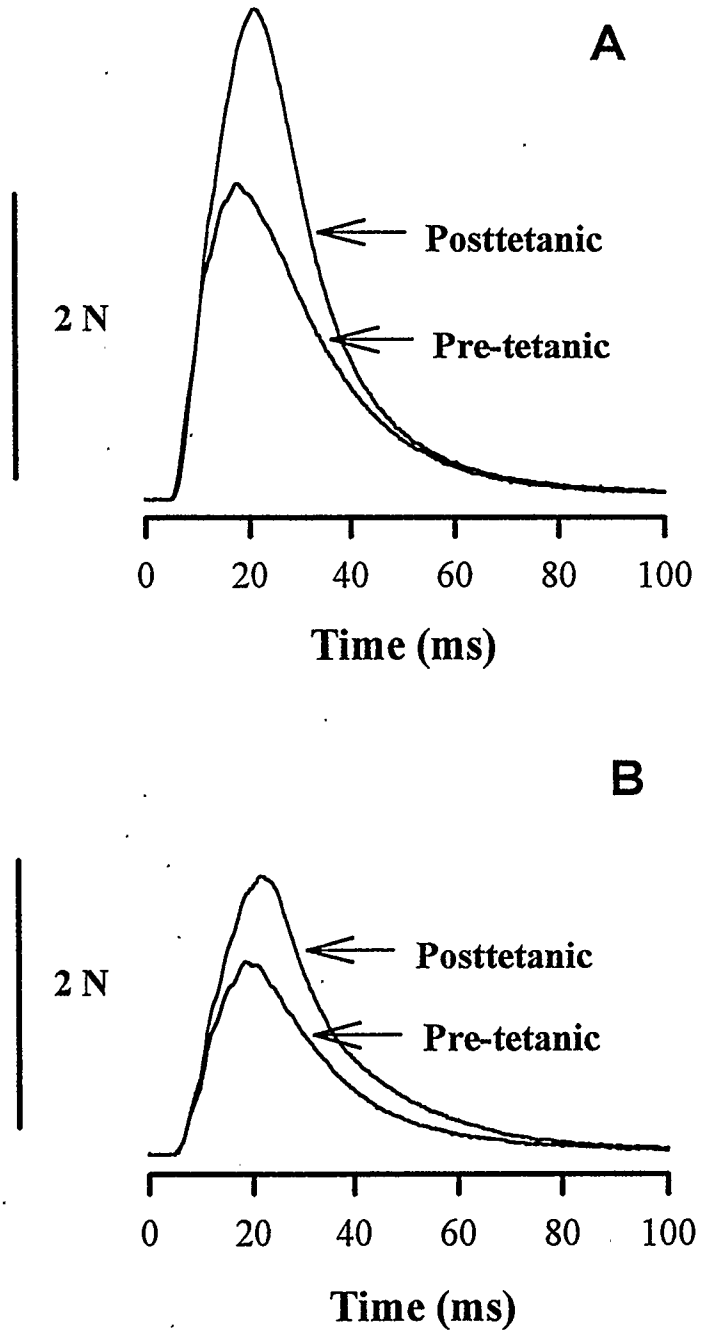

Figure. 11. A recording of twitches from two typical experiments with a sham-treated muscle (A) and an atrophied muscle (B). The trace from the posttetanic twitch is superimposed on the trace from the pre-tetanic twitch in both cases. The posttetanic twitch follows a 2 s tetanic stimulation (200 Hz) and demonstrates potentiation in both muscles, but to a greater degree in the sham-treated muscle. The vertical calibration bar is 2 newtons.

Table 3. Pre-tetanic and posttetanic twitch, and tetanic contractile characteristics.

	Atrophied	Sham-Treated	Control
Pre-tetanic Twitch:			
DT (N)	**1.61 ± .16**	**1.89 ± .15**	**1.67 ± .17**
C_t (ms)	**13.3 ± .71**	**13.8 ± .68**	**13.5 ± .75**
$DT \cdot C_t^{-1}$ ($N \cdot ms^{-1}$)	**0.12 ± .01**	**0.14 ± .01**	**0.12 ± .01**
Posttetanic Twitch:			
DT (N)	**2.06 ± .16***	**2.82 ± .15**	**2.36 ± .17**
C_t (ms)	**16.6 ± .71**	**16.1 ± .68**	**15.2 ± .75**
$DT \cdot C_t^{-1}$ ($N \cdot ms^{-1}$)	**0.13 ± .01#**	**0.18 ± .01**	**0.16 ± .01**
Tetanic DT:			
Absolute (N)	**5.50 ± 0.3***	**13.3 ± 0.9***	**10.7 ±1.1***
Normalized ($mN \cdot g^{-1}$)	**28.0 ± 2.0***	**65.0 ± 5.0**	**56.0 ± 4.9**
Twitch:Double Pulse Ratio	**0.56 ± .03***	**0.44 ± .01**	**0.44 ± .02**
Twitch:Tetanus Ratio	**0.30 ± .02***	**0.14 ± .01**	**0.16 ± .01**

Abbreviations: DT, developed tension; C_t, contraction time; $DT \cdot C_t^{-1}$, average rate of force development; N, newtons; ms, milliseconds; mN, millinewtons; and g, grams (body weight). Values are means ± SE. * Significantly different from other groups; # significantly different from sham-treated group.

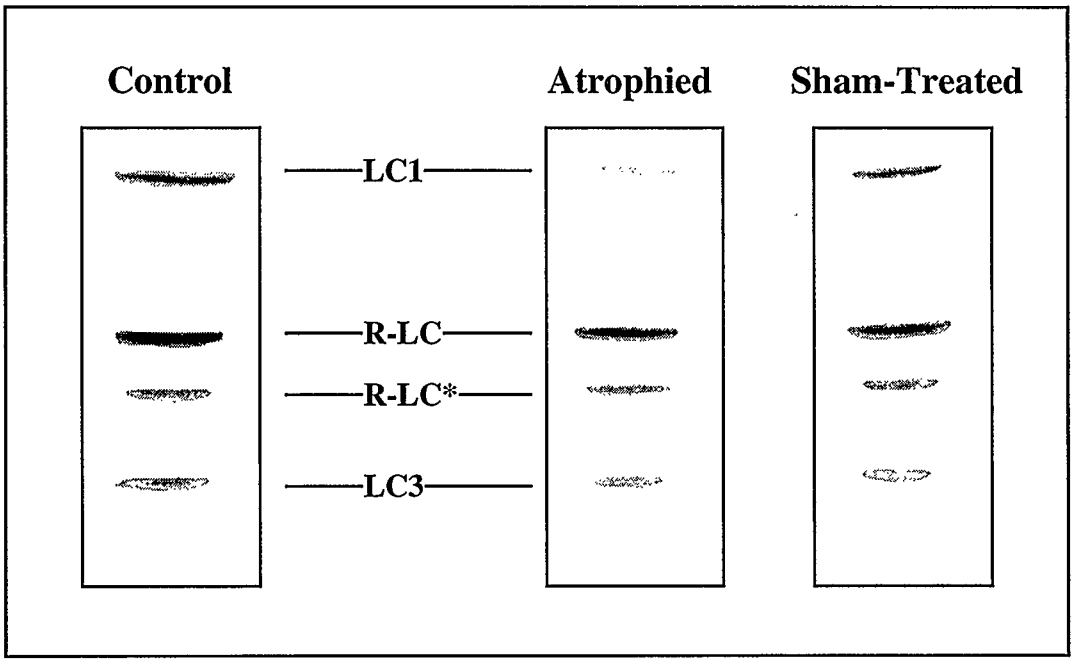

Figure. 12A. Pyrophosphate-polyacrylamide isoelectric focussing gels illustrating the separation and identification of the three myosin light chains of rat fast-twitch skeletal muscle. The muscles represented in this diagram were frozen in the resting state. A sample from each treatment group is represented by a single lane, from left to right: control, atrophied and sham-treated. The abbreviations are as follows: LC1, light chain 1; R-LC, non-phosphorylated form of the regulatory light chain; R-LC*, phosphorylated form of the regulatory light chain; and LC3, light chain 3. A comparison of the lanes indicates that in resting muscles the proportion of phosphorylated R-LC appears to be lower in the atrophied versus non-atrophied muscles.

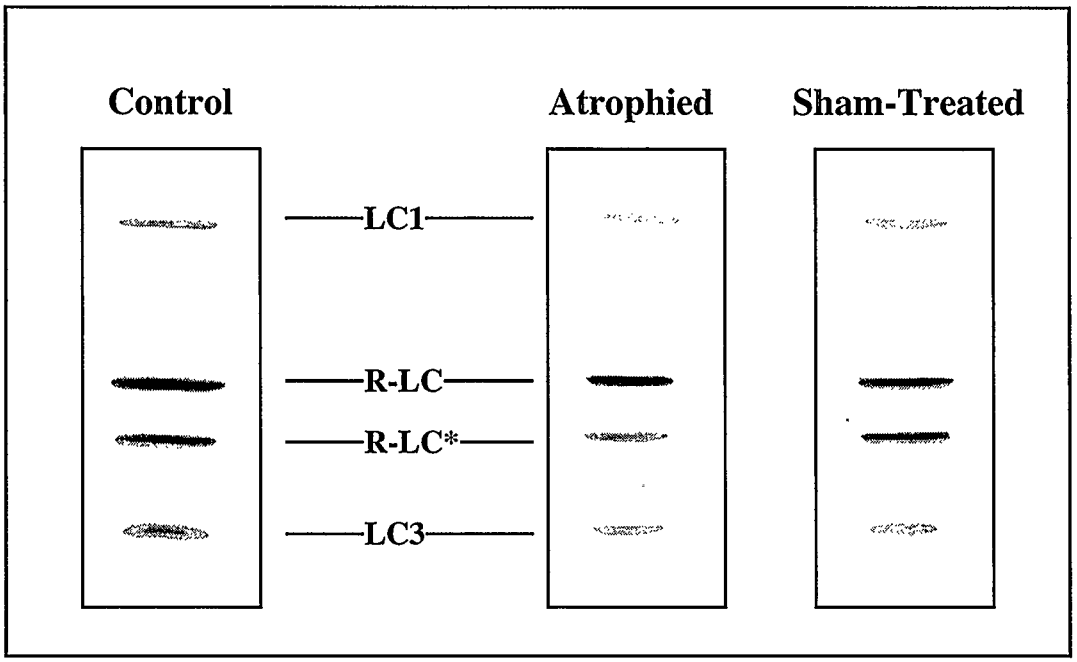

Figure. 12B. The muscles represented in this diagram were frozen 20 s following a 2 s tetanic contraction (stimulated condition). A sample from each treatment group is represented by a single lane, from left to right: control, atrophied and sham-treated. The abbreviations are as follows: LC1, light chain 1; R-LC, non-phosphorylated form of the regulatory light chain; R-LC*, phosphorylated form of the regulatory light chain; and LC3, light chain 3. A comparison of the lanes indicates that under stimulated conditions the proportion of phosphorylated R-LC appears to be lower in the atrophied versus non-atrophied muscles.

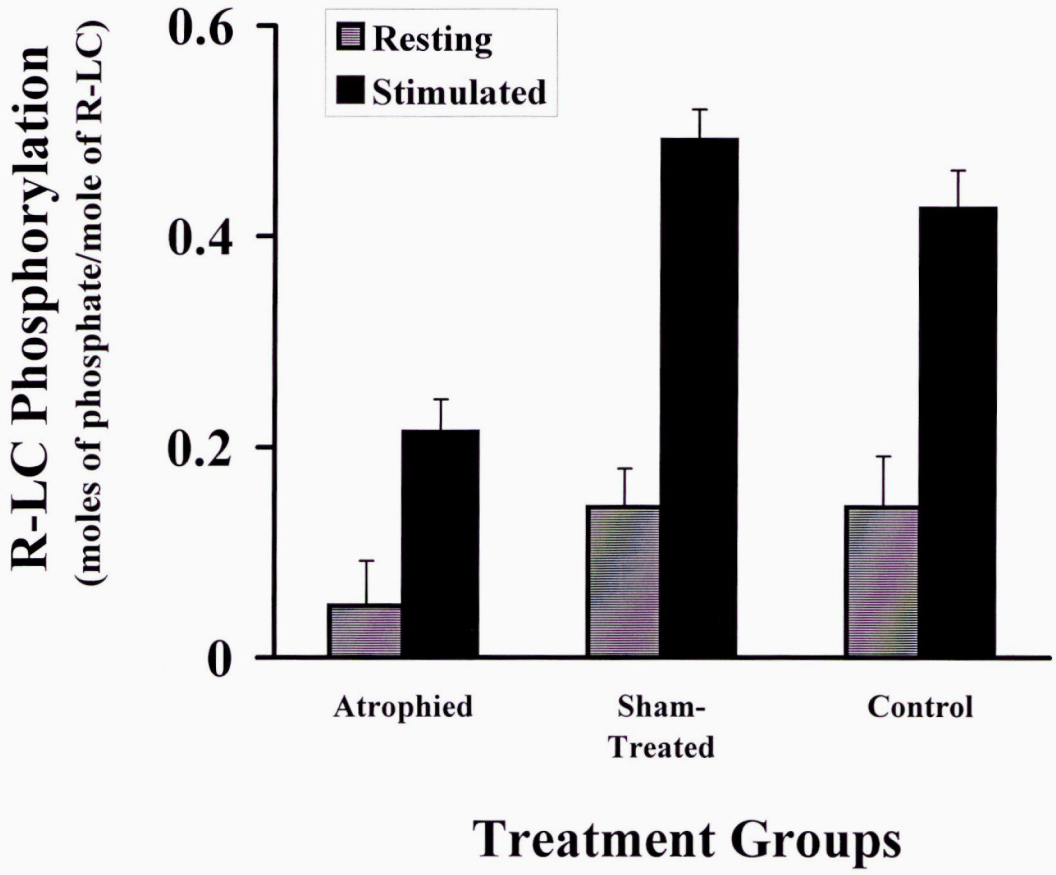

Figure. 13. Bar graph illustrating the R-LC phosphorylation values (moles of phosphate per mole of R-LC) for the atrophied (resting, n=5 and stimulated, n=10), sham-treated (n=4, n=9) and control (n=6, n=11) groups under both conditions: resting and stimulated. Values are means and the vertical bars represent SEM. Statistical comparison is presented in the text.

DISCUSSION

The major finding in the present study was that tetanic stimulation produced a significant increase in R-LC phosphorylation from resting levels in atrophied rat gastrocnemius muscle, and this was accompanied by significant PTP. R-LC phosphorylation levels for atrophied muscles have not been previously measured following tetanic stimulation using an *in situ* preparation. These results are in contrast to the observation that low frequency stimulation (10 Hz), which does not result in staircase in atrophied rat gastrocnemius muscles, does not increase the R-LC phosphorylation from resting levels (Tubman et al., 1996).

The consequences of disuse as a result of a spinal hemisection can be described by the specific contractile characteristics of the atrophied muscles. Since tetanic force development is dependent upon the muscle cross-sectional area (Close, 1972; Powell et al., 1984), it was expected that disuse atrophy of the gastrocnemius muscle would result in a depressed tetanic DT. The tetanic DT, when normalized for animal weight, was significantly lower for the atrophied muscles than for the non-atrophied muscles.

Previous observations showing a higher twitch:tetanus ratio in atrophied versus non-atrophied muscles (Gardiner et al., 1992; St-Pierre et al., 1987) have been confirmed in this study. This is clearly due to a lower tetanic DT and a relatively stable twitch DT. Further, the twitch:double pulse contraction ratio was also significantly elevated in the atrophied muscles showing that the increment in tension development with a second pulse of stimulation is much less in atrophied muscles, as previously reported (MacIntosh et al., 1988). The high twitch:tetanus and twitch:double pulse contraction ratios have been used

to support the contention that, in atrophied muscles, a single stimulation pulse results in relatively enhanced Ca^{2+} release and activates a greater proportion of the available contractile protein than occurs with a single pulse in non-atrophied muscles.

In the present study, the degree of posttetanic twitch potentiation following a 2 s tetanic stimulation (200 Hz) was significantly greater in the non-atrophied muscles (~ 45%) than in the atrophied muscles (~ 30%). Depression in posttetanic potentiation of atrophied muscle has been previously reported (Dulhunty, 1985; Matsushita et al., 1988). Dulhunty (1985) studied the effects of denervation on contractile properties in rat fast-twitch (EDL) muscle fibers at 21°C. After 14 days of inactivity, PTP decreased by 50% in denervated muscles when compared to control values. These results are qualitatively similar to the present study where the atrophied muscles showed a 33% decrease in potentiation.

Matsushita and coworkers (1988) reported a greater reduction in PTP 7 days after denervation in rat EDL muscles (35°C). PTP decreased from 51% (in the contralateral limb) to 3%, and was associated with R-LC phosphorylation values of 51% and 10%, respectively (Matsushita et al., 1988). Short-term denervation appears to result in a greater magnitude of change in both PTP and phosphorylation, compared to hemisection. This may be because residual activation of the muscle may occur following spinal hemisection due to the intact spinal reflexes. However, it is possible that disuse atrophy results in a rapid decline in both potentiation and R-LC phosphorylation (i.e., by 7 days after denervation) which is then reversed, permitting some potentiation (Dulhunty, 1985) and R-LC phosphorylation by 14 days after denervation.

If it is assumed that R-LC phosphorylation is the principal mechanism for

posttetanic potentiation as suggested by others (Moore and Stull, 1984; Manning and Stull, 1982; Sweeney et al., 1993), then the lower magnitude of R-LC phosphorylation seen in this study would contribute to the lower magnitude of potentiation in atrophied muscles. Since R-LC phosphorylation is thought to cause potentiation via an enhanced rate of force development (Vandenboom et al., 1995), this theory is consistent with our findings. We observed that the change in the rate of force development from pre-tetanic to posttetanic conditions was much greater in non-atrophied versus atrophied muscles, while the change in C_t was similar for all muscles. It is interesting to note that C_t did increase from the pre-tetanic to posttetanic twitch in all groups. Although most studies on twitch kinetics during potentiation demonstrate no change in C_t (Close and Hoh, 1968; Hanson, 1974), results similar to ours have been reported previously (Olson and Swett, 1971), and may be the result of the relatively long duration tetanic contraction used in these studies. Thus an increased C_t may have contributed to PTP in all groups, but it does not account for the difference in PTP between atrophied and non-atrophied muscles.

It is also possible that the relative effect of R-LC phosphorylation on potentiation of a twitch may be less in atrophied muscles. Considering atrophied muscles have a higher twitch amplitude (pre-tetanic) relative to the tetanic contraction, the potential for relative enhancement of the twitch for a given level of R-LC phosphorylation would be less. Persechini and coworkers (1985) studied the effects of R-LC phosphorylation on isometric twitch tension in skinned fibers (fast-twitch rabbit) at low (0.6 μM) and high (10 μM) Ca^{2+} concentrations. At a low [Ca^{2+}] R-LC phosphorylation potentiated the twitch by 50%, while at a high [Ca^{2+}], R-LC phosphorylation was observed to have little

effect on twitch tension. Also, Palmer and Moore (1989) reported a threefold increase in the slope of the phosphorylation-potentiation relationship during attenuated Ca^{2+} availability. Thus, the magnitude of twitch potentiation was inversely proportional to Ca^{2+} concentration. These studies suggest that the effects of R-LC phosphorylation on twitch potentiation would be less during conditions of enhanced Ca^{2+} availability, which may be the case in atrophied muscles.

While these observations may explain the diminution of PTP in atrophied muscles, the explanation for the attenuated R-LC phosphorylation observed in the atrophied muscle is less clear. Although moderate phosphorylation (0.21 moles of phosphate/mole of R-LC) was achieved following a tetanic contraction in atrophied muscles, it was significantly less than that observed for the sham-treated and control muscles (0.49 and 0.43 moles of phosphate/mole of R-LC, respectively). It appears that some component of the phosphorylation process is altered in atrophied muscles.

Phosphorylation of the 18,500-dalton regulatory light chain is catalyzed by the Ca^{2+}-dependent enzyme myosin light chain kinase (MLCK). The activation of MLCK first requires the binding of Ca^{2+} to calmodulin, and then the binding of the Ca^{2+}-calmodulin complex to the inactive form of MLCK. This binding produces a conformational change in MLCK and converts the enzyme to the active form (Klug et al., 1982). Dephosphorylation of the light chains occurs via a phosphatase. Therefore the extent of R-LC phosphorylation is dependent upon the relative activities of the two enzymes, MLCK and a protein phosphatase. A lower phosphate content of R-LC in atrophied muscle could be due to: i) a decreased rate of phosphorylation due to

impairment of the Ca^{2+}-calmodulin-MLCK system, or ii) an increase in dephosphorylation via the phosphatase.

It is probable that the impairment of the Ca^{2+}-calmodulin-MLCK system is responsible for the diminution of R-LC phosphorylation. However, it is difficult to determine the exact mechanism. Klug and coworkers (1992) demonstrated that chronic stimulation of rabbit fast-twitch muscle resulted in a reduced MLCK activity with no change in phosphatase activity. These authors attributed this depression in MLCK activity to a disruption in the Ca^{2+}-handling properties. However, the observation that the twitch: tetanus ratio and twitch:double pulse ratios are elevated in atrophied muscles suggests that Ca^{2+} availability is not limited for each single stimulus. Also, *in vitro* studies have shown that the mean open time of the Ca^{2+} release channels (Delbono and Chu, 1995), and the number of Ca^{2+}-release channels of sarcoplasmic reticulum (Zorzato et al., 1989), are greater in denervated muscle. These findings are consistent with the theory that Ca^{2+} availability is not a limitation in atrophied muscle (chapter 3). Attenuated R-LC phosphorylation may be caused by a decrease in the activity or concentration of MLCK.

Regardless of the mechanism responsible for the evident impairment of the regulatory light chain phosphorylation, the association between potentiation and R-LC phosphorylation was preserved in this study. Atrophied muscles demonstrated a depressed PTP that was similar to the attenuation of stimulation-induced R-LC phosphorylation. These observations, together with the previous findings that severe attenuation of staircase potentiation is associated with a virtual absence of R-LC phosphorylation (Tubman et al., 1996), support the theory that R-LC phosphorylation is the primary mechanism for twitch

potentiation.

SUMMARY

Previously we have demonstrated that the absence of staircase potentiation in atrophied rat gastrocnemius muscle is accompanied by significantly attenuated phosphorylation of the regulatory light chains (R-LC) of myosin. It was our purpose in the present study to determine if potentiation and R-LC phosphorylation were also attenuated in disuse-atrophied muscles following tetanic stimulation. Two weeks after a spinal hemisection (T12), twitch and tetanic contractile characteristics were measured *in situ* in control, sham-treated and atrophied (hemisected) muscles. Posttetanic potentiation 20 s after a 2 s tetanic contraction (200 Hz) was depressed in atrophied muscles (128.7 $\pm$ 2.6%; mean $\pm$ SEM) when compared to sham-treated (149.9 $\pm$ 2.4%) and control (142.9 $\pm$ 2.7%) muscles. The change in rate of force development from the pre-tetanic to the posttetanic twitch, was significantly greater for non-atrophied versus atrophied muscles. Atrophied muscles demonstrated a significant increase in R-LC phosphorylation from rest (0.05 $\pm$ .04) to posttetanic conditions (0.21 $\pm$ .03 moles of phosphate/mole of R-LC), and less phosphorylation than control and sham-treated muscles (0.43 $\pm$ .06 and 0.49 $\pm$ .03 moles of phosphate/mole of R-LC, respectively) after tetanic stimulation. The preservation of the potentiation-phosphorylation relationship in atrophied muscles is consistent with the hypothesis that R-LC phosphorylation may be the principal mechanism for twitch potentiation.

Chapter 6

Summary and Future Directions

Summary

The purpose of this dissertation was to examine more closely the relationship between twitch potentiation and myosin light chain phosphorylation. Although the potentiation-phosphorylation relationship has been well documented in the literature (Klug et al., 1992; Manning and Stull, 1979; Manning and Stull, 1982; Moore and Stull, 1984), the assessment of these two variables together in atrophied muscles has only been reported using a denervation model with an *in vitro* preparation (Matsushita et al., 1988). The attenuation of twitch potentiation in atrophied muscle both during repetitive stimulation and after a tetanic contraction was confirmed. This depressed potentiation was accompanied by a significantly reduced R-LC phosphorylation. The hypotheses stated in the introductory portion of this dissertation were therefore supported.

One of the limitations in this research project was that the extent of atrophy could

not be directly evaluated. While the best method for assessing atrophy is by weighing the muscle following disuse, this was not possible as the muscles were quick-frozen immediately following the experiment for later biochemical analysis. Alternatively, analysis of twitch and tetanic contractile characteristics of atrophied muscles also provides a valid method of confirming disuse atrophy (MacIntosh et al., 1988; St-Pierre and Gardiner, 1985; Gardiner et al., 1992).

The consequences of disuse include an elevation of both twitch:tetanus ratio (St-Pierre et al., 1987; Gardiner et al., 1992) and twitch:double pulse contraction ratio (MacIntosh et al., 1988; Rassier et al., 1996). The TTX-treated animals (chapter 3) demonstrated an elevated twitch:double pulse contraction ratio compared to control animals indicating less enhancement of DT with the second pulse of activation. This observation is consistent with previous studies of TTX-atrophied muscles (MacIntosh et al., 1988) for which atrophy is associated with a loss of myofibrillar protein (St-Pierre and Gardiner, 1985). In muscles atrophied via hemisection, the diminution of tetanic DT was accompanied by the preservation of twitch amplitude and resulted in an elevation of the twitch:tetanus ratio. An increase in this ratio has commonly been observed in cases of marked atrophy (Gardiner et al., 1992; St-Pierre et al., 1987). In both the *in situ* studies atrophy was confirmed by the contractile measurements.

The first study using repetitive stimulation (10 Hz for 10 s) showed a complete abolishment of twitch potentiation in TTX-atrophied muscles (chapter 3). This loss of potentiation was associated with a virtual absence of R-LC phosphorylation substantiating hypothesis #1 of this dissertation. Notably, in several of the atrophied muscle samples

the phosphorylated band of the R-LC was completely absent. These results strongly suggest that the process of R-LC phosphorylation is impaired in atrophied muscles.

In the second *in situ* study, the change in PTP in atrophied muscles (hemisection) was examined concomitant with R-LC phosphorylation measurement (chapter 5). In contrast to the initial study, PTP was depressed but still evident in atrophied muscles. The second hypothesis: that an attenuated PTP would be associated with an attenuated R-LC phosphorylation in muscles atrophied via spinal hemisection was verified.

PTP of approximately 28% was associated with R-LC phosphorylation of ~21% in atrophied muscles, compared to ~ 45% PTP associated with ~ 45% R-LC phosphorylation in non-atrophied muscles. Matsushita and coworkers (1988) also demonstrated an attenuation of R-LC phosphorylation in denervated rat EDL muscle fibers following a tetanic contraction (200 Hz for 1 s). However, 7 days after denervation, a more severe decrement in both variables was observed in that study, with a R-LC phosphorylation of ~10% and ~3% twitch potentiation. The reduction of PTP and R-LC phosphorylation from 0-7 days after denervation showed a high correlation ($r = 0.96$) (Matsushita et al., 1988), and is consistent with the proportional changes in these two parameters observed in the present *in situ* study.

The observation that the potentiation-phosphorylation relationship appears to be preserved in atrophied muscles is consistent with the hypothesis that R-LC phosphorylation has a primary role in twitch potentiation. This is suggested in Figure 14, which illustrates the potentiation-phosphorylation relationship from the data obtained in both *in situ* studies. During repetitive stimulation (10 Hz for 10 s), control muscles

demonstrated the greatest degree of twitch potentiation and the largest extent of R-LC phosphorylation. Alternatively, in TTX-atrophied muscles 10 Hz stimulation resulted in a twitch of lesser magnitude than the pre-stimulation twitch (~75%). This attenuation of twitch amplitude may be due to fatigue or other factors influencing the developed tension in TTX-treated muscles within this short period of repetitive stimulation. Although the absence of twitch potentiation was not observed in the PTP study, potentiation was severely attenuated, and this was accompanied by significantly less R-LC phosphorylation (chapter 5). In the PTP study the alterations in degree of potentiation and R-LC phosphorylation appear to be proportional and a close relationship between these two variables appears to be maintained.

While Figure 14 provides some information regarding the potentiation-phosphorylation relationship, further information could be revealed by examining the relationship between R-LC phosphorylation and force development relative to muscle cross-sectional area. Because the twitch may already be enhanced in atrophied muscles, presumably as a result of alterations in the Ca^{2+}-handling properties of the muscle cell, it may be relevant to examine changes in tension development relative to muscle cross-sectional area. The theory that there may be a higher level of Ca^{2+} activation for a single twitch in atrophied muscles has been previously discussed but is based primarily on the observations that the twitch:tetanus ratio and twitch:double pulse contraction ratio are elevated in atrophied muscles. These observations have been attributed to an already enhanced twitch force, as a result of increased Ca^{2+} availability with the first pulse of stimulation.

To determine if any differences in the potentiation-phosphorylation relationship are present between atrophied and non-atrophied muscles, strain is reported as developed tension relative to the estimated cross-sectional area of the muscle (and will be referred to as normalized developed tension). Taking into consideration the known fiber type distribution of the rat gastrocnemius muscle (Armstrong and Phelps, 1984) and the apparent fiber type specific atrophy with TTX-treatment (Gardiner et al., 1992) it was estimated that muscle fiber cross-sectional area was decreased by 62%. For the muscles atrophied via spinal hemisection (chapter 5), the measurement of tetanic DT was used as an estimate of relative change in cross-sectional area. Tetanic force development is dependent upon the muscle cross-sectional area. The ratio for atrophied versus non-atrophied muscles for maximal tetanic DT was 0.42.

Using these methods, strain (normalized developed tension) could be estimated for all stimulation schemes in atrophied and non-atrophied muscles. Accordingly Figure 15 demonstrates the relationship between strain and R-LC phosphorylation for all conditions examined in this dissertation. In Figure 15 the data lines join the mean resting and stimulated values (strain and corresponding R-LC phosphorylation) for the control muscles in the staircase study (CS), the control muscles in the PTP study (CP), the atrophied muscles in the staircase study (AS) and the atrophied muscles in the PTP study (AP).

If R-LC phosphorylation is the only factor which effects potentiation and atrophy does not change this relationship then, one would expect a similar increase in force per change in phosphorylation regardless of staircase or PTP, and regardless of atrophy or absence of atrophy. To investigate this theory standardized correlation coefficients were

calculated for each data set, and subsequently the data sets were compared using 95% confidence intervals. Confidence intervals for r were calculated using the Fisher Z-transformation.

By examining the data in this way, new observations are evident. The data lines representing the control muscles during two different stimulation conditions (staircase and PTP), and the atrophied muscles (hemisection) are very similar in slope. The correlation coefficient (r) for the staircase data is 0.74, for the PTP data r=0.76, and for the atrophied (hemisection) data r=0.43. Calculation of the confidence interval *(r=.19 to r=.95)* for the control staircase data indicates that there is no statistical difference between the three conditions/treatments. The potentiating effect of R-LC phosphorylation on strain or force development does not appear to differ during low frequency repetitive stimulation and following a 2 s tetanic contraction for control muscles or for muscles atrophied by spinal cord hemisection. Alternatively, it is obvious that the strain-phosphorylation relationship is different in atrophied muscles (TTX-treatment) during low frequency repetitive stimulation. The AS data set shows a negative slope compared to the positive slopes of all other data lines.

It appears from Figure 15 that the normalized DT observed for a single twitch without previous activation, is much greater in the atrophied versus control muscles. This enhancement in normalized twitch DT is consistent with the higher twitch:tetanus ratio discussed earlier. As previously noted, the TTX-atrophied muscles appear to demonstrate a negative slope for the DT versus phosphorylation relationship. It seems unlikely that phosphorylation of the R-LC would result in attenuation of DT. Therefore it appears that

the potentiation-phosphorylation relationship has shifted downward, indicating an unknown influence on the magnitude of the twitch DT during staircase.

The AS data strongly suggest that some factor other than R-LC phosphorylation must be responsible for modulating twitch force. Fatigue may be a greater factor in muscles which have a greater degree of atrophy, or fatigue may occur more readily as a result of repetitive 10 Hz stimulation versus tetanic stimulation. Unfortunately the design and purpose of this study limits the conclusions to a speculative nature. There is no way to determine if factors such as attenuated Ca^{2+} availability, impaired Ca^{2+} binding, and/or increased inorganic phosphate are responsible for the decrement in force evident in the AS data.

The principal finding in this dissertation was that the marked differences in extent of R-LC phosphorylation in atrophied versus non-atrophied muscles observed in both stimulation schemes suggests an impairment of the R-LC phosphorylation process as a result of atrophy. Further investigations revealed that the cellular content of calmodulin did not change with atrophy (chapter 4). This finding eliminates the possibility that diminished CaM causes a reduction in MLCK activation and, in turn, a decreased extent of phosphorylation. However, the specific mechanism resulting in attenuated R-LC phosphorylation cannot be fully elucidated by these data.

In summary the major findings in this dissertation were: (1) absence of twitch potentiation during repetitive stimulation is accompanied by attenuated R-LC phosphorylation, (2) the impaired phosphorylation of R-LC in atrophied muscles is not due to a decrease in the CaM content of the muscle, (3) depressed PTP is associated with

diminished R-LC phosphorylation, and (4) while the close relationship between potentiation and R-LC phosphorylation observed for non-atrophied muscles is maintained in muscle atrophied via spinal hemisection following a tetanic stimulation, a similar potentiation-phosphorylation relationship is not evident in TTX-atrophied muscles during repetitive 10 Hz stimulation. Together, these observations cannot disprove the theory that regulatory light chain phosphorylation is the principal mechanism for twitch potentiation. However, the possibility that other mechanisms contribute cannot be ruled out.

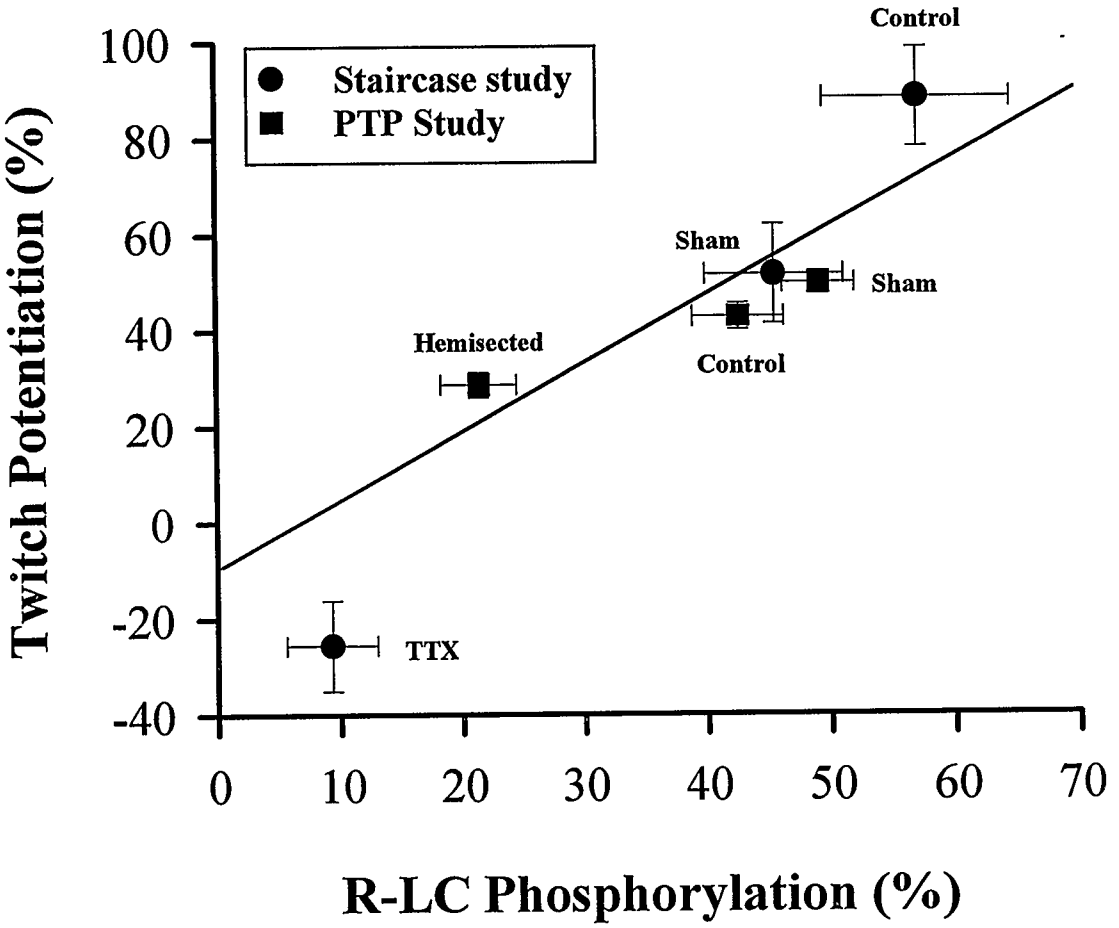

Figure. 14. The relationship between twitch potentiation and R-LC phosphorylation from data obtained during 10 Hz stimulation (staircase study) and following a tetanic contraction (PTP study). Mean values ± SEM are presented for each treatment group.

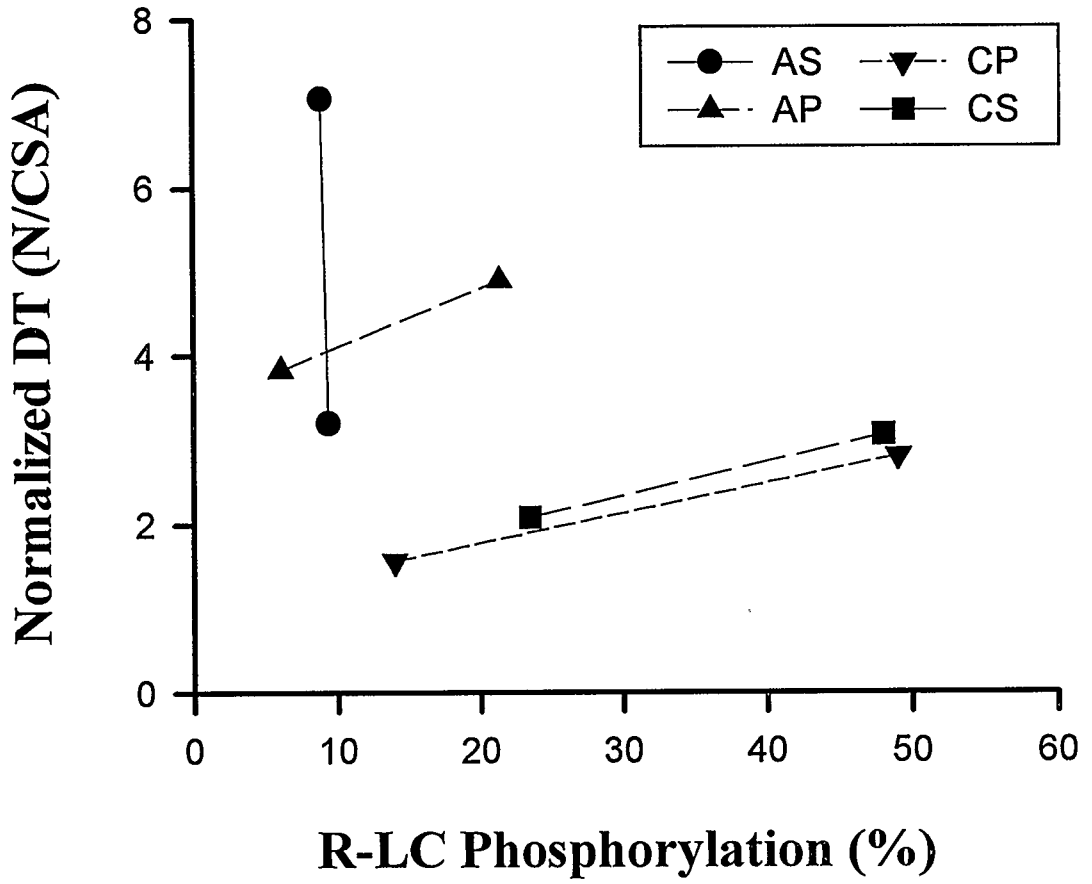

Figure 15. The relationship between normalized DT (developed tension) and R-LC phosphorylation for different conditions. N/CSA is the developed tension (newtons) of all groups normalized relative to the change in CSA from control to atrophied conditions. Therefore control=DT/1.0 and atrophied=DT/0.42 for hemisection and DT/0.38 for TTX-treatment. The lines represent data for control muscles in the staircase study (CS), control muscles in the PTP study (CP), atrophied muscles in the staircase study (AS) and atrophied muscles in the PTP study (AP).

Future Directions

Modulation of twitch amplitude and time course due to previous muscle activation is a very complex issue. Further insight into the primary mechanism for twitch potentiation could be attained from clarification of the role of Ca^{2+} modulation in potentiation. An investigation recently completed in our laboratory demonstrated that dantrolene treatment restores potentiation in atrophied muscles, presumably via an attenuated Ca^{2+} release (Rassier et al., 1996). This observation is consistent with the idea that atrophied muscles have a disproportionate release of Ca^{2+} during the first pulse of stimulation and suggests a role for Ca^{2+} in potentiation. Although indirect techniques such as dantrolene provide useful information concerning Ca^{2+}- handling properties of the muscle, a direct assessment of Ca^{2+} transients during potentiation would be valuable. Unfortunately existing measurement techniques are not capable of accurate resolution, ie. the Ca^{2+} transient is thought to be too fast for the fluorescent probes to respond during a twitch contraction.

The findings from the present study are also of interest from the perspective of the physiological consequences of disuse. Disuse atrophy can occur as a result of spinal cord injury, peripheral nerve damage and limb immobilization. Knowledge of the biochemical and contractile changes within the muscle are essential for development of effective rehabilitation strategies. Absent or diminished potentiation in disuse atrophied muscle could contribute to greater apparent fatigue with repetitive stimulation. This attenuation of potentiation appears to be a consequence of impaired R-LC

phosphorylation, although there may be interaction with other factors.

Since the extent of R-LC phosphorylation is regulated by two key enzymes, it would be useful to measure their relative activities in a disuse atrophy model. An accelerated phosphatase activity and thus an increased rate of dephosphorylation may explain the decreased extent of R-LC phosphorylation in disuse atrophied muscles. Alternatively, assessment of the MLCK activity would establish whether attenuation of this kinase activation is responsible for diminished phosphate incorporation into the R-LC. These investigations would provide insight into the mechanism for absent or diminished R-LC phosphorylation.

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