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Length-dependence of twitch potentiation in skeletal muscle

by

Dilson Jose Etcheverry Rassier

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Abstract

Staircase potentiation is an increase in developed tension (DT) during the first seconds of low-frequency repetitive muscle stimulation. Evidence suggests that phosphorylation of the regulatory light chain (R-LC) of myosin is responsible for this activity-dependent twitch potentiation in skeletal muscle. The degree of potentiation depends on the muscle length, but the mechanism of this length-dependence is not clear. Two hypotheses were tested in this dissertation: (1) length-dependence of myosin regulatory light chain (R-LC) phosphorylation is responsible for length-dependence of staircase potentiation, and (2) mechanisms related to length-dependence of muscle activation are responsible for the length dependence of staircase potentiation. Five independent projects were conducted to investigate the length-dependence of staircase potentiation. Two projects were completed with small bundles of the extensor digitorum longus (EDL) muscle from the mouse *in vitro*, and three projects used the whole gastrocnemius muscle *in situ*. The results confirmed the presence of length-dependence of staircase potentiation, in both preparations. Hypothesis (1) was rejected, as R-LC phosphorylation proved to be independent of muscle length, despite the length-dependence of force enhancement during staircase potentiation. The studies suggest that the mechanisms of length-dependence of muscle activation (Hypothesis 2) are related to the mechanisms of length-dependence of staircase potentiation. Interactions between filament spacing and length-dependence of Ca^{2+} sensitivity, as a result of changes in muscle length may explain these findings.

Preface

The chapters 2, 3, 4, 5, 6 and 7 of this dissertation are based on the following manuscripts:

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List of abbreviations

C_t	contraction time
$dP \bullet dt^{-1}_{min}$	peak rate of relaxation
DT	developed tension
$dP \bullet C_t^{-1}$	average rate of force development
EDL	extensor digitorum longus
L_o	optimal length, defined using double pulse contractions
PTP	posttetanic potentiation
MLCK	myosin light chain kinase
P_t	twitch developed tension
P_t^*	twitch developed tension after a conditioning stimulus
P_t^*/P_t	fractional activity-dependent change of twitch developed tension
½ R_t	half-relaxation time
R-LC	regulatory light chains of myosin
SL	sarcomere length
SR	sarcoplasmic reticulum

Chapter 1

Introduction

For many years, it has been recognized that muscle length affects force production in skeletal muscle. The comprehensive study by Gordon et al. (1966a) showed that, by changing the sarcomere length, and consequently the degree filament overlap, force production is changed in a related way. Consistent with the original cross-bridge model of muscle contraction (Huxley, 1957), it was proposed that changing filament overlap alters the number of interactions between myosin cross-bridges and actin active sites. Consequently, force decreases as muscle is stretched beyond optimal overlap between the filaments.

The force-length relationship is currently accepted as one the most important features supporting the sliding filament theory of muscle contraction, and has fundamental importance in the study of muscle physiology and muscle mechanics. However, some questions regarding the force-length relationship still remain to be investigated. In particular, this dissertation concentrates on the length-dependence of staircase potentiation in fast twitch skeletal muscle. Staircase potentiation is a progressive enhancement of twitch developed tension that occurs during the early phase of repetitive low frequency stimulation (Desmedt and Hainaut, 1968; Krarup, 1981a; Krarup, 1981b; MacIntosh, 1991). In an abstract presented to the Physiological Society, Walling-de Jonge (1979) reported that the magnitude of staircase potentiation in rat extensor digitorum longus muscle (EDL) was less at lengths longer than optimal. However, in a subsequent study Moore and Persechini (1990) reported that the magnitude of posttetanic potentiation (PTP) in mouse EDL was greater at long muscle length.

Since these two studies do not agree, the first objective of this dissertation was to investigate the relationship between muscle length and activity-dependent staircase potentiation in mammalian skeletal muscle. We anticipated that staircase potentiation would be inversely related to muscle length, based on the fact that Wallinga-de Jonge (1979) reported this. In keeping with prior observations, we anticipated that PTP would be proportional to muscle length. The second objective of this dissertation was to investigate possible mechanisms responsible for the length-dependence of staircase potentiation. Two hypotheses were tested: (1) length-dependence of myosin regulatory light chain (R-LC) phosphorylation is responsible for length-dependence of staircase potentiation, and (2) mechanisms related to length-dependence of muscle activation are responsible for the length dependence of staircase potentiation.

The first hypothesis, proposed by Moore and Persechini (1990), is based on overwhelming evidence suggesting that twitch potentiation is related to R-LC phosphorylation (Houston et al. 1985; Klug et al. 1982; Manning and Stull, 1979; Manning and Stull, 1982; Moore et al. 1985). Moore and Persechini (1990) suggested that the difference between their results and the results of Wallinga-de Jonge (1979) could be due to differences in the protocol used in the two studies. While they chose to deliver the conditioning stimulus always at optimal length, changing the length during the subsequent twitches, Wallinga-de Jonge (1979) performed a low -frequency stimulation at different lengths to obtain the staircase response. Moore and Persechini (1990) suggested that the strategy used by Wallinga-de Jonge (1979) would have different results because R-LC phosphorylation may be length-dependent. This would not have been the case in their study. Since R-LC phosphorylation is strongly correlated to twitch potentiation, this hypothesis could explain the different results.

The second hypothesis tested in this dissertation was that the mechanisms of length-dependence of muscle activation are related to the mechanisms of length-dependence of staircase potentiation. Studies have shown that the force-length relationship for twitch contractions has a peak force at a muscle length longer than the optimal degree of

filament overlap (Close, 1972a; Rack and Westbury, 1969). Therefore, the apparent level of activation during a single stimulus (twitch contraction) is increased with increasing muscle length. Also, studies with skinned fibers have shown a leftward shift in the force-pCa^{2+} relationship at long muscle lengths (Endo, 1973; Endo, 1972; Martyn and Gordon, 1988; Stephenson and Williams, 1982), similar to what happens with R-LC phosphorylation. Therefore the twitch is already potentiated at a long length when compared to a short length. Further increases in potentiation due to repetitive stimulation are likely to be less than when the muscle is at a short length.

This dissertation is organized in the following way. A review of literature will be presented, with information concerning staircase potentiation in skeletal muscle, and also the force-length relationship for maximally and sub-maximally activated muscles. Following this review, the dissertation is composed of five sequential projects, that were designed to test the stated hypotheses.

The first two projects (chapters 3 and 4) describe the force-sarcomere length relationship for mammalian skeletal muscle, since few studies have concentrated on this issue. Specifically, it was the purpose of these two projects to investigate the relationship between sarcomere length and twitch potentiation. For this purpose, an *in vitro* preparation was used with small fiber bundles of the extensor digitorum longus muscle from the mouse. This preparation was similar to that described by ter Keurs et al. (1984), and allowed measurement of sarcomere length. The results confirmed the length dependence of activity-dependent potentiation. An inverse relationship was found between sarcomere length and twitch potentiation for both staircase and PTP.

The next three projects focussed on the second purpose of this dissertation; to investigate possible mechanisms of the length-dependence of staircase potentiation. The first project focussed on hypothesis (1) stated above, studying the possible role of R-LC phosphorylation in this relationship. The second and third projects were designed to test hypothesis (2), that mechanisms related to length-dependence of Ca^{2+} activation are

responsible for the length-dependence of staircase potentiation. These three studies were performed with an *in situ* experimental preparation, where the whole gastrocnemius muscle length was changed during experiments, when needed. This preparation has been used extensively (MacIntosh, 1991; MacIntosh and Kupsh, 1987; Tubman et al. 1996b; Tubman et al. 1996a; Roszek et al. 1994; MacIntosh and Gardiner, 1987) to investigate twitch potentiation in skeletal muscle.

Chapter 2

Review of Literature

ACTIVITY-DEPENDENT TWITCH POTENTIATION

When skeletal muscle is activated with a single pulse of stimulation, it produces a twitch contraction. The twitch is the most elementary event of muscle contraction, and it has received great attention in the literature as it can be modulated by many factors, such as temperature (Krarup, 1981c; Moore et al. 1990), muscle length (Close, 1972a; Rack and Westbury, 1969), and previous activity (Close and Hoh, 1968a; Krarup, 1981b), among others. Previous activity refers to muscle activation before the twitch contraction is elicited, that may either decrease the contraction force (fatigue), or enhance the contraction force (twitch potentiation). As this dissertation is concerned with twitch potentiation, this will be the focus of this section of the review of literature.

Twitch potentiation may be described as staircase potentiation or posttetanic potentiation (PTP). Staircase potentiation is the increase in DT that occurs during the first seconds of low-frequency stimulation (usually 5-20 Hz) (Close and Hoh, 1968a; MacIntosh, 1991; MacIntosh and Kupsh, 1987). PTP is the force enhancement during a twitch contraction elicited following a tetanic contraction (Close and Hoh, 1968a; Krarup, 1981c; Krarup, 1981b; MacIntosh and Gardiner, 1987). In both cases, the DT is greater during a twitch elicited after a conditioning stimulus when compared to the twitch prior to the conditioning stimulation.

It has been recognized that staircase twitch potentiation occurs without a significant

increase in the contraction time (MacIntosh and Kupsh, 1987; MacIntosh, 1991). This suggests that the enhancement in force during repetitive muscle stimulation is not due to a prolongation of muscle activation processes, since this would be associated with an increased contraction time (Desmedt and Hainaut, 1968). Instead, staircase potentiation is accomplished with an increased rate of force development during twitches, implying that during twitch potentiation there is a greater intensity of activation of the muscle.

Twitch potentiation and R-LC phosphorylation. Although the mechanisms responsible for activity-dependent twitch potentiation are still under investigation, there is overwhelming evidence that phosphorylation of the myosin R-LC is the main mechanism by which twitch potentiation is attained. R-LC phosphorylation is a Ca^{2+} dependent process, where the Ca^{2+}-binding protein calmodulin is responsible for activating the enzyme myosin light chain kinase (MLCK). Light chain specific phosphatase is responsible for dephosphorylation of R-LC. The balance between the activities of these enzymes will determine the extent of R-LC phosphorylation. The maximal rate of phosphorylation of the R-LC is more than twenty times faster than the rate of R-LC dephosphorylation (Moore and Stull, 1984).

At rest, the calmodulin-Ca^{2+} binding domain of MLCK is in a position of partially blocking the binding of R-LC to the active site of the MLCK (Kennely et al. 1990; Kennelly et al. 1990). Each transient increase in intracellular Ca^{2+} concentration during muscle activation leads to the formation of Ca^{2+}-calmodulin complex. This complex binds to the MLCK, converting the enzyme to an active form (Stull et al. 1985; Sweeney et al. 1993). The active form of the enzyme phosphorylates a specific residue in the amino-terminal portion of the R-LC [serine-15 in R-LC from the rabbit fast-twitch muscle (Sweeney et al. 1993)], putting negative charges in that region. During muscle relaxation, dissociation of the Ca^{2+}-calmodulin complexes causes inactivation of the MLCK (Stull et al. 1990). The low activity of myosin light chain phosphatase results in a slow rate of dephosphorylation of R-LC (Moore and Stull, 1984).

Studies with skinned fiber preparations have shown that, at submaximal levels of Ca^{2+}, the force is potentiated when calmodulin and MLCK are added to the bath solution, and the myosin R-LC are phosphorylated (Persechini et al. 1985; Persechini and Stull, 1984; Sweeney and Stull, 1986). The force-pCa^{+2} relationship presents a leftward shift when the R-LC are phosphorylated, indicating an increased Ca^{2+} sensitivity of force generation. Consequently, at a given submaximal Ca^{2+} concentration, there is a greater force development when there is a net increase in phosphate content of the R-LC. When fibers are maximally activated with Ca^{2+}, force is not affected by R-LC phosphorylation (Persechini et al. 1985).

Studies with intact muscle fibers have also shown that R-LC phosphorylation is associated with twitch potentiation (Houston et al. 1985; Klug et al. 1982; Manning and Stull, 1979; Manning and Stull, 1982; Moore et al. 1985). Basically, these studies have shown a strong correlation between the extent of R-LC phosphorylation and the degree of twitch potentiation. Furthermore, it has been observed that, under conditions in which twitch potentiation is absent (Tubman et al. 1996b) or diminished (Tubman et al. 1997), the R-LC phosphorylation is also absent or diminished.

Based on the mechanisms described previously, it is not difficult to understand that R-LC phosphorylation may be the responsible for twitch potentiation in intact muscles. During a tetanic contraction, as a result of very fast activation of MLCK (Manning and Stull, 1979), the levels of R-LC phosphorylation increase rapidly. At the end of the tetanus, although Ca^{2+} concentration decreases rapidly to initial levels, R-LC phosphorylation decreases slowly (Manning and Stull, 1979; Manning and Stull, 1982), due to the low activity of myosin light chain phosphatase. Therefore a twitch elicited just after the tetanus will be potentiated when compared to the pre-tetanic twitch. This is known as posttetanic potentiation (PTP). The situation during staircase potentiation may be similar. During one single twitch contraction, a Ca^{2+} transient likely activates just a small portion of the MLCK. As low frequency muscle stimulation persists, additional fractions of MLCK would be activated, resulting in a net increase in R-LC phosphorylation during

repeated muscle stimulation. This would in turn potentiate force of contraction during the period of repetitive stimulation.

The studies cited above show a strong association between R-LC phosphorylation and twitch potentiation. The actual mechanism by which R-LC phosphorylation increases Ca^{2+} sensitivity and potentiates a twitch contraction has been proposed. Studies with skinned fiber preparations and with x-ray diffraction support a model to explain twitch potentiation by R-LC phosphorylation which will be described below.

Mechanism of R-LC phosphorylation-induced potentiation. The possible explanation for the effects of R-LC phosphorylation on twitch potentiation is that an increase in Ca^{2+} sensitivity is associated with a greater probability of cross-bridges entering the force-generating state (Metzger and Moss, 1992; Sweeney and Stull, 1990). In one of these studies, Sweeney and Stull (1990) measured force, stiffness, rate of force redevelopment and ATPase activity in skinned fibres from the rabbit psoas. These measurements were done before and after R-LC phosphorylation (induced by addition of MLCK to a bath solution). Sweeney and Stull (1990) observed that the force generation, either before or after R-LC phosphorylation, was always directly proportional to stiffness and ATPase activity. Therefore, increased force generation was accomplished with an increased number of cross-bridges binding to actin but without an increase in the time for which each cross-bridge was interacting with actin. Furthermore, they observed that R-LC phosphorylation increased force generation and rate of force redevelopment.

Using a two-state cross-bridge cycle model as proposed by Brenner (1988), they concluded that, while the rate at which cross-bridges enter the force-generating state is increased by R-LC phosphorylation, the opposite (from force-generating to non-force generating state transition) is not affected. This conclusion is based on the assumption that the number of cross-bridges in the strong-binding state is dependent on the two rate constants (rate of transition into and out of the strong-binding state). An increase in the rate at which cross-bridges enter the strong-binding state (force-generating state) is

consistent with their observations. Based on these results, Sweeney et al. (1993) proposed a mechanism by which the myosin cross-bridge is moved away from the myosin backbone filament, increasing the probability of myosin-actin interaction.

In accepting the model proposed by Sweeney et al. (1993), the mechanism that induces the myosin cross-bridge moving away from the myosin backbone may be explained by a greater mobility of the cross-bridges when phosphorylated [see also Levine et al. (1996)]. The phosphorylatable region (serine-15) of the R-LC is situated adjacent to positively charged residues in the N-terminal region (Saraswat and Lowey, 1991). These positively charged residues likely form ionic interactions with negatively charged regions on the underlying myosin rod in the filament backbone. These interactions may stabilize the myosin cross-bridges close to the filament backbone. When phosphorylation occurs, this overall positive charge of the R-LC N-terminus is neutralized, releasing the myosin cross-bridges from their alignment with the filament backbone (Sweeney et al. 1994). In this way the myosin cross-bridges would be more free to interact with actin and enter the force generating state.

This model of the release of the cross-bridges, as explained in the previous paragraph, has received support from studies with x-ray diffraction in skeletal (Levine et al. 1996) and limulus (Levine et al. 1991) muscles. Using rabbit skeletal muscles, Levine et al. (1996) have demonstrated that the cross-bridges become disordered when the R-LC are phosphorylated. This disorder represents a disturbance in the orientation of myosin heads. In this case, each myosin cross-bridge associated with R-LC phosphorylation can spend more time in the vicinity of the myosin-binding sites on the actin filaments, increasing the probability of cross-bridge attachment. It is important to note that these experiments were performed at room temperature (23-25°C), and it has been suggested that the cross-bridges are wrapped around the filament backbone at this temperature in non-phosphorylated conditions (Malinchik and Yu, 1997).

Evidence for these mechanisms in intact muscles is still lacking, but these models are

consistent with the results found with skinned fiber preparations, and with the high correlation between R-LC phosphorylation and twitch potentiation in intact muscles.

Length-dependence of twitch potentiation. The degree of twitch potentiation is affected by muscle length in skeletal muscle. The few studies that have investigated the length-dependence of potentiation show contradictory results. At a conference of the Physiological Society, Wallinga-de Jonge (1979) presented observations demonstrating the length-dependence of staircase potentiation in the EDL muscle from the mouse. These results are consistent with the study by Roszek et al. (1994), who observed that when the rat gastrocnemius muscle was stimulated at 15 and 30 Hz, the degree of potentiation was higher at short muscle lengths than at long lengths.

Similarly, Lopez et al. (1981) have shown that when single fibers from the frog are exposed to potentiating agents (caffeine, Zn^{2+}, NO_3^- and tetraethylammonium ions), the degree of force enhancement is length dependent. These authors (Lopez et al. 1981) found a greater chemical potentiation at short muscle lengths when compared to longer lengths, consistent with the studies with mammalian skeletal muscle cited above (Wallinga-de Jonge, 1979; Roszek et al. 1994).

Different results were reported by Moore and Persechini (1990) when investigating PTP. They observed a greater degree of PTP at lengths longer than optimal, opposite to the results reported in the other studies (Wallinga-de Jonge, 1979; Roszek et al. 1994). As stated in the introduction of this dissertation, they suggested that this difference could be a result of length-dependence of R-LC phosphorylation. The different results obtained in the studies described above are conspicuous and deserve further investigation. The nature of length-dependence of staircase may give important insights into additional mechanisms responsible for twitch potentiation.

Since the length-dependence of potentiation is the main subject of this dissertation, it is important to describe how muscle length affects force production in skeletal muscle.

Furthermore, the force-length relationship is a fundamental property of the sliding filament theory of muscle contraction, and will be described next in this review.

THE FORCE-LENGTH RELATION IN SKELETAL MUSCLE

Single fiber experiments

It is generally agreed that the shape of the force-length relation for isometric contractions includes an ascending limb, a plateau, and a descending limb. This general shape has been reported numerous times, in single cell as well as multicell preparations, and intact muscles. The mechanism responsible for this shape of the force-length relation has been attributed to the sliding filament theory of muscle contraction. On the plateau of this relationship, force is maximal because there is optimal overlap of filaments and therefore the highest number of cross-bridges can interact. At lengths shorter than optimal length, force decreases, and this has been explained by probable interference of thin filaments as these filaments now overlap from opposite ends of the sarcomere. When sarcomere length is increased beyond the optimal length, filament overlap decreases, and this decreases the opportunity for cross-bridge interaction resulting in less developed tension.

Theoretical predictions based on the sliding filament theory, and assuming the cross-bridges behave as independent force generators, clearly indicate that there should be a linear decrease in force from some maximal value, as observed by Ramsey and Street (1940). Also, the linear decrease should begin at the sarcomere length corresponding to the longest length which permits maximal overlap of the thick and thin filaments, and should reach zero developed force at a sarcomere length corresponding with the sum of the lengths of the filaments (myosin filament plus 2 actin filaments, resulting in no overlap of filaments). Predictions concerning the ascending limb are less clear, and for the most part this aspect of the force-length relation is ignored. One of the problems associated with study of the ascending limb is that a muscle at lengths less than optimal is often "slack", and in short duration contractions, some of the time of the contraction is required for just taking up the slack. This makes interpretation of such contractions

difficult.

There are two general approaches which have been used in studying the force-length properties of muscle, and only one of these provides direct evidence in support of the predictions described above. The two approaches include fixed end contractions, and sarcomere or segment length controlled contractions. The observations related to these two approaches are given below. Although fixed end contractions do not provide direct evidence in support of the sliding filament theory, the results can be explained within the sliding filament framework.

Studies using length or segment control. Gordon et al. (1966a) used a spot following device to provide feedback for servo-control of the length of a segment of a single frog skeletal muscle fiber. This device maintained a constant average sarcomere length in a small mid-section of a fiber during contractions. They observed a plateau in developed tension within the sarcomere length range from 2.0 μm to 2.2 μm. Beyond the plateau, as the amount of filament overlap was decreased by increasing sarcomere length, tetanic developed tension decreased in a linear fashion, reaching zero developed tension at a sarcomere length of 3.6 μm, which corresponds with the sarcomere length at which filament overlap would cease. This study offered solid support for the sliding filament theory of muscle contraction. It should be pointed out that in addition to the feedback control of sarcomere length, these authors also used a back extrapolation technique to obtain the apparent developed tension after the initial (fast) rise in tension of a tetanic contraction. This approach avoided the need to interpret the slow increase in tension (creep) which has been attributed to a redistribution of sarcomere length (see below).

Edman and Reggiani (1987) used the segment isometric contraction technique, but rather than a strictly linear decrease in developed tension, they observed a sigmoidal decline in developed tension along the descending limb of the force-length relation. They suggest that such an observation is consistent with a small distribution of sarcomere lengths within the controlled length segment. This distribution would be expected to diminish

the magnitude (width) of the plateau region of the force-length relation, and also permit some force development at sarcomere lengths apparently beyond the point where overlap of filaments would cease. These two deviations from the anticipated linear decline in developed tension give the descending limb of the length tension relation a sigmoidal shape.

Although there are minor discrepancies between studies which have used segment length control in the investigation of the force-length properties of muscle, in general the agreement is quite good, indicating support for the sliding filament theory of muscle contraction (Granzier and Pollack, 1990; Edman and Reggiani, 1987). It should be pointed out however, that it has been suggested that other theories of muscle contraction can be devised, which can be supported by these observations (Granzier and Pollack, 1990).

Studies using fixed-end contractions. In contrast to the results described above, studies using fixed-end contractions (no length feedback and servo-control) have reported levels of force that are considerably greater at a given average sarcomere length than the corresponding forces measured with clamped segment or sarcomere length (Granzier and Pollack, 1990; ter Keurs et al. 1978). The force-length relationship for tetanic contractions observed in these studies has an extended plateau, and a descending limb that is highly deviated to the right when compared to the theoretical force-length relationship. This difference has been a focus of criticism of the sliding filament theory by Pollack and colleagues (Pollack, 1988; Pollack, 1983; Pollack, 1995; ter Keurs et al. 1978) as it does not agree with predictions based on this theory. However, it is recognized that inhomogeneity of sarcomere lengths can account for this deviation from the predicted shape of the force-length relationship, and Pollack et al. (1990) have apparently accepted that this feature is not inconsistent with the sliding filament theory. The manner by which inhomogeneity of sarcomere length can account for this feature of the force-length relation is described below.

Hill (1953) first proposed that, in a stretched muscle, redistribution of length occurs during contractions, and this was responsible for the slow phase of rise in force (creep). This idea was advanced by Gordon et al. (1966a; 1966b), who proposed that this tension creep was due to a redistribution of sarcomere lengths during a tetanus, leading to sarcomere length inhomogeneity. In a series of elegant experiments, Edman and colleagues (1987; 1984) have provided evidence that sarcomere length is not generally uniform along the length of an isolated muscle fiber, neither at rest, nor during activation. In fact, inhomogeneity has been shown to exist during tetanic contractions elicited on the descending limb of the force-length relationship by several investigators (Gordon et al. 1966b; Gordon et al. 1966a; Julian et al. 1978a; Julian et al. 1978b; Julian and Morgan, 1979a; Julian and Morgan, 1979b), where sarcomeres situated near the end of the fibers shorten while sarcomeres are stretched in central parts of the fiber. This idea is consistent with findings in highly stretched fibers. The sarcomere length is shorter near the ends of fibers when compared to sarcomere length in the middle part of the fibers (Huxley and Peachey, 1961; Julian et al. 1978a; Carlsen et al. 1961).

If it is assumed that each sarcomere follows a force-length relation according to the sliding filament theory, and that sarcomeres in series have some distribution of length about a mean value, then on the descending limb any sarcomere which is at a relatively long length will be weaker than sarcomeres which are at shorter (closer to optimal) lengths. Therefore, on activation, long (weaker) sarcomeres should lengthen, and short sarcomeres should shorten. Complimentary to this theory is the notion that the force-velocity relation permits lengthening sarcomeres to resist stretching with greater force than they can generate isometrically. Therefore, the apparently greater isometric force which occurs on the descending limb in fixed-end contractions can be accomplished by lengthening long sarcomeres and shortening short sarcomeres. In fact, it has been demonstrated that a very slow rate of lengthening of the long sarcomeres would be consistent with the greater force generated by the fiber (Edman and Reggiani, 1984). In this way, it could be said that the muscle force (strength) is determined not by the weakest link (sarcomere) but by the strongest one.

ter Keurs et al. (1978), without using feedback for segment length control, observed a linear decline in developed tension consistent with the sliding filament theory of muscle contraction. They employed the extrapolation method to obtain developed tension after a fast phase of tension development, similar to the technique used by Gordon et al. (1966a). However, they observed a rightward shift in the length-developed tension relation when they measured tension later in the contraction, when a force steady state appeared to have been reached. In selecting fibers for this study, these authors excluded any fibers which exhibited a resting sarcomere length dispersion greater than 0.2 μm along its length. By this exclusion criterion, these authors claimed to have effectively controlled sarcomere length, at least during the rapid phase of force development. It is therefore not surprising that they obtained results similar to those obtained with segment length control when using the extrapolation method. In general, it can be concluded that fixed-end contractions yield a force-length relation which demonstrates enhanced force on the descending limb of this relation. The enhanced force can be explained by inhomogeneity of sarcomere lengths along a single fiber.

Force-length relation in submaximal contractions. The shape of the force-length relation as described above, has been determined in single muscle cells which have been maximally activated (tetanic contractions). Submaximal activation of single fibers results in incompletely fused tetanic contractions. With each level of stimulation, the shape of the force-length relation is altered (Balnave and Allen, 1996). In particular, maximal tension for a given level of activation is developed at a length longer than the length associated with maximal overlap of the filaments [see also Stephenson and Wendt (1984), p.254].

This observation is particularly interesting, since the change in apparent optimal length is in the opposite direction of that which would be anticipated if there was an impact of series elastic properties of muscle. The Hill model would predict that optimal muscle length would be longer for tetanic contractions than for submaximal contractions. It can be assumed that muscle length is the sum of the contractile element length (representing

the sarcomeres in series), and the length of any elastic structure in series with the contractile element. At optimal length, the contractile element should be at the length representing optimal overlap of the filaments. The series elastic component should be extended to a greater length during a tetanic contraction than during a submaximal contraction. This should result in a shorter optimal muscle length for submaximal contractions. This is in contrast with what is known to be the case. Optimal length for submaximal contractions is longer than optimal length for contractions with maximal activation (Close, 1972a; Rack and Westbury, 1969).

Since a greater developed force is actually obtained during submaximal contractions when available cross-bridges are declining in number, this property of muscle has been assumed to be the result of enhanced activation at longer lengths and has been referred to as length-dependent activation. Such an effect could conceivably result from either a length-dependent enhancement of Ca^{2+} release, or a length-dependent enhancement of sensitivity to Ca^{2+}. Increased sensitivity to Ca^{2+} could result from any factors which permit more force at a given level of Ca^{2+} concentration.

Studies using skinned fibre preparations have been particularly important in the investigation of length-dependent muscle activation. These studies have shown a leftward shift in the force-Ca^{2+} relationship when the muscle is stretched to lengths beyond optimum (Endo, 1972; Endo, 1973; Martyn and Gordon, 1988; Stephenson and Williams, 1982; Moisescu and Thieleczek, 1979), suggesting that the length-dependence of muscle activation is due to a length-dependence of Ca^{2+} sensitivity to force. In this case, a greater force at a given submaximal Ca^{2+} concentration is obtained when measurements are made at longer sarcomere lengths. Over the past years this has been an exciting area of investigation [for extensive reviews of some of the aspects involving this issue see Moss (1992) and Fuchs (1974)], and now it is believed that changes in interfilament spacing as a consequence of changes in muscle length may be responsible for length-dependent changes in Ca^{2+} sensitivity (Godt and Maughan, 1981; Moss et al. 1983; Fuchs, 1985; Fuchs and Wang, 1991; Wang and Fuchs, 1995; Patel et al. 1997).

Length-dependence of activation can be explained on the basis of mechanical features of the myofilaments. It has been shown that intermyofilament spacing decreases as skeletal muscle is stretched (Rome, 1968; Matsubara and Elliott, 1972; Rome, 1972). Using skinned fibers of the soleus muscle from the rabbit, Godt and Maughan (1981), confirmed that compression of the myofilament lattice with high molecular weight dextran, resulted in enhanced Ca^{2+} -sensitivity. These results have been confirmed by other investigators (de Beer et al. 1988b; Stienen et al. 1985; Wang and Fuchs, 1995).

The conditions within the myofilament space which effect length-dependence of Ca^{2+} sensitivity are still unknown. One possible explanation is that in order to engage in a force-generating state the myosin cross-bridge must first move away from the thick filament backbone (Rayment et al. 1993). The greater the distance the thin filament is from the thick filament, then the less the likelihood of actin-myosin binding. According to this model, reducing intermyofilament distance by stretching (or osmotic compression), would increase the probability of cross-bridge interaction with actin thereby increasing the number of cross-bridges engaged in the strong-binding state and thus the amount of tension production.

Another somewhat related hypothesis, presented by Zhao and Kawai (1993) is that lattice compression of muscle fibers helps to stabilize the strong-binding actin-myosin-ADP complex, which is thought to be the force-generating cross-bridge state in current models of muscle contraction [see work by Brenner et al. (1988; 1986; 1991)]. After compression of skinned fibers, the authors observed that the rate of transition from weak to strong-binding states of cross-bridge attachment was increased, while other steps involving detachment and weak-binding states were not affected. Interestingly, this effect is similar to the consequence of R-LC phosphorylation, which is also thought to bring the cross-bridges closer to the thin filament.

Generally, the studies done using single fibers support the sliding filament theory of muscle contraction. Studies with fixed-end contractions show an extended plateau and

higher forces than expected by theoretical predictions, but the results can still be explained by the sliding filament theory. At sub-maximal levels of activation, the force-length relationship shows a different shape, and this change may be due to length-dependence of Ca^{2+} sensitivity.

Force-length relation in isolated whole muscle

It is difficult to use the segment or sarcomere length control approach to the study of the force-length properties of multicellular muscle preparations, which includes bundles of fibers as well as whole isolated or semi-isolated muscles. With the exception of experiments done by ter Keurs et al. (1984), using small bundles (6-20 fibers) from the EDL muscle of the rat, such experiments fall into the category of fixed end contractions. The results of ter Keurs et al. (1984) are not significantly different from those found with single fiber preparations. Therefore we will not further discuss segment controlled experiments in this section.

Shape of the whole muscle force-tension relation. The sarcomere force-length relation has not been studied in detail in multicellular preparations partially because it is very difficult to measure sarcomere length in such preparations. In general these studies use muscle length as an indicator of sarcomere length, and the results agree mostly with observations made in single cell preparations with sarcomere length measurement. In one of these studies, Rack and Westbury (1969) have shown an extended plateau in the force-length relation for tetanic contractions, where the developed force did not start to decrease significantly until a muscle length that was estimated to give a sarcomere length of $\sim 3.0\ \mu m$.

Although using fixed-end contractions, Close (1972a) has reported a force-length relation similar to that observed in studies using segment control (Gordon et al. 1966a; Granzier and Pollack, 1990; Edman and Reggiani, 1987), i.e., the force decreased linearly with decreasing filament overlap. There is not a clear explanation for this difference.

Force-length relation in submaximal contractions. As in studies with single cells, studies with whole muscles have revealed that the force-length relationship may be dependent on level of muscle activation. Several studies show an extended plateau in the force-length relation for twitch contractions (Close, 1972a; Rack and Westbury, 1969; Roszek et al. 1994) and sub-maximally activated muscle (Roszek et al. 1994), and the descending limb of the force-length relation was shifted to longer muscle lengths.

Consistent with the hypothesis that this higher force at a longer length is due to a length-dependence of muscle activation, Wendt and Barclay (1980) have observed that dantrolene, a drug that decreases Ca^{2+} release from the sarcoplasmic reticulum, therefore decreasing the level of muscle activation, shifted the peak tetanic tension to longer lengths either in soleus or EDL muscles from the mouse.

These results agree with the studies cited previously in this review, using intact and skinned single skeletal muscle cells, that besides the degree of filament overlap, the levels of muscle activation also influence the shape of the force-length relation in skeletal muscle.

SUMMARY

Twitch potentiation is a complex phenomenon not completely understood yet. Studies investigating length-dependence of twitch potentiation are contradictory and further investigation is needed in this area. Based on the literature regarding mechanisms of twitch potentiation and the force-length relation in skeletal muscle, two hypotheses are suggested to explain the length-dependence of staircase potentiation: (1) length-dependence of R-LC phosphorylation is responsible for length-dependence of staircase potentiation, and (2) mechanisms of length-dependence of muscle activation are related to the length-dependence of staircase potentiation. The next chapters consist of studies performed to investigate the length-dependence of staircase potentiation.

Chapter 3

Force-sarcomere length relationship for twitch and tetanic contractions of the mouse extensor digitorum longus muscle

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INTRODUCTION

The sliding filament and cross-bridge theories of muscle contraction (Huxley, 1957; Huxley, 1969) predict that the amount of tension produced by a fully activated muscle is proportional to the number of independently-acting cross bridges attached to actin filaments within each half-sarcomere. According to the sliding filament theory, developed tension should be proportional to the degree of overlap between actin and myosin filaments. This theory obtained substantial support from the study by Gordon et al. (1966a), who investigated the force-length relationship in single frog skeletal muscle fibres. These authors used a spot following device that maintained isometric sarcomere length in a small portion in the mid-section of a fibre during contractions. It was observed that, beyond the plateau of the force-length relationship, as the amount of filament overlap was decreased tetanic developed tension decreased proportionally.

The force-length relationship provides an important experimental evidence of the sliding filament theory of muscle contraction, and it represents a fundamental property of skeletal muscle. However, few studies have investigated the force-sarcomere length relationship in mammalian skeletal muscle. ter Keurs et al. (1984) studied the extensor digitorum longus (EDL) muscle of the rat, using a sarcomere length clamping technique

similar to that used by Gordon et al. (1966a). They showed that the descending limb of the force-length relationship was deviated to the right compared to theoretical predictions according to expected filament overlap.

Phillips and Woledge (1992), who studied the omohyoideus muscle from the mouse, and Stephenson and Williams (1982), who measured the force response to Ca^{2+} activation in skinned EDL and soleus muscles from the rat, investigated the force-length relationship without controlling the sarcomere length. Instead, they used fixed-end contractions, in which the length of the muscle-tendon unit rather than the sarcomere is maintained constant during contraction. Even with small differences from the study done by Gordon et al. (1966a), the force-length relationship observed in these two studies is reasonably consistent with the predictions of the cross-bridge model of muscle contraction.

However, some features of the force-length relationship of mammalian skeletal muscle still need to be investigated. For example, the force-sarcomere length relationship has not been studied in a large range of sarcomere lengths in intact mammalian muscles in conditions of fixed-end contractions. In this case, stretch of any series elastic component, or weak sarcomeres, may permit shortening of other sarcomeres, resulting in increased sarcomere inhomogeneity (Edman, 1966; Paolini et al. 1977). Therefore, the force-sarcomere length relationship in this condition may be different than the force-sarcomere length relationship when the sarcomere length is controlled. In fact, studies on frog striated muscle using fixed-end contractions have reported levels of force that are considerably greater than the corresponding forces measured when the sarcomere length is regulated (Granzier and Pollack, 1990; ter Keurs et al. 1978).

Furthermore, there is no report in the literature investigating the force-sarcomere length relationship for mammalian muscle twitch contractions. The force-sarcomere length relation for twitch contraction is known to be different from that for tetanic contractions in frog muscle (Close, 1972a). The frog muscle twitch force reaches a peak amplitude

at lengths longer than the optimal length for tetanic contractions, suggesting that the levels of muscle activation influence the force-length relationship in these muscles. Rack and Westbury (1969) have found similar results using the whole soleus muscle from the cat, but they did not measure sarcomere length. The results of these two studies are interpreted as evidence that there is a length-dependence of muscle activation in skeletal muscle, similar to that known to occur in cardiac muscle, as observed by Wang and Fuchs (1994).

A better understanding of the force-length relationship in mammalian muscles is needed. The aim of this study was to investigate the force-sarcomere length relationship of the mouse EDL muscle for tetanic and twitch contractions using fixed-end conditions. We were particularly interested in testing the idea that the force-length relationship for twitch contractions is different from the force-length relationship for tetanic contractions. Also, since the studies reported previously were conducted at room temperature, we performed experiments at both room (22°C) and physiological (35°C) temperatures, to assess possible differences between these two conditions.

METHODS

Male mice weighing approximately 30 grams were housed in a room with a 12:12h light:dark cycle. Standard mouse chow and water were provided *ad libitum*. Care and treatment of these animals were in accordance with the Canadian Council on Animal Care, and all procedures were approved by the an ethics committee of the University of Calgary.

Muscle preparation. The animals were deeply anaesthetized with an intraperitoneal injection of pentobarbitol (60 $mg \bullet kg^{-1}$). The right hindlimb was shaved and an incision was made along the surface of the hindlimb. The tibialis anterior muscle was removed to expose the EDL muscle. The EDL muscle was transferred to a dissecting chamber, through which Krebs Henseleit solution (NaCl, 125 mM; KCl, 4.7 mM; NaH_2PO_4, 1.78

mM; $MgCl_2$, 1 mM; $CaCl_2$, 1.9 mM; $NaHCO_3$, 24 mM; Dextrose, 10 mM) was pumped. This solution was bubbled continuously with 95% O_2 and 5% CO_2 (pH = 7.4, temperature = 22°C).

The muscle of the third digit was selected for these experiments. Using a pair of iris scissors, forceps and small probes under a microscope with dark-field illumination at 40X magnification (maximum), a fibre bundle was carefully dissected from the selected muscle. Experiments were performed with small fibre bundles (approximately 6 to 30 fibres, 10-12 mm long) of the EDL muscle. Care was taken to clear the fibres from connective tissue as much as possible.

After dissection, the tendons of the dissected fibres were gripped with small pieces of T-shaped aluminum foil as close to the end of the fibres as possible. The bundle of fibres was then transferred to an experimental chamber, with continuous superfusion of the same solution as in the dissection chamber. One tendon clip was attached to a transducer hook and the other to a Cambridge motor arm (model 300B), allowing the fibre bundle to be suspended horizontally and positioned to a specific sarcomere length, as desired during the experiment. Experiments were performed at 22°C and 35°C.

Sarcomere length measurements. The sarcomere length was measured by laser diffraction. A 10 mW He-Ne laser (Model 1125, Uniphase, Manteca, California) with a beam diameter of 0.8 mm was directed through the experimental chamber perpendicular to the long axis of the muscle fibres. The sarcomere length was established in a clear region of the bundle, close to the force transducer. Although it is known that irregularities in sarcomere length may occur along the fibers (Huxley and Peachey, 1961; Julian et al. 1978a; Carlsen et al. 1961), this region was chosen because in most experiments it was not possible to obtain a clear diffraction pattern in all regions of the fibre due to adherent damaged fibres and/or connective tissue.

The position and intensity distribution of the first-order diffraction pattern was monitored

by a photodiode array which was scanned electronically every 0.5 ms. The diffraction pattern coming from the muscle was collected in a 10X objective lens (N.A. 0.5, Nikon) and reflected through an access port in the microscope. The transmitted laser was projected via a telescopic lens and three other cylindrical lenses onto a photodiode array (RL-128A, Reticon Corp., Sunnyvale, California). An amplifier was used to produce an analog signal, the voltage of which was proportional to the sarcomere length, based on the median intensity profile of the first-order diffraction pattern. The system was calibrated before every experiment with test gratings (12.5 μm). The intensity distribution of the first-order diffraction pattern was monitored during the experiments with an oscilloscope.

Muscle bundles were adjusted to the desired length during experiments with the servo Cambridge motor. Muscle tension and median sarcomere length were displayed on the computer screen during the experiments.

Muscle force measurements and stimulation procedures. Muscle force was measured with a semiconductor strain gauge transducer (ENTRAN, ESU-060-350, 360Ω impedance, Intertechnology Inc., Calgary) connected to an amplifier in a half-bridge configuration. The position of the force transducer was maintained constant during the experiments.

Stimulation was with supramaximal (10-50V) square pulses, 0.5 ms duration, through two platinum wires which were arranged in parallel to the muscle fibre bundle in the experimental chamber. Twitches and tetanic contractions (200 Hz stimulation rate; 200-400 ms duration) were elicited at sarcomere lengths that were changed in steps of 0.1 μm to 0.4 μm, with 2 min rest periods between contractions. The developed tension (DT) was measured as the difference between the passive force and the peak force during a contraction.

Muscles were studied first at 22°C and then at 35°C. A 20-30 min equilibration period

was allowed at each temperature. Sometimes during the course of the experiments, the muscle bundles were set at a reference sarcomere length where maximal force was obtained to verify absence of fatigue and/or damage in the preparation. In all cases, the force was not decreased when compared to previous contractions recorded at this same reference length, indicating that fatigue or damage did not occur in our experiments.

RESULTS

Sarcomere length measurements. In many preparations, after obtaining a clear pattern of sarcomere length at rest with the laser diffraction, the tracing was lost during the contractions. This was probably caused by translations of the muscle which resulted in connective tissue and/or adherent damaged fibres moving into the laser beam and interfering with the pattern. When the sarcomere length was clear during the contractions, the tracings showed a varied pattern of change. The variability of sarcomere length was more evident at long sarcomere length, as observed by ter Keurs et al. (1984). This is clear in Figure 1, which shows results from a bundle of fibres stimulated close to the plateau region of the force-length relationship (in this case at 2.46 μm), and results from the same bundle of fibres stimulated at 3.2 μm. At 2.46 μm, the sarcomere length shows an initial shortening, and then stabilizes until the end of contraction. At the longer sarcomere length, however, the pattern of change is more complicated. After an initial stretch it keeps changing during the contraction. This variability of sarcomere length change was observed often when the tracing was clear during the contraction. Due to the variability of sarcomere length during contractions, all results presented herein are related to sarcomere length readings made at the beginning of the contractions (passive sarcomere length), at one position during fixed-end contraction. Results of a given contraction were included only when the sarcomere length after the contraction was the same as it was in the beginning (i.e., the tracing was still clear and without apparent change).

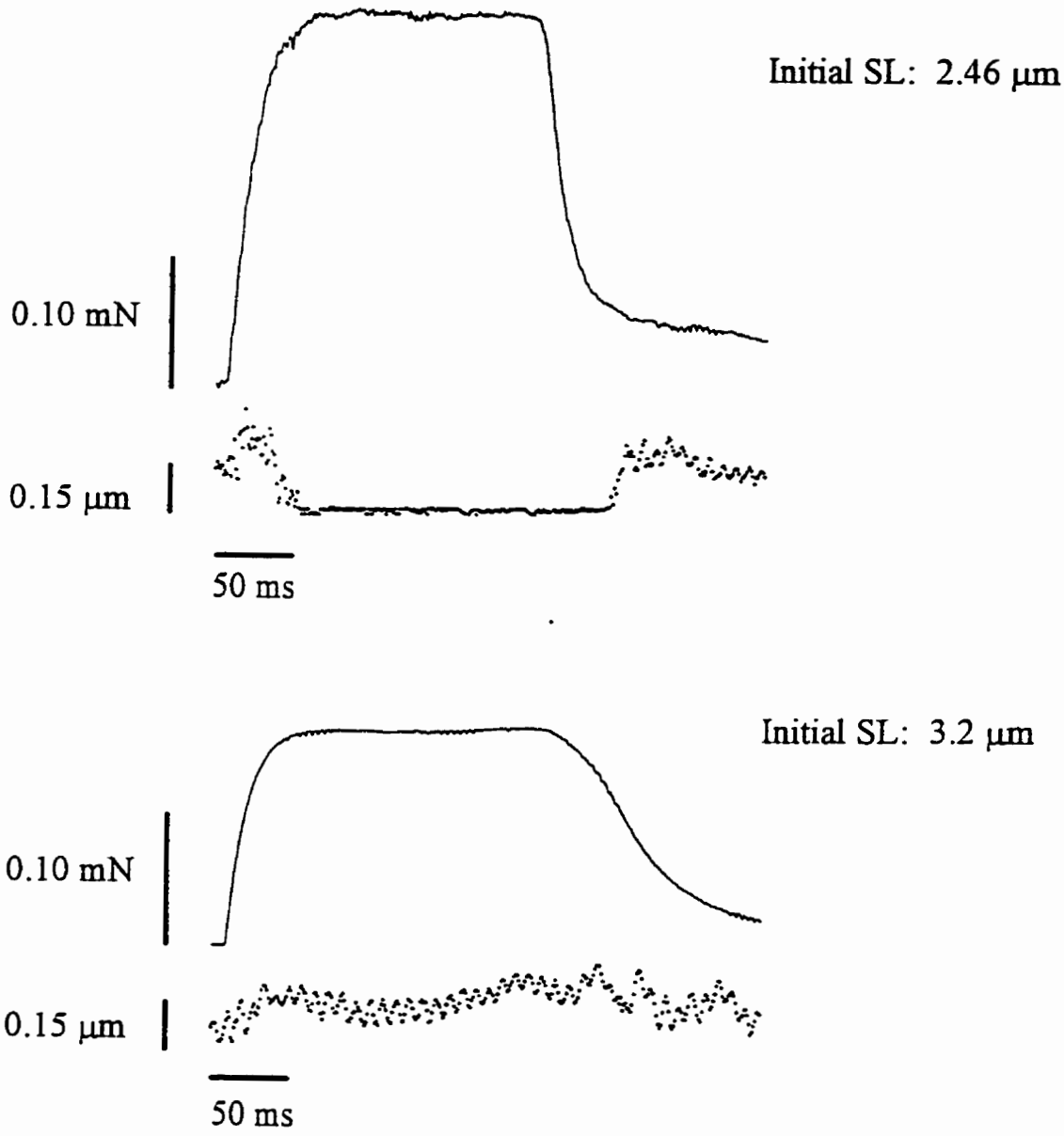

Figure 1. Tetanic contractions recorded at initial sarcomere lengths of 2.46 μm and 3.2 μm. In each panel, upper trace represents developed force and bottom trace shows the sarcomere length. In both cases, the fast and slow phases of increase in force are apparent. At an initial sarcomere length of 2.46 μm , there is first sarcomere shortening (downward deflection), but during the force plateau the sarcomere length is relatively stable. In the lower panel, the pattern is more variable. After an initial stretch it keeps changing during the contraction. Note also the lower developed force when the initial sarcomere length is at 3.2 μm.

Figure 1 also shows the expected decrease in force produced by setting the muscle at a long length. In this case, force was about 60% of maximal at a sarcomere length of 3.2 μm. Also, the two phases of rise in force, described by Gordon (1966a), i.e., the fast and the slow components of rise in force, are visible in these examples.

Effects of temperature. The effects of temperature on typical twitch and tetanic contractions recorded during the experiments are shown in Figure 2. As expected (Moore et al. 1990; Kossler and Kuchler, 1987), twitches elicited at 22°C presented a greater force development than twitches elicited at 35°C. This difference becomes smaller at longer sarcomere lengths (Figure 2B), possibly because the degree of activation during a twitch contraction appears to be greater at long compared to shorter sarcomere lengths (see below). Figure 2C shows three tetanic contractions, one recorded at 22°C, and two contractions recorded at 35°C (with a 5 min interval between the second and third contractions). Contrary to twitch contractions, the tetanic force magnitude does not change significantly after increasing the temperature, however the time course of the contractions is different at the two temperatures. The time to reach peak force and time for relaxation is faster at 35°C than at 22°C. In some cases, the force was slightly greater at 35°C than at 22°C, but the difference was very small.

Force-sarcomere length relationship of tetanic contractions. The force-sarcomere length relationship for tetanic contractions is presented in Figure 3. Since the results obtained at the two temperatures used in this study depict virtually the same force-length relationship [$DT = -1.20 + 1.86 \bullet SL - 0.39 \bullet SL^2$ ($r^2 = 0.87$) and $DT = 0.85 + 0.55 \bullet SL - 0.20 \bullet SL^2$ ($r^2 = 0.86$), for 22°C and 35°C respectively], the pooled results are shown, with the force plotted relative to the highest force obtained at any length in a given experiment. By extrapolating the results obtained on the descending limb of the force-sarcomere length relationship, the force appears to decrease to zero at approximately 3.8 μm - 3.9 μm, consistent with previous findings with intact (ter Keurs et al. 1984) or skinned fibres (Stephenson and Williams, 1982) of EDL muscle of the rat

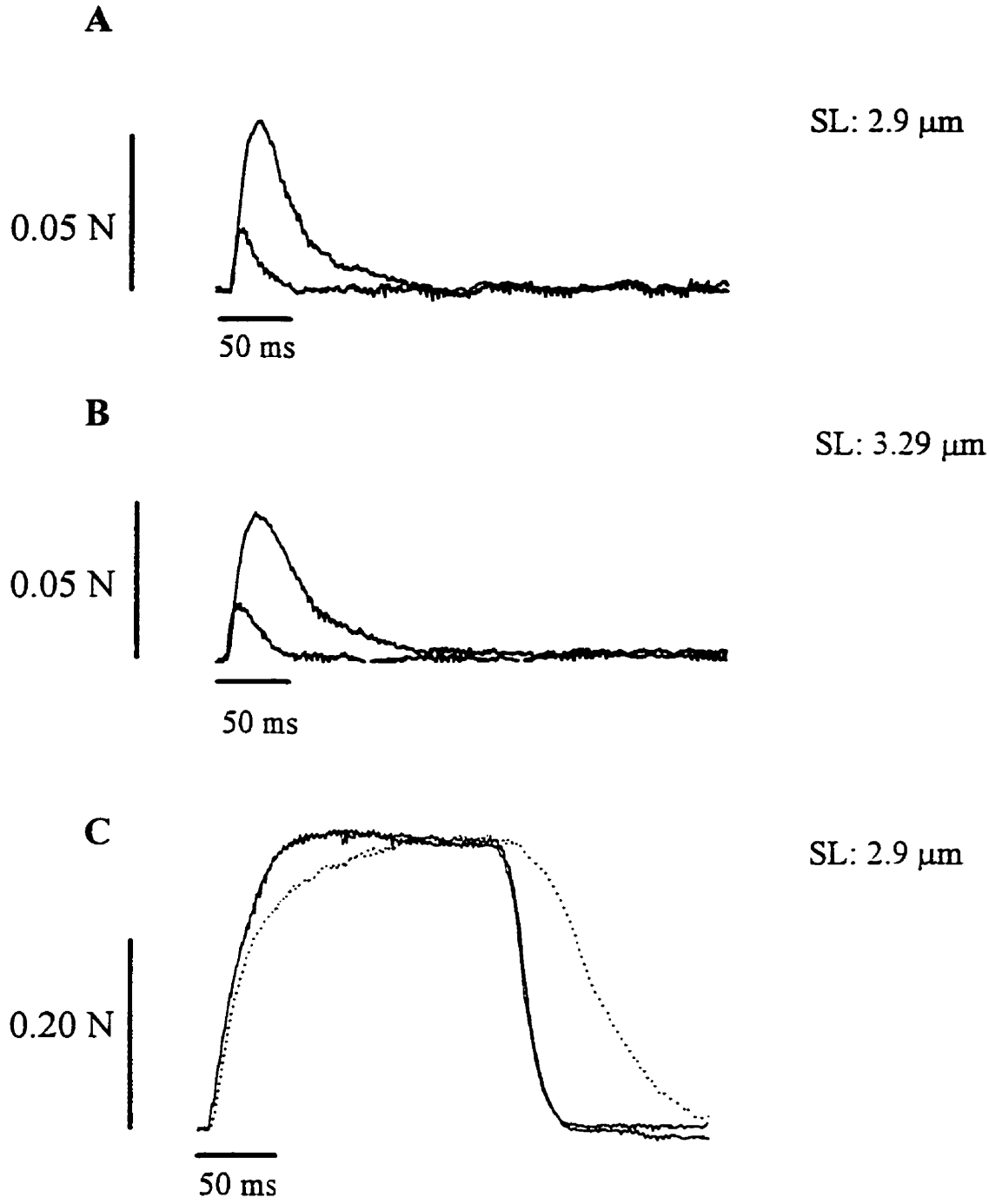

Figure 2. Typical traces of twitch and tetanic contractions recorded at 22°C and 35°C. Panel A shows twitch contractions recorded at a sarcomere length of 2.9 μm, and it is clear that the force recorded at 22°C (upper twitch) is higher than the force recorded at 35°C. Panel B shows the same bundle of fibres and the same conditions, but recorded at a sarcomere length of 3.29 μm. The force at 22°C is still higher than at 35°C, but the difference is less. Panel C shows a tetanic contraction recorded at 22°C and two tetanic contractions recorded at 35°C (sarcomere length: 2.9 μm). The magnitude of force is virtually the same in these conditions. The two tetanic contractions recorded at 35°C show that, during the period of equilibration after changing temperature, the force did not change.

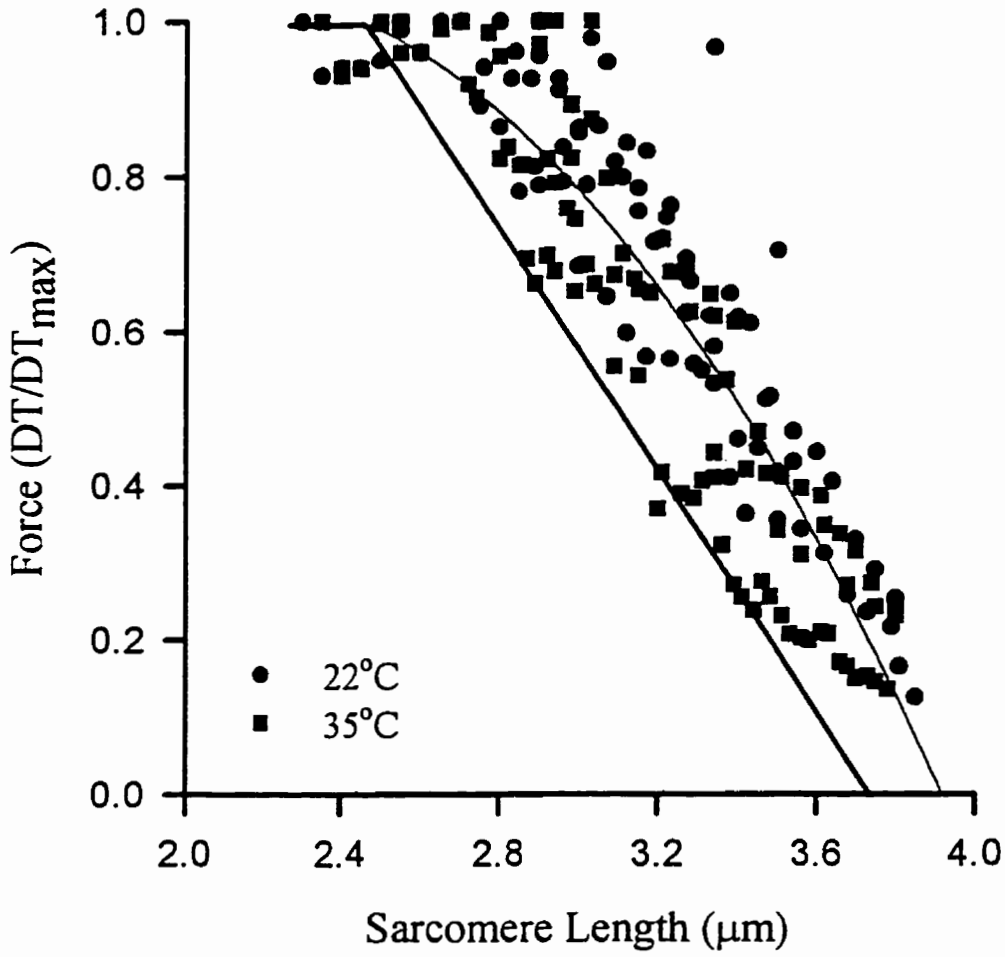

Figure 3: Force-length relationship for tetanic contractions (n=10). The theoretical (thick line) relationship is obtained from ter Keurs et al. (1984) with a plateau extended from 2.26 μm to 2.43 μm and a zero overlap intercept on the descending limb occurring at 3.79 μm. The force (DT) is given relative to the maximum force obtained, and the passive tension has been subtracted, as explained in methods. Note that the shape of the force-length relationship does not change substantially with temperature. Force appears to drop to zero at a sarcomere length of approximately 3.9 μm (thin line, representing 2nd order polynomial least squares fit).

(3.79 μm and 3.93 μm, respectively). The descending limb of the force-length relationship, with the sarcomere length measured at the beginning of the contractions, shows a shift to the right when compared to the theoretical predictions based on the length of myofilaments, obtained from ter Keurs et al. (1984).

Force-sarcomere length relationship of twitch contractions. The force-length relationship of the twitch contractions was much more variable than that for tetanic contractions when data from all fibres were plotted together. This was due mainly to variations in the twitch:tetanus ratio observed in the different experiments. However, in all experiments performed the force plateau is extended to longer sarcomere lengths, when compared to the force plateau of tetanic contractions. In order to evaluate this phenomenon objectively, we considered the right corner of the force plateau as the last twitch contraction detected before the force decreased to 95% (or less) of the value of the maximum force obtained in each experiment. The tetanic force started to decrease at a sarcomere length of 2.87 $\pm$ 0.05 μm (mean $\pm$ SEM) and the twitch force at 3.22 $\pm$ 0.08 μm; this difference was statistically significant ($p < 0.01$). This extended plateau which is consistent with length-dependence of muscle activation is evident in Figure 4. This is similar to results reported by Close (1972a) for frog muscles and Rack and Westbury (1969) for the cat soleus muscle. This seems to be a feature of skeletal muscles, independent of temperature, since we observed this phenomenon at both 22°C and 35°C. In the examples displayed in Figure 4, the twitch:tetanus ratios at 2.9 μm were 0.31 and 0.11, for 22°C and 35°C, respectively. The twitch:tetanus ratio is maximal at a sarcomere length of 3.64 μm and 3.42 μm (0.59 and 0.25, respectively), for 22°C and 35°C.

In Figures 5 and 6, results of representative experiments at 22°C and 35°C are shown. In these figures, the twitch contraction is normalized relative to the tetanic force presented in the figure. Clearly the twitch:tetanus ratio is greater at longer sarcomere lengths. This is because the decline in twitch force begins to occur at a sarcomere length longer than the decline in tetanic force.

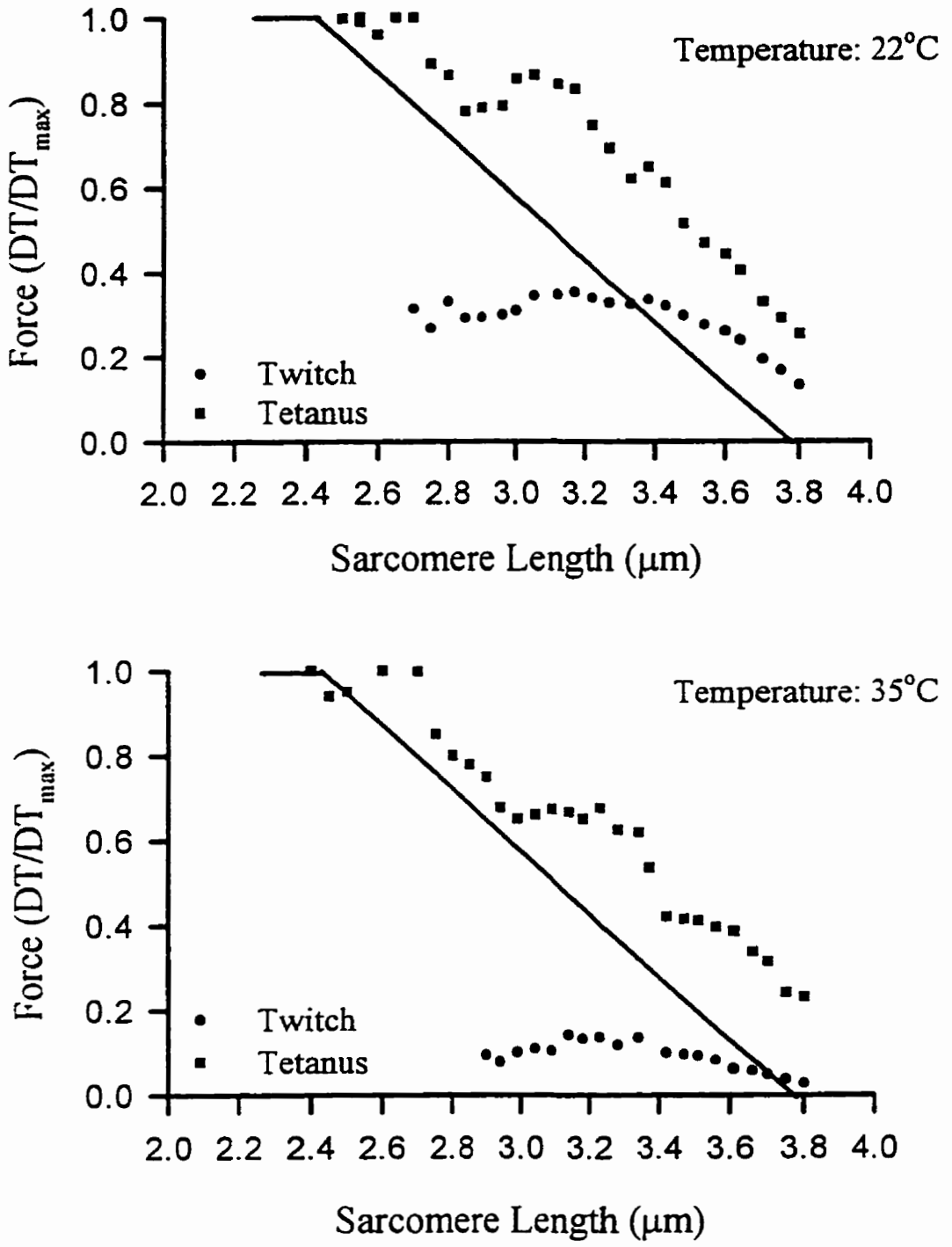

Figure 4. Force-length relationship for tetanic and twitch contractions for one representative muscle, at 22°C (A) and 35°C (B). The force is given relative to the maximum force obtained, after subtracting the passive tension. The plateau of twitch force is extended to longer sarcomere lengths when compared to tetanic contractions at both temperatures. Note also that the twitch:tetanus ratio is smaller at 35°C than at 22°C.

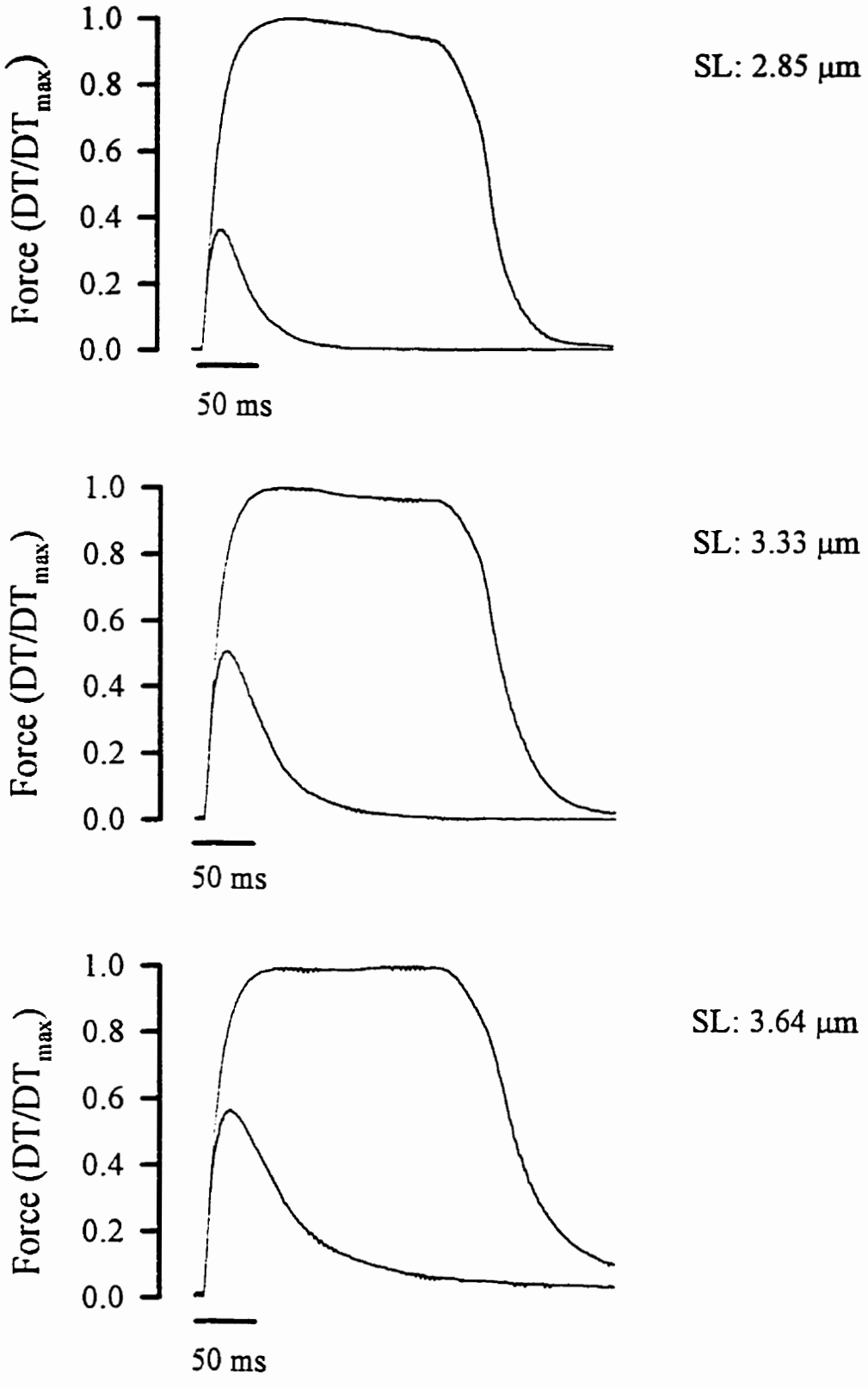

Figure 5. Twitch and tetanic contractions at 2.85, 3.33 and 3.64 μm recorded during one typical experiment performed at 22°C. Force is normalized for the tetanic contraction.

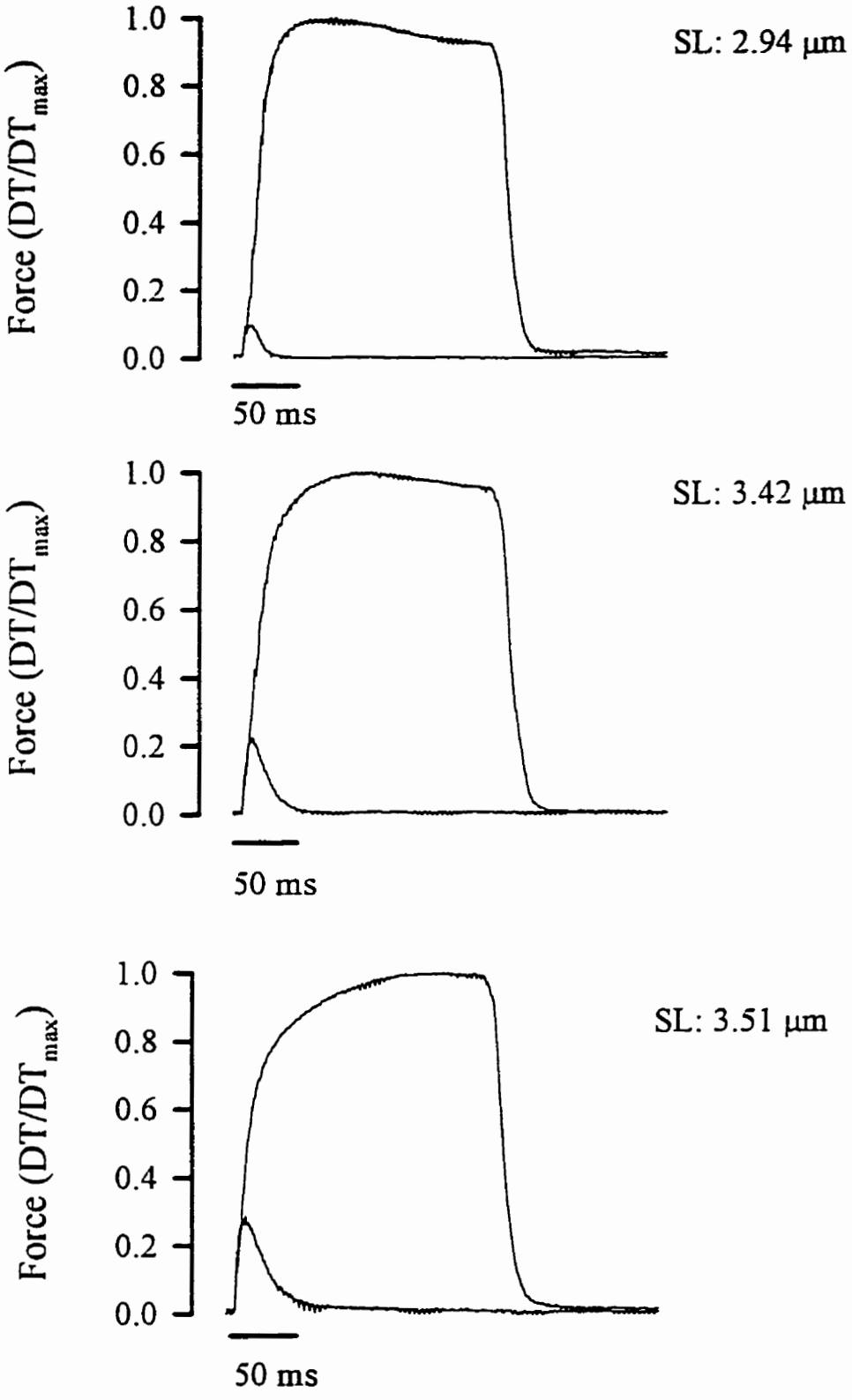

Figure 6. Twitch and tetanic contractions at 2.94, 3.42 and 3.51 μm recorded during one typical experiment performed at 35°C. Force is normalized for the tetanic contraction.

In Figure 6, the twitch:tetanus ratio is also increased with increasing sarcomere lengths, but this ratio is always smaller than that observed at room temperature. As stated before, this finding is apparently a result of a smaller twitch contraction recorded at 35°C, with virtually no change in the amplitude of the tetanic contraction.

DISCUSSION

The main purpose of this study was to describe the force-sarcomere length relationship for the mouse EDL muscle, and special consideration was given to this relationship for twitch contractions. In this regard, this study agrees with previous studies using single fibres from the frog (Close, 1972a) or the whole muscle from the cat (Rack and Westbury, 1969), showing that there is a length-dependence of muscle activation.

Effects of temperature. When the temperature was changed from 22°C to 35°C, force production in the EDL muscle decreased for twitch contractions but there was no significant change in the tetanic force, as reported previously (Moore et al. 1990; Close and Hoh, 1968b; Krarup, 1981c; Lannergren and Westerblad, 1987; Westerblad et al. 1997). Some studies show an increase in maximum tetanic force at a high temperature when compared to room temperature (Westerblad et al. 1997; Kossler and Kuchler, 1987), but in most cases this increase is very small. Therefore, the finding that tetanic force did not change significantly in our preparation is not surprising and is consistent with previous investigations (Close and Hoh, 1968b).

Assuming that tetanic contractions represent maximal activation of the muscle, our results suggest that the higher force observed at 22°C during a twitch contraction (when compared to 35°C) is associated with a higher level of muscle activation attained with a single pulse. The level of muscle activation may be enhanced by increases in Ca^{2+} concentration in the myoplasm or increased sensitivity of the filaments to Ca^{2+}, i.e., higher force attained at a given Ca^{2+} concentration.

Our results would be consistent with either of these two hypotheses, and evidence for both ideas are found in the literature. Characteristics of twitch contractions (longer time course) suggest that Ca^{2+} transients may be prolonged at cold temperatures when compared to physiological temperatures. These include an increased time to peak and an increased half-relaxation time at room temperature relative to 35°C (Moore et al. 1990; Kossler and Kuchler, 1987). These two factors could be dependent on the rate of Ca^{2+} re-uptake during a twitch, and this is probably slower at room temperature (Kossler and Kuchler, 1987). The slower rate of Ca^{2+} re-uptake would also contribute to the observed larger twitch amplitude.

It has been reported that Ca^{2+} sensitivity of contractile proteins of skinned fibre preparations from the rat (Stephenson and Williams, 1985) and frog (Godt and Lindley, 1982) decreases at high temperatures. As a result, a given submaximal Ca^{2+} concentration would lead to a greater force at 22°C when compared to 35°C. This may be a result of a decrease in the affinity of troponin C for Ca^{2+} with increasing temperatures. This could be expected if we assume that Ca^{2+} binding to the troponin C is an exothermic reaction (Potter et al. 1977) and this would result in a lower Ca^{2+} binding rate at higher temperatures. Whatever the mechanism responsible for a higher twitch force at 22°C is, our results support previous studies looking at this issue (Moore et al. 1990; Close and Hoh, 1968b; Krarup, 1981c; Lannergren and Westerblad, 1987; Westerblad et al. 1997).

The force-length relationship of tetanic contractions. The force-length relationship for tetanic contractions observed in this study had a descending limb which was deviated to the right when compared to the theoretical force-length relationship (Figure 3). It also showed an extended plateau where the force did not decrease until well beyond estimated optimal filament overlap.

This deviation from the theoretical force-length relationship, first described by Gordon et al. (1966a), has been observed in different preparations. Studies using clamped

sarcomere length have found a small deviation from theoretical predictions, but the force still decreases linearly with increase in sarcomere length (ter Keurs et al. 1978; Granzier and Pollack, 1990; ter Keurs et al. 1984). Preparations that used fixed-end contractions have observed a more extended plateau, and a descending limb that is deviated further from that predicted by the degree of filament overlap (ter Keurs et al. 1978; Granzier and Pollack, 1990; Phillips and Woledge, 1992). This higher force observed at a given sarcomere length (compared to the theoretical degree of filament overlap) has been investigated, but there is still uncertainty over the cause of this phenomenon.

ter Keurs et al. (1978) have proposed that the force-length relationship obtained with steady-state plateau values of a tetanic contraction represents the state of Ca^{2+} activation that is needed to produce maximum force. They suggest that, if cross-bridges in the central part of the thick filament do not contribute significantly to force production, and/or the probability of cross-bridge attachment increases with distance from the centre of the sarcomere, this could account for higher force in a region when little filament overlap would be expected. This would also explain our results where we observed some force production in a region where the overlap of filaments should be minimal.

Higher forces than predicted could also be explained if there is an inhomogeneity of sarcomeres during force development, as proposed by Gordon et al. (1966a). Inhomogeneity has been shown to occur during tetanic contractions elicited on the descending limb of the force-length relationship (Edman, 1966; Paolini et al. 1977), where some sarcomeres are being stretched while others are shortening. The sarcomeres that are shortening are likely situated near the end of the fibres. In our study, sarcomeres near the tendons may have been operating at a shorter sarcomere length during the contraction, when compared to the passive sarcomere length that we actually measured. Though numerous studies have found very small sarcomere length dispersion in frog skeletal muscle (Edman, 1966; Paolini et al. 1977), this question remains unanswered.

Our results are also in agreement with some studies that used skinned fibre preparations to determine the force-length relationship. Using skinned fibres from the EDL muscle, Stephenson and Williams (1982) observed an extended plateau and data points that deviate slightly from the theoretical force-length relationship. Their results are important in the discussion of this paper, because they measured sarcomere length while the fibres were at rest. This is also what we did, and despite a larger variation found in our study, force was predicted by extrapolation to be zero at a similar sarcomere length. Our results are also in good agreement with Endo (1973) who used skinned toad muscle fibres. He also found an extended plateau (from 2.0 μm to 2.8 μm) and force was about 50% of maximal at about 3.4 μm.

This study did not investigate the mechanisms responsible for the extended plateau of force production, but if we bear in mind the nature of the procedures used, these results are consistent with previous studies in the literature using intact frog (Granzier and Pollack, 1990; Gordon et al. 1966a; ter Keurs et al. 1978) and rat (ter Keurs et al. 1984) fibres, and also with results of skinned fibre preparations (Endo, 1973; Stephenson and Williams, 1982).

Length-dependent activation. As stated in the introduction, the length-dependence of muscle activation in intact muscles has been observed before (Rack and Westbury, 1969; Close, 1972a), but without consistent measures of sarcomere length. This length dependence is characterized by the fact that the optimum length for twitch contractions is longer than it is for tetanic contractions, either in preparations using the whole soleus muscle from the cat (Rack and Westbury, 1969) or single fibres from the frog (Close, 1972a). Furthermore, Balnave and Allen (1996) have used single fibres from the mouse, and observed a graded shift in the force-length relationship to longer lengths as stimulation frequency decreases.

We did not use different stimulation frequencies, but we did observe an increased twitch:tetanus ratio as sarcomere length increased to a certain point, and we suggest that

this phenomenon is due to a length-dependence of muscle activation. Length-dependent activation in skeletal muscle is supported by many studies using skinned fibre preparations. Endo (1972; 1973) used skinned frog skeletal muscle and showed that the Ca^{2+} threshold for contraction was shifted toward lower Ca^{2+} concentrations with increasing sarcomere length. Subsequently, it was shown that the entire force-pCa relationship is shifted to the left with increasing sarcomere length in amphibian twitch-fibres (Moisescu and Thieleczek, 1979) and mammalian fast and slow-twitch muscles (Stephenson and Williams, 1982).

The mechanisms responsible for this length-dependent activation are not yet completely understood. While it has been established that cardiac muscle length-dependence of Ca^{2+} sensitivity operates via an increased Ca^{2+} affinity of troponin C when the muscle is stretched (Wang and Fuchs, 1994), much evidence now suggests that interactions between myofilament spacing and cross-bridge cooperativity (Guth and Potter, 1987) or intermyofilament spacing alone (Patel et al. 1997; Fuchs and Wang, 1991) may be responsible for this phenomenon in skeletal muscle.

It appears that the increased activation that happens at room temperature is not big enough to modify the general force-length relationship, as the plateau for the twitch contractions measured at room temperature is similar to that obtained at 35°C. It may be that, different mechanisms may be operating in these two situations.

The sarcomere force-length relationship for bundles of EDL muscle from the mouse is similar to that reported for frog muscle when stimulated with fixed-ends. Force is higher than that predicted by apparent degree of filament overlap on the descending limb of the force-length relationship. There is a length-dependence of activation in these muscles. The plateau force for twitch contractions is broader and extends to longer sarcomere lengths than the plateau force for tetanic contractions.

SUMMARY

Few studies have investigated the force-sarcomere length relationship in mammalian skeletal muscles. The purpose of this study was to describe the force-sarcomere length relationship for tetanic and twitch contractions for the extensor digitorum longus (EDL) muscle from the mouse. *In vitro* fixed-end contractions of small bundles of EDL fibres were elicited at 22°C and 35°C at average sarcomere lengths ranging from 2.35μm to 3.85 μm (measured by laser diffraction technique). Changes in temperature did not affect the force-length relationship. The force decreased linearly with increasing sarcomere length beyond an extended plateau. This linear force decrease was shifted to the right when compared to theoretical predictions based on the degree of filament overlap, with a zero intercept between 3.8μm and 3.9μm. Twitches had a force plateau which extended to longer sarcomere lengths (started to decrease at 3.22 $\pm$ 0.08μm) than the plateau of tetanic contractions (started to decrease at 2.87 $\pm$ 0.05μm), but the force dropped to zero at similar lengths. This shift in force plateau suggests that the level of activation influences the force-length relationship in mammalian skeletal muscle.

Chapter 4

Sarcomere length-dependence of activity-dependent twitch potentiation in mouse skeletal muscle

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INTRODUCTION

Activity-dependent potentiation is the enhancement of contractile response which occurs as a consequence of prior activation. Staircase, the progressive increase in developed tension during low frequency stimulation, and posttetanic potentiation, the enhancement of twitch developed tension following tetanic stimulation are two common forms of activity-dependent potentiation (Close and Hoh, 1968a; Close, 1972b; Krarup, 1981b). It is generally considered that the enhancement of force during staircase and following a tetanic contraction occurs as a result of phosphorylation of the light chains of myosin (Grange et al. 1993; Sweeney et al. 1993).

It has been reported that there is a length-dependence of potentiation in skeletal muscle. Experiments conducted at 37°C have demonstrated that the magnitude of staircase potentiation is inversely related to muscle length (Wallinga-de Jonge, 1979; Close, 1972a).

Length-dependence of posttetanic potentiation has been evaluated *in vitro* at room temperature, and it has been reported that there is a length-dependence of potentiation, with the fractional enhancement of force being proportional to muscle length (Moore and Persechini, 1990). It would appear that the impact of muscle length on the potentiation

of a twitch is different between staircase and posttetanic potentiation. This difference in length-dependence could be a function of the different temperatures at which the experiments were conducted, or there may be a fundamental difference in the mechanism of posttetanic potentiation relative to staircase.

In this study, the length-dependence of staircase and posttetanic twitch contraction amplitude was evaluated *in vitro*, at room temperature with measurement of sarcomere length. In addition, staircase was assessed at a warmer temperature, to permit comparison with the responses previously done with staircase. The results indicate that there is a length-dependence of force modulation in the twitch contraction for both staircase and posttetanic response, and that in both cases the twitch amplitude after the conditioning stimulus (P_t*) when expressed relative to a control twitch (P_t*/P_t) is inversely proportional to sarcomere length. These experiments confirm that there is no justification for ascribing a mechanism for posttetanic response which is any different from the mechanism for staircase.

METHODS

Male mice weighing approximately 30 grams were housed in a room with a 12:12 h light:dark cycle, and standard mouse chow and water were provided *ad libitum*. Care and treatment of these animals were according to the Canadian Council on Animal Care and all procedures were approved by a University committee for the ethical use of animals for research.

Muscle preparation. The animals were deeply anaesthetized with an intraperitoneal injection of pentobarbitol (60 $mg \cdot kg^{-1}$). The entire extensor digitorum longus muscle (EDL) was dissected from the animals and transferred to a dissecting chamber, through which Krebs Henseleit solution flowed (mM concentrations: NaCl, 125; KCl, 4.7; NaH_2PO_4, 1.78; $MgCl_2$, 1; $CaCl_2$, 1.9; $NaHCO_3$, 24; dextrose, 10). This solution was bubbled continuously in the reservoir with 95% O_2 and 5% CO_2 (pH=7.4, temperature

= 22°C). Small fibre bundles (6-30 fibers were dissected from the EDL, and transferred to an experimental chamber, with continuous flow of the same oxygenated solution. Experiments were then conducted at room temperature (22°C) or the superfusate was warmed to 35°C using a water jacketed heat exchanger positioned close to the experimental chamber.

Procedures. Sarcomere length was measured by laser diffraction, as described in Chapter 3). The sarcomere length was established in a clear region of the bundle close to the force transducer, and it was measured at the beginning and the end of contractions. The advantage of measuring sarcomere length, rather than just setting the muscle at different initial lengths, is that a given change in muscle fibre-tendon unit length does not necessarily reflect a proportional change in sarcomere length. Muscle force was measured with a semiconductor strain gauge transducer (ENTRAN, Intertechnology Inc., Calgary) connected to an amplifier in a half-bridge configuration. Stimulation (Grass S88, Grass Instruments), was with supramaximal (10-50V) square pulses, 0.5 ms duration, through two platinum wires in the experimental chamber parallel to the muscle fibres.

Each fiber bundle was used to assess either staircase (10 Hz for 10s) or posttetanic response (75 Hz for 1.5 s). When staircase was tested, sarcomere length was set, then the stimulation was applied. Three to four sarcomere lengths were tested in each preparation (10 min intervals between each period of repetitive stimulation. The first (P_t) and last (P_t*) contraction in each series were collected (4000 Hz analogue to digital conversion) for assessment of the effects of the conditioning stimulus (P_t*/P_t). The sarcomere length was always the same after the 10 s of stimulation as it had been prior to the stimulation. It is known that R-LC phosphorylation is not different at different lengths following 10 s of 10 Hz stimulation *in situ* (Chapter 5). During the 10 minute intervals between stimulation, control twitch contractions were elicited to verify absence of force decline due to fatigue and/or fiber damage.

To assess the length-dependence of posttetanic response, twitch contractions at 10 s intervals were obtained at a variety of sarcomere lengths both prior to (P_t) and following (P_t*) a tetanic contraction which was elicited at optimal length (the length which gave the strongest tetanic contraction). The first twitch contraction was obtained 30 s after the tetanic contraction, and the subsequent twitches were collected with 10 s intervals. These procedures follow closely what was done by Moore and Persechini (1990), who have shown that at room temperature R-LC phosphorylation and P_t*/P_t following a tetanic contraction return to baseline very slowly.

RESULTS

Postactivity twitch amplitude was evaluated after 10 Hz for 10 s at 35°C, 10 Hz for 10 s at room temperature, and 75 Hz for 1.5 s at room temperature. In each case, the amplitude of developed tension expressed relative to a control twitch (P_t*/P_t) was proportional to sarcomere length, with a negative slope.

When stimulation was 10 Hz for 10 s, at 35°C, there was a staircase potentiation (P_t*/P_t > 1) which was evident at all sarcomere lengths studied (range = 2.4 μm- 3.6 μm). Figure 7A illustrates the force-length relation for twitch contractions obtained at the start and end of 10 s of 10 Hz stimulation at each of 4 different sarcomere lengths in a given muscle preparation. Two features of the force-length relationship should be noted. The obvious point is that enhancement of developed tension, whether expressed in absolute or relative (P_t*/P_t) terms, is much greater at short sarcomere lengths than at long sarcomere lengths. The second point is that the shape of the force-length relation relative to sarcomere length has shifted, such that optimal length (peak of the force-length relation) for potentiated contractions is less than optimal length for nonpotentiated contractions. This pattern is apparent as an increase in developed tension going from 2.4 μm to 3.1 μm prestaircase, as opposed to a decrease over this length range for poststaircase twitch contractions.

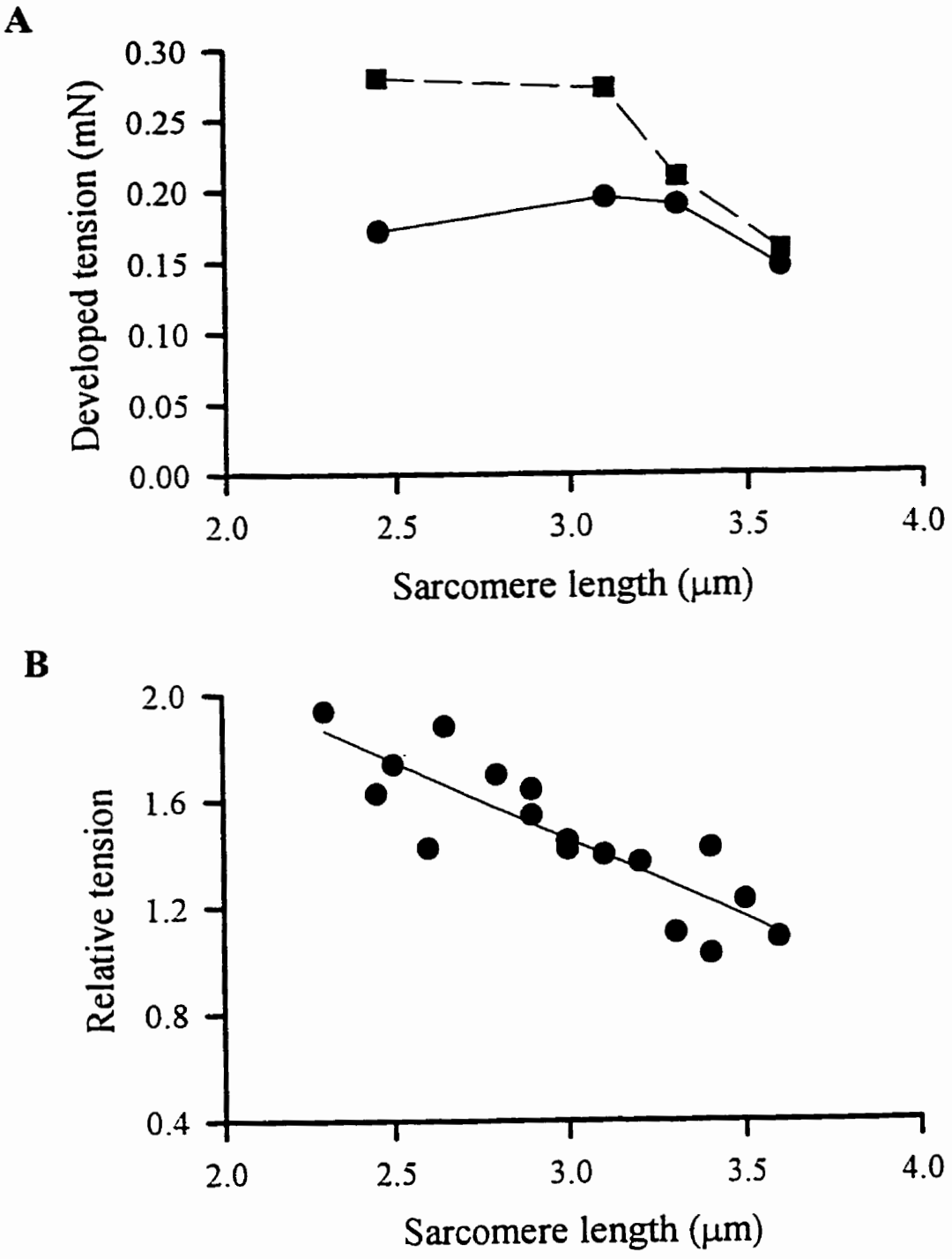

Figure 7. An example of the length-tension relation observed in the experiments conducted at 35°C. Each pair of points (prestaircase, solid line vs. poststaircase, dashed line) was obtained at the start (P_t) and end (P_t*) of 10 s of 10 Hz stimulation at a given sarcomere length. In the lower panel (B), the relative tension (P_t*/P_t) is presented as a function of sarcomere length. Data from 4 muscles are shown. Regression analysis gave r^2=0.74 and P_t*/P_t = -0.59•SL +3.22 (where SL is sarcomere length).

The relative enhancement of force (P_t^*/P_t) is presented in Figure 7B, showing the strong length-dependence of the staircase response, and the virtual absence of potentiation at long sarcomere lengths.

Stimulation at 10 Hz for 10 s at 22°C always resulted in potentiation at short sarcomere lengths, but potentiation was not always observed at long sarcomere lengths. A representative experiment is shown in Figure 8A. Again there appears to be a shift to the left in the length at which the peak developed tension occurs. Figure 8B presents P_t^*/P_t for staircase at a variety of sarcomere lengths in muscles incubated at 22°C. The magnitude of potentiation is less than the magnitude at 35°C, but the length-dependence is still quite evident.

Twitch contractions following tetanic contractions were potentiated only at short sarcomere lengths (22°C). At longer sarcomere lengths, posttetanic depression was apparent. That is, the posttetanic twitch was of a smaller amplitude than the pretetanic twitch ($P_t^*/P_t < 1$). This is clearly evident in the representative experiment illustrated in Figure 9A. In studying posttetanic potentiation, the length tension relation for twitch contractions was obtained in sequential contractions at different lengths, both prior to and after a tetanic contraction at optimal length. For this reason, the force-length relation is more clearly defined in these experiments than in the staircase experiments, for which twitch contractions were obtained at a given length prior to and after repetitive stimulation at that sarcomere length. In Figure 9A, the length associated with maximal twitch amplitude is clearly shifted to the left in the posttetanic contractions. In spite of the posttetanic depression which was evident in these experiments, there was a marked length-dependence of the posttetanic twitch amplitude (see Figure 9B).

DISCUSSION

The primary purpose of this study was to determine if there was a fundamental difference in the length-dependence of staircase and posttetanic twitch contractions.

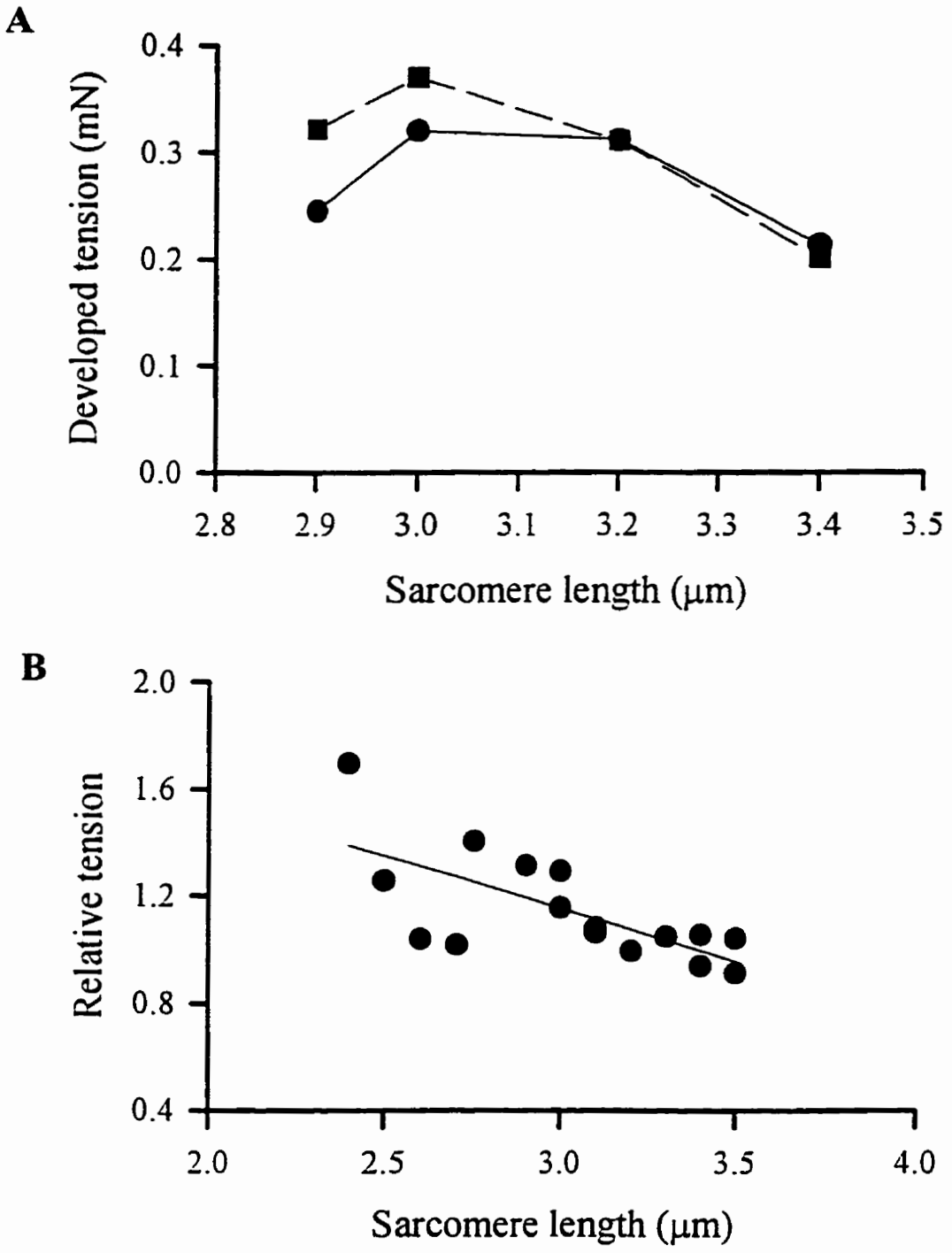

Figure 8. An example of the length-tension relation observed in the experiments for staircase at 22°C. Individual points represent twitch contractions either prior to (P_t, solid line) or after 10 s of 10 Hz stimulation (P_t*, dashed line) at selected sarcomere lengths. Similar to the situation at 35°C, potentiation was greater at short sarcomere lengths than at long sarcomere lengths. In B, the results from 5 muscles are shown. In this case (as in Figure 2B), the tension is expressed as poststaircase developed tension divided by prestaircase developed tension. Regression analysis gave r^2= 0.48 and Tension= -0.39•SL + 2.34 (where SL is sarcomere length).

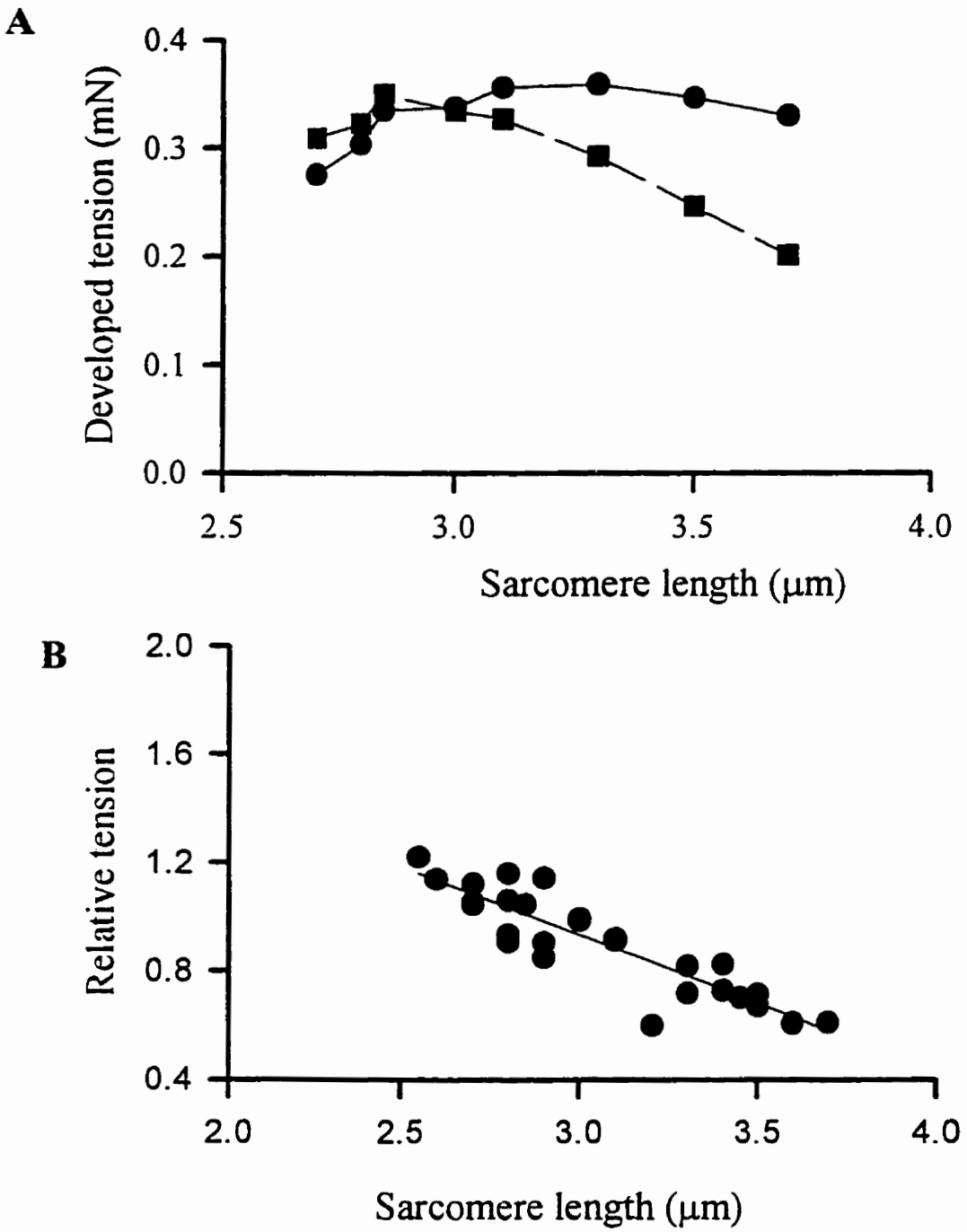

Figure 9. The twitch developed tension at various sarcomere lengths is shown in A for contractions obtained prior to and after a 1.5 s tetanic contraction at 75 Hz when bath solution temperature was 22°C. In this case, like for staircase, there was enhancement of developed tension at short sarcomere lengths, but unlike the case for staircase, there was a clear force depression at long sarcomere lengths. As seen in B, the posttetanic developed tension when expressed relative to the pretetanic developed tension at that sarcomere length decreases with increases in sarcomere length. The results from 4 muscles are shown. Regression analysis gave $r^2=0.80$ and Tension= -0.50•SL + 2.45 (where SL is sarcomere length).

The results of the experiments reported in this paper confirm that both staircase and the posttetanic response have a length-dependence, and that the activity-dependent change in twitch amplitude is inversely proportional to sarcomere length in both cases. These observations for staircase at 35°C represent a confirmation of the length-dependence which has been reported previously at physiological temperatures (Wallinga-de Jonge, 1979; Roszek et al. 1994). Close and Hoh (1968a) also observed a length-dependence of potentiation after 10 Hz repetitive stimulation in frog muscle at 20°C.

The observation that the magnitude of the posttetanic twitch response (P_t*/P_t) is related to length with a negative slope is in direct contrast with a previous report (Moore and Persechini, 1990), but is consistent with data of Roszek et al. (1994), who reported that in the medial rat gastrocnemius muscle (at 27°C) decreasing stimulation frequency resulted in enhanced low frequency responses in comparison with 1 s of stimulation at a given frequency. They observed that the apparent enhancement was greater at short muscle lengths. The similarity of the response with staircase and posttetanic potentiation indicates that the two forms of activity-dependent potentiation probably occur via a similar mechanism. It is generally thought that the mechanism of potentiation is associated with regulatory light chain phosphorylation.

The fact that length-dependence of staircase at 22°C was similar to the length-dependence of staircase at physiological temperature, confirms that there is not a temperature-dependent change in the apparent mechanism which effects the length-dependence of staircase potentiation. In staircase, the slope and the intercept of the relationship between P_t*/P_t and sarcomere length was lower at 22°C than when staircase was conducted at 35°C. These differences were such that there was always greater potentiation at 35°C at any length. This is consistent with previous reports that greater activity-dependent potentiation is obtained at warm temperature than at room temperature (Close and Hoh, 1968b; Krarup, 1981c; Walker, 1951).

It is interesting that the twitch contraction after a brief tetanic contraction at 22°C

demonstrated potentiation when the response was measured at short sarcomere lengths, and depression when the response was measured at long sarcomere lengths. This is consistent with the observation of Krarup (1981c) that in the poststimulation period at room temperature, there are both factors which enhance subsequent contractile response and factors which diminish the contractile response. Krarup (1981c) reported that the dissipation of the depression of force was faster than the dissipation of enhancement, such that after a period of time, the enhancement became evident. In the case of the posttetanic contractions reported here, the enhancement apparently predominated at a short length, while depression was evident at long sarcomere length. This indicates that the factor(s) resulting in depression may have a mechanism which is length-dependent. Alternatively, the depression may be similar at all lengths, and the potentiating effects may be the only factor which is length-dependent.

The depression of twitch amplitude after a tetanic contraction may be a consequence of hydrogen ion or inorganic phosphate (Pi) accumulation. It is known that decreased pH or increased Pi result in a rightward shift in the force-pCa^{2+} relation (Cooke and Pate, 1985; Cooke et al. 1988; Fabiato and Fabiato, 1978). It has been demonstrated at least in the case for pH that the shift is more evident at room temperature than at physiological temperature (Westerblad et al. 1997; Wiseman et al. 1996). This factor may also explain why potentiation is less during staircase at 22°C than at 35°C.

The mechanism for the length-dependence of potentiation may be related to length-dependence of activation. It is known that the force-length relation of submaximally activated muscle is shifted to the right in comparison with maximally activated muscle (Rack and Westbury, 1969; Roszek et al. 1994). This rightward shift is apparently due to enhanced sensitivity to Ca^{2+} as muscle length is increased (Endo, 1973; Stephenson and Williams, 1982). Since regulatory light chain phosphorylation also results in increased sensitivity to Ca^{2+} (Persechini et al. 1985), then it may be that there is a ceiling effect, and the two mechanisms are not simply additive. Light chain phosphorylation may be less effective in enhancing the contractile response at a length

(Yang et al. 1992) when it is already enhanced by length-dependent activation.

The data available in this study cannot discern the possible mechanisms which are operating to result in a length-dependence of P_t^*/P_t. However, the observations in this paper clearly demonstrate that this length-dependence is operative at the sarcomere level, and P_t^*/P_t is proportional to sarcomere length with a negative slope, whether it is studied after high frequency (tetanic contraction) or after low frequency (staircase) stimulation.

SUMMARY

It has been reported that potentiation of a skeletal muscle twitch response is proportional to muscle length with a negative slope during staircase, and proportional to muscle length during posttetanic potentiation. This study was done to permit direct comparison of the properties of staircase and posttetanic responses with measurement of sarcomere length to confirm the nature of the length-dependence of these properties. Mouse extensor digitorum longus muscles were dissected to obtain small bundles of fibers which permit measurement of sarcomere length (SL) by laser diffraction. Twitch contractions were assessed prior to and after 1.5 s of 75 Hz stimulation at 22°C or during 10s of 10 Hz stimulation at 22°C or 35°C. The magnitude of staircase potentiation was greater at 35°C than 22°C, and in all cases the magnitude of the twitch contraction relative to that prior to the conditioning stimulus (P_t^*/P_t) was inversely proportional to sarcomere length, over the range 2.3 μm - 3.7 μm. Linear regression was used to evaluate the length-dependence of the response, which yielded the following: $P_t^*/P_t = -0.59 \bullet SL + 3.27$ ($r^2=0.74$); $P_t^*/P_t = -0.39 \bullet SL + 2.34$ ($r^2=0.48$); and $P_t^*/P_t = -0.50 \bullet SL + 2.45$ ($r^2=0.80$) for staircase at 35°C, and 22°C as well as posttetanic response respectively. After the tetanic contraction, depression rather than potentiation was present at long SL. This indicates that there may be at least two processes operating in these muscles to modulate the force response: one factor which enhances and a second which depresses the force. Either or both of these processes may have a length-dependence of its mechanism.

Chapter 5

Length-dependent potentiation and myosin light chain phosphorylation in rat gastrocnemius muscle

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INTRODUCTION

There is evidence that phosphorylation of the regulatory light chains of myosin (R-LC) is directly related to activity-dependent potentiation of an isometric twitch contraction of fast-twitch skeletal muscle (MacIntosh et al. 1993; Moore et al. 1985; Moore and Stull, 1984; Palmer and Moore, 1989). During repetitive stimulation, each transient increase in cytosolic free Ca^{2+} results in the formation of Ca^{2+}-calmodulin complex. This complex subsequently activates the enzyme myosin light chain kinase (MLCK). This enzyme in turn increases the fraction of phosphorylated R-LC (Barsotti and Butler, 1984; Moore et al. 1985; Moore and Stull, 1984), which is known from skinned muscle fibres to increase the Ca^{2+} sensitivity of force generation (Persechini et al. 1985; Sweeney and Stull, 1986). Hence, at a given submaximal Ca^{2+} concentration, there is a greater force development when there is a net increase in phosphate content of the R-LC.

There is a length-dependence of twitch potentiation. Wallinga-de Jonge (1979) observed that the magnitude of staircase potentiation in rat extensor digitorum longus muscle (EDL) decreased at lengths longer than optimal. This result was confirmed in our laboratory (Chapter 4). In contrast, Moore and Persechini (1990) observed a positive relationship between muscle length and magnitude of posttetanic potentiation in mouse EDL at a given level of R-LC phosphorylation. These authors (Moore and Persechini,

1990) suggested that the observation reported by Wallinga-de Jonge (1979) could be explained if the magnitude of R-LC phosphorylation after staircase at a long muscle length was less than at a short length. This would be consistent with their own observation that R-LC phosphorylation was less following a 1.5 s tetanic contraction at a long length than after similar tetanic contraction at optimal length. However, Barsotti and Butler (1984) observed no difference in R-LC phosphorylation after a 1 s tetanic contraction at optimal length compared to a 1 s tetanic contraction at lengths longer than optimum. Furthermore, because activation of MLCK is Ca^{2+} dependent, and it has been suggested that the Ca^{2+} transients are smaller at short lengths than at long lengths (Lopez et al. 1981; Ridgway and Gordon, 1975), it would be expected that R-LC phosphorylation would be less at short lengths, not greater as suggested by Moore and Persechini (1990).

Clearly, there is a need to evaluate the length-dependence of staircase, and in particular the magnitude of R-LC phosphorylation following a brief period of repetitive stimulation at different lengths. In this study, we tested the hypothesis proposed by Moore and Persechini (1990) that length-dependent staircase potentiation is modulated directly by phosphorylation of R-LC. To evaluate this hypothesis, the extent of potentiation and R-LC phosphorylation in the rat gastrocnemius muscle was measured after 10 s of 10 Hz repetitive stimulation at different muscle lengths.

METHODS

Female Sprague-Dawley rats weighing 160-200 grams were used for this study. Rats were housed in a room with a 12:12h light:dark cycle, and standard rat chow and water were provided *ad libitum*. 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 (University of Calgary).

Muscle length determination *in vivo*. The animals were deeply anesthetized with

intramuscular injections of ketamine-xylazine (ketamine 100 mg/ml, xylazine 100 mg/ml, mixed 85:15 IM; dosage: 0.22 ml). A deep level of anesthesia was maintained throughout the procedures with supplemental injections when needed. *In vivo* length of the gastrocnemius muscle in each animal was measured before isolation of this muscle for contractile assessment. The gastrocnemius muscle length was defined as the distance between the most proximal and the most distal ends of the muscle fibres, and it was measured with a vernier scale (calipers model Measy 2000, Switzerland, error < 0.03 mm). The mean ($\pm$ SEM) muscle length measured *in vivo* was 27.87 ± 0.23 mm. This measured length was used to calculate 10% of the muscle length, to allow adjustment of the muscle length during the experiment, relative to an experimentally determined optimum.

***In situ* muscle preparation.** The triceps surae were exposed and the gastrocnemius muscle was cleared from connective tissue. Insertions of the soleus and plantaris muscles were detached from the Achilles tendon and were partially separated from the gastrocnemius muscle. The calcaneus was cut and the Achilles tendon was connected to an isometric force transducer (model Grass FT10 with black/blue springs) via a thin stainless steel wire and the sciatic nerve was severed close to the spine. The distal stump of the cut sciatic nerve was placed across a pair of stainless steel wire electrodes for indirect stimulation of the left gastrocnemius muscle. The hindlimb was immobilized with two pins: one placed perpendicularly into the femur and the other axially in the severed tibia. The loosened skin from the hindlimb was stretched and anchored to form a pocket which was filled with mineral oil. Rectal and oil bath temperatures were monitored and regulated at $\sim 37°C$ throughout the experiment using radiant heat. At the end of the experiment, the rats were sacrificed with an intracardiac injection of KCl.

Muscle length adjustments during the experiments. Optimal muscle length (L_o) was set during the experiment at the length which gave the greatest developed tension (DT) in response to paired pulses of stimulation (5 ms delay). Paired pulses were used

because it is considered more likely to result in optimal overlap of the myofilaments than using the maximal response to a single pulse of stimulation, which is thought to overestimate the optimal length (Lopez et al. 1981).

The experiments were carried out at L_o - 10%, L_o and L_o + 10%. These changes in muscle length were based on the *in vivo* muscle length measurement. The force transducer was mounted on a rack and pinion device which allowed fine adjustments in the position of the transducer (4 mm per rotation), and thus in muscle length. Prior to each sequence of repetitive stimulation, L_o was determined with a series of double pulse contractions, and length was subsequently changed ($\pm$ 10%) as necessary, relative to this experimentally determined optimum.

In the rat gastrocnemius muscle a large percentage ($\sim$ 60-65%) of the distance between the most proximal and the most distal end of the muscle fibres consists of tendon (Huijing et al. 1994). In this study, we assumed that the tendon during passive stretch is relatively inextensible, resulting in no significant contribution to change in muscle length at rest. Also, we assumed that a 10% change in the estimated muscle length would result in a similar sarcomere length change in all of the animals, i.e., geometry of the gastrocnemius muscle is similar from one animal to another. It was expected that changes of 10% in the muscle tendon complex as used in this study would change the length of muscle fibres by approximately 21%. This estimation assumes that about 13% of the length change is explained by change in the angle of pinnation (Huijing et al. 1994).

Force measurements at different muscle lengths. Single isometric twitch contractions prior to repetitive stimulation and twitches during 10 s of 10 Hz stimulation were assessed at three different time/conditions during each experiment: L_o - 10%, L_o and L_o + 10%. The order in which the lengths were studied in a given animal was randomized in a balanced design.

Immediately following the last repetitive stimulation protocol, the gastrocnemius muscle was frozen with clamps precooled in liquid nitrogen for subsequent R-LC phosphorylation analysis. The muscle was cut from the animal with the clamps in place, and the entire assembly (clamps and muscle) was plunged into the liquid nitrogen. A significant portion of the gastrocnemius muscle was rapidly frozen in this process, and the parts of the muscle tissue that were not completely covered by the precooled clamps was discarded before storing the remaining muscle sample at a temperature of -70°C. The freezing procedure (clamp, cut and plunge) was completed in a few seconds, and it is reasonable to expect that the portion of the muscle between the tongs was frozen before the muscle was cut from the animal. This procedure has been used successfully before with this *in situ* preparation (MacIntosh et al. 1993; Tubman et al. 1996a; Tubman et al. 1996b).

Thirty-six animals were used in this part of the study, giving 12 muscles frozen after staircase at each length (L_o - 10%, L_o and L_o + 10%). Three additional groups were formed with animals that did not undergo repetitive stimulation. The muscles were frozen for analysis of R-LC phosphorylation after 10 minutes of inactivity at each of the length conditions (n=6 at each length).

Muscle stimulation procedures. Stimulation (Grass Model S88) was done with supramaximal square pulses, 50 μs in duration (1-10 V). In these experiments, there were three periods of stimulation, one including staircase at each designated length. In each period of stimulation, paired pulses were initially used to set the muscle at L_o. Subsequently, a twitch contraction was recorded to permit assessment of fatigue. The muscle length was then adjusted to the required position and four twitch contractions (20 s interval) were elicited. The last of these contractions was used to assess the twitch characteristics associated with each length condition. The muscle was then stimulated (single stimuli) for 10 s at 10 Hz, and twitch contractions were recorded at 0, 5 and 10 s. After the repetitive stimulation, the muscle was permitted 10 min of inactivity (rest) to allow dissipation of the potentiating factors associated with the repetitive stimulation,

and more specifically to permit R-LC phosphorylation to return to resting levels.

In addition to the procedures described above, when L_o + 10% was the first length to be assessed, twitch contractions at L_o were recorded prior to and 10 min after the repetitive stimulation. These twitch contractions were used to determine if muscle fibre damage resulted from this condition, since this may occur as a result of stimulation of a stretched muscle (Brooks and Faulkner, 1990).

Digitized (4000 Hz) contractions were analyzed by computer for the following parameters: developed tension (DT), measured as the amplitude difference between the resting tension and the peak of the contraction; contraction time (C_t), measured from the first detectable increase in tension to the peak of the twitch; average rate of force development ($dP \bullet C_t^{-1}$), determined as the developed tension divided by contraction time; half-relaxation time ($\frac{1}{2}R_t$), the time measured from the peak of the contraction until tension decreased half way to the initial tension; and peak rate of relaxation ($dP \bullet dt^{-1}_{min}$), determined as the maximal rate of tension decline over 10% of the relaxation phase of the twitch.

Myosin regulatory light chain phosphorylation. The frozen muscle samples were analyzed to determine the proportion of phosphorylated regulatory light chains (R-LC). The procedure used for analysis of R-LC phosphorylation has been described previously (MacIntosh et al. 1993; Tubman et al. 1996a; Tubman et al. 1996b; Moore and Stull, 1984; Silver and Stull, 1982). The muscle was first pulverized by percussion in an aluminum device precooled in liquid nitrogen, then approximately 8-10 mg of frozen tissue was homogenized at 0°C with a Polytron which remained in a cold chamber, and subsequently centrifuged (15 min. in the cold at 1500 X g). The resulting supernatant was removed and stored in a freezer (-70°C) for subsequent analysis.

The supernatant was subjected to pyrophosphate polyacrylamide gel electrophoresis to isolate the myosin (gel composed of 40 mM sodium pyrophosphate, 20% glycerol, 200

mM ethylenediaminetetraacetate, 0.375 M sodium fluoride and 3% bisacrylamide, at a pH of 8.8). Gels underwent electrophoresis (90 V) for 3.5 hours at O°C. The myosin band was identified with Coomassie Blue stain (R-250, Sigma), cut from the gel, and stored in a freezer (-70°C), usually overnight.

The isolated myosin was subsequently denatured and subjected to isoelectric focusing over a pH range of 4.5 to 6 to separate the phosphorylated from the nonphosphorylated light chains. Gels from isoelectric focusing were fixed with 15% trichloroacetic acid, and then silver stained to identify the bands representing the light chains. The gels were subsequently scanned using a laser densitometer (LKB 2202 Ultroscan), and the phosphate content was expressed as moles of phosphate per mole of total R-LC.

Statistical analysis. A one-way analysis of variance with repeated measures was used to compare single twitch contractions at L_o recorded after adjustment to the three lengths (L_o - 10%, L_o and L_o + 10%) to detect potential fatigue effects. A one-way analysis of variance with repeated measures was also used to compare the twitch contractions at L_o before and after the muscle underwent repetitive stimulation at L_o + 10%, when this was done at the first intervention during the experiments. This procedure allowed us to check for a possible decrease in DT that may have resulted from tissue damage.

Contractions obtained during repetitive stimulation for each of the three conditions (L_o - 10%, L_o and L_o + 10%) were analyzed for DT, C_t, $\frac{1}{2}R_t$, $dP \bullet C_t^{-1}$ and $dP \bullet dt^{-1}{}_{min}$. A two-way analysis of variance (muscle length, time) with repeated measures, was done for each of these contractile parameters. For comparison of R-LC phosphorylation among the three muscle length conditions and the different activity states (resting and stimulated), a two-way analysis of variance was used.

When appropriate, within condition and between condition comparisons established a priori were done with Student Newman-Keuls test. A significance level of $p < 0.05$ was used in all comparisons.

RESULTS

Single twitch contraction. The magnitude of the twitch DT was not different when measured at L_o at different times during the experiment (i.e., before the muscle underwent repetitive stimulation at the various lengths). This finding suggests that there was not a fatigue effect during the experiments. Also, for those muscles exposed to repetitive stimulation at L_o + 10% in the first condition, there was no difference in DT between twitch contractions elicited before and 10 min after 10 s of 10 Hz stimulation. Therefore, intra-animal differences in force magnitude were not apparently due to damage in muscle fibres because of stimulation at elongated muscle lengths.

The characteristics of a single twitch contraction at different muscle lengths are presented in Table 1. The differences found between muscle length conditions for DT, C_t, $\frac{1}{2}R_t$, $dP \bullet C_t^{-1}$ and $dP \bullet dt^{-1}{}_{min}$, were similar to those reported previously (Wallinga-de Jonge et al. 1980). The decrease in DT at muscle lengths other than optimal, together with the prolongation of C_t and $\frac{1}{2}R_t$ during twitch contractions at lengths beyond optimal, supports observations of others who used the whole EDL of the rat (Wallinga-de Jonge et al. 1980) or the soleus muscle from the cat (Rack and Westbury, 1969). A decrease in force of 17.2% at long muscle lengths is somewhat less than results from studies that measured the sarcomere length directly, where a decrease of ~30% is reported for tetanic contractions at the same apparent fiber length (Stephenson and Williams, 1982; ter Keurs et al. 1984). However, this result is not surprising, since it has been shown that preparations that use fixed length contractions give greater developed tension in the descending limb of the force-length relationship than contractions where sarcomere length is controlled during the contraction. For example, Granzier and Pollack (1990) observed a decrease as small as 10% at a similar fiber length, using fixed-end tetani in the semitendinosus muscle from the frog. Furthermore, since length dependence of activation permits an increase of twitch amplitude as length is extended beyond optimal overlap, it is not surprising that the decrease in DT at the long length was not as great as the decrease at the short length.

TABLE 1

Parameters of a twitch contraction at different muscle lengths (mean ± SEM).

	L_o - 10%	L_o	L_o + 10%
DT (N)	1.02 ± 0.06	1.63 ± 0.06	1.35 ± 0.07
$dP \bullet C_t^{-1}$ ($N \bullet ms^{-1}$)	0.09 ± 0.01	0.13 ± 0.01	0.10 ± 0.01
$dP \bullet dt^{-1}_{min}$ ($N \bullet ms^{-1}$)	0.08 ± 0.01	0.09 ± 0.01	0.06 ± 0.01
C_t (ms)	10.6 ± 0.3	12.8 ± 0.34	14.1 ± 0.4
½R_t (ms)	8.4 ± 0.3	11.8 ± 0.3	16.9 ± 0.8

DT, developed tension; $dP \bullet C_t^{-1}$, average rate of force development; $dP \bullet dt_{min}^{-1}$, peak rate of relaxation; C_t, contraction time; ½R_t, half-relaxation time.

L_o, optimal length, L_o - 10%; optimal length less 10%; L_o + 10%, optimal length plus 10%

Repetitive stimulation. Stimulation at 10 Hz for 10 s resulted in a staircase response at all three muscle lengths studied. The results of a typical experiment are presented in Figure 10, showing superimposed traces of twitch contractions recorded at 0, 5 and 10 s of 10 Hz stimulation. It is clear from these traces that the degree of potentiation is inversely related to the muscle length. At L_o - 10%, the increase in twitch DT from 0 s to 10 s of stimulation is greater than at the other lengths. Furthermore, the pattern of relaxation differs in the three conditions studied. When the muscle was stretched beyond L_o the relaxation time became longer and the $dP \bullet dt^{-1}_{min}$ became slower.

The effects of length on potentiation of the isometric twitch are shown in Figure 11A (absolute values) and Figure 11B (expressed as the developed tension relative to the first twitch contraction). Similar to that shown from a typical experiment (Figure 10), a staircase response was present in all conditions, where the mean DT increased during 10 s of 10 Hz repetitive stimulation. When these values were expressed relative to the first twitch contraction (figure 11b), the length dependence of staircase potentiation was more evident. In agreement with Wallinga-de Jonge (1979), the degree of potentiation was inversely related to the muscle length; the magnitude of enhancement of the DT was greater at L_o - 10% and smaller at L_o + 10%. These results also agree with findings of Roszek et al. (1994), who reported a higher degree of potentiation during low frequency stimulation (15 and 20 Hz) at lengths below optimum, when compared to optimum, in the rat gastrocnemius muscle. However, these data are contrary to results observed during posttetanic potentiation (Moore and Persechini, 1990), where muscles at lengths greater than optimum presented a higher degree of force enhancement. These conflicting results may be due to differences in the conditioning stimuli, since Moore and Persechini (1990) used a fixed tetanic contraction at optimal length, and measured posttetanic potentiation by changing lengths during subsequent twitches. Conversely, we have used repetitive stimulation at each muscle length.

The increase in DT during repetitive stimulation was accompanied by an increase in $dP \bullet C_t^{-1}$ in the three length conditions (Figure 12A).

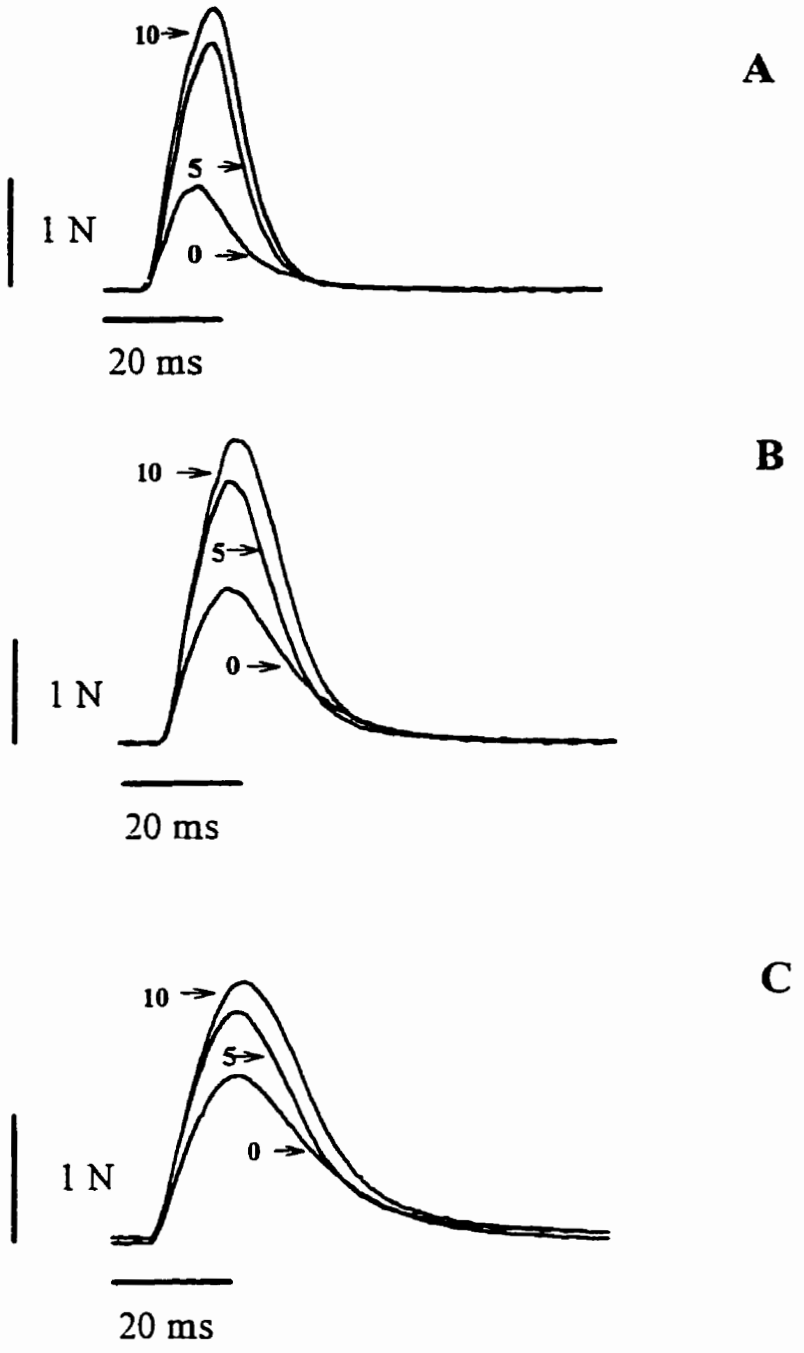

Figure 10. Twitch contractions recorded at 0, 5 and 10 s of 10 Hz stimulation for the: (A) L_o -10%, (B) L_o and (C) L_o + 10% conditions, during one typical experiment.

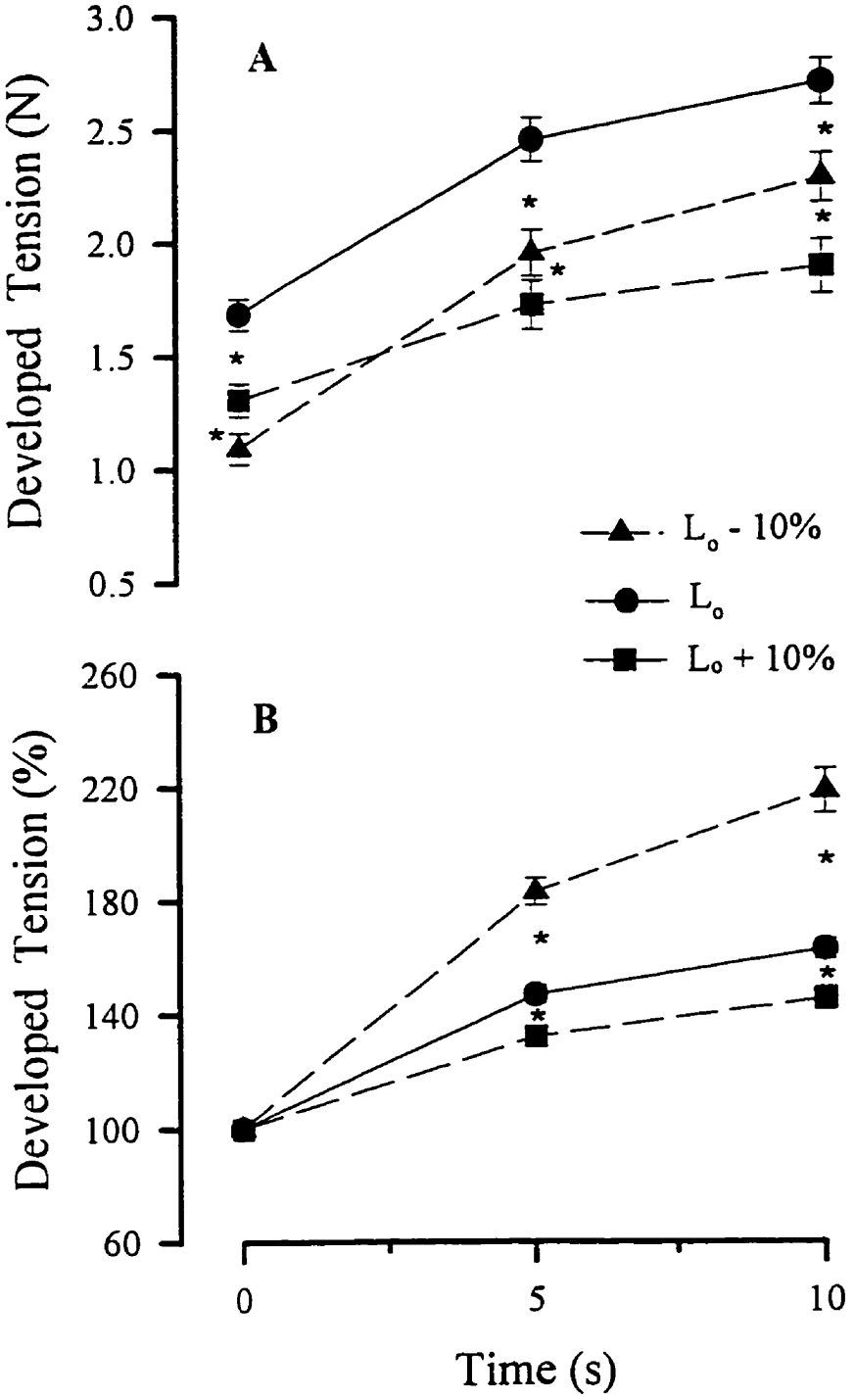

Figure 11. Developed tension vs time for 10 s of repetitive stimulation at 10 Hz. Values of developed tension are in newton (A), or expressed as % of the first twitch contraction recorded at 0 s (B). Each symbol represents mean ± SEM, and asterisks (*) represent a significant difference ($p < 0.05$) between groups at a given time.

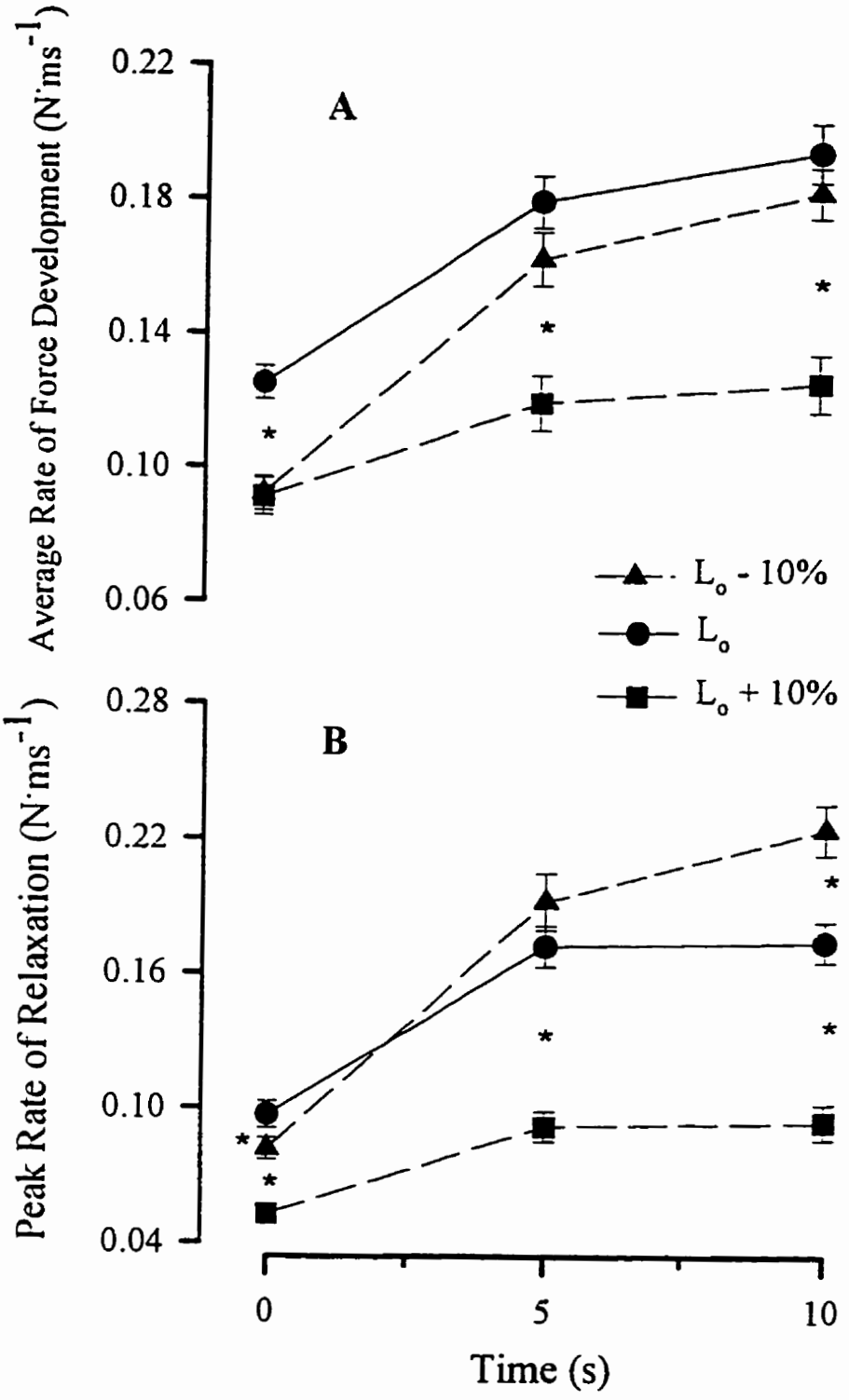

Figure 12. Average rate of force development (A) and peak rate of relaxation (B) vs time for 10 s of repetitive stimulation at 10 Hz. Each symbol represents mean ± SEM, and asterisks (*) represent a significant difference ($p < 0.05$) between groups at a given time.

This parameter followed the same pattern of change as DT, with a greater increase observed at short lengths. This close relationship between increases in DT and rate of force development has been observed previously in studies that show staircase potentiation of mammalian skeletal muscle (MacIntosh et al. 1988). When normalized for the first twitch contraction recorded, the relative increases in $dP \bullet C_t^{-1}$ observed at 10 s were different between all lengths (101.6 $\pm$ 7.7%, 55.6 $\pm$ 4.1% and 37.2 $\pm$ 4.4%, for L_o - 10%, L_o and L_o + 10%, respectively). At L_o and L_o + 10%, the $dP \bullet dt^{-1}_{min}$ increased from 0 s to 5 s then plateaued, but at L_o - 10% a further increase in $dP \bullet dt^{-1}_{min}$ was observed from 5 s to 10 s (Figure 12B). When expressed in relative terms, changes of 201.1 $\pm$ 21.7%, 90.5 $\pm$ 9.2% and 85.3 $\pm$ 6% were observed over the 10 s of repetitive stimulation at L_o - 10%, L_o and L_o + 10%, respectively.

Values for C_t and $\frac{1}{2}R_t$ of the isometric twitch contractions during 10 Hz stimulation are presented in Figure 13. The values of C_t did not change during the staircase response at any muscle length, as shown previously at optimal length (MacIntosh et al. 1988). In all conditions (L_o - 10%, L_o and L_o + 10%) there was a decrease in $\frac{1}{2}R_t$ from 0 to 5 s, and no further change from 5 to 10 s. At 10 s, the values of $\frac{1}{2}R_t$ normalized for the first twitch contraction were not different between these length conditions (78.4 $\pm$ 2.8%, 85.7 $\pm$ 2.4% and 80.8 $\pm$ 2.7%).

Figure 14 shows the gel analysis, and the scan results from typical muscles frozen at rest (A) and under potentiated (B) conditions (in this case at L_o). It is clear that the bands representing the phosphorylated light chains are different at these two different conditions, as are the peaks collected from the densitometer used in quantification of R-LC phosphorylation. Figure 15 shows the mean values of R-LC phosphorylation for the stimulated (n=36) and rested muscles (n=18). Although the poststimulated muscles showed a higher R-LC phosphorylation than the rested muscles, as expected (MacIntosh et al. 1993; Moore et al. 1985; Moore and Stull, 1984; Palmer and Moore, 1989), no differences were observed among L_o - 10%, L_o and L_o + 10% for either the resting or after 10 s of 10 Hz stimulation.

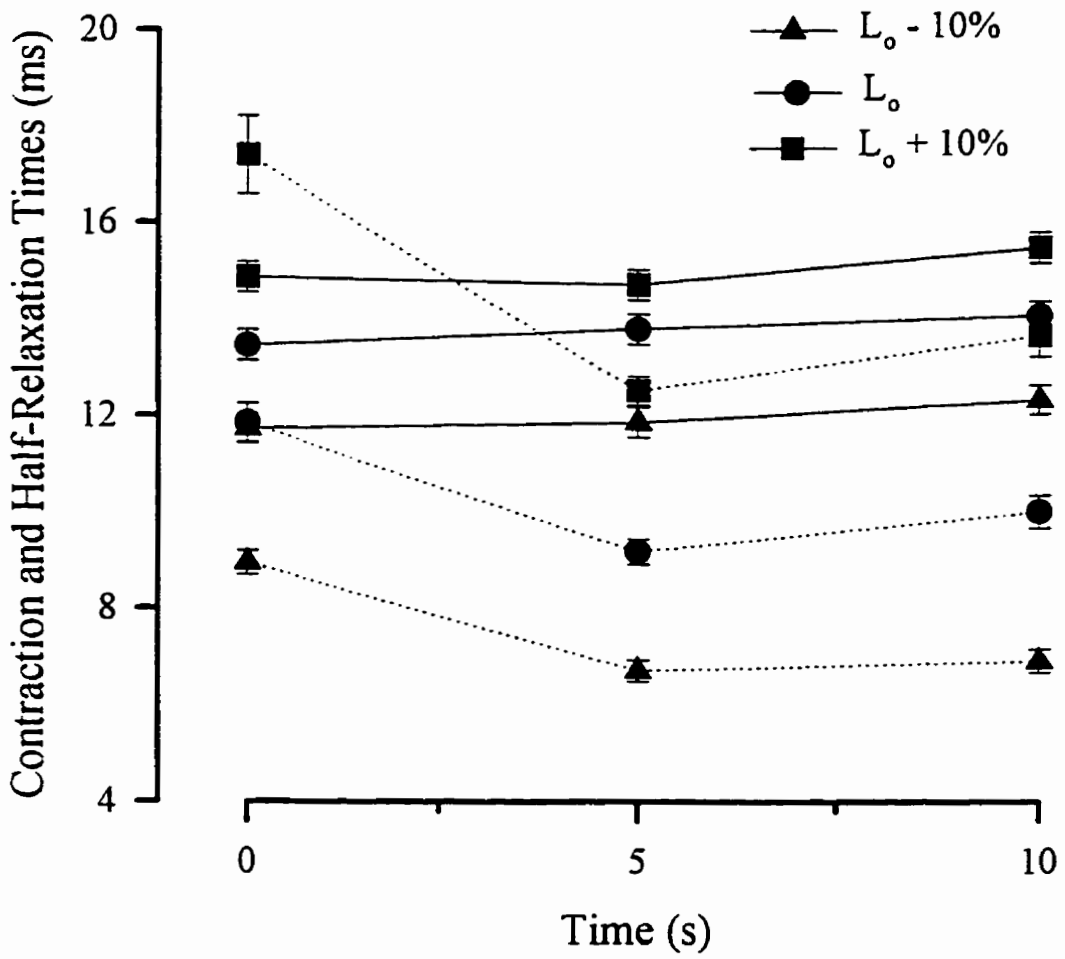

Figure 13. Contraction time and half-relaxation time vs time of 10 s of repetitive stimulation at 10 Hz. The solid lines show contraction time in the different conditions, and the dotted lines show the half-relaxation time. Each symbol represents mean ± SEM.

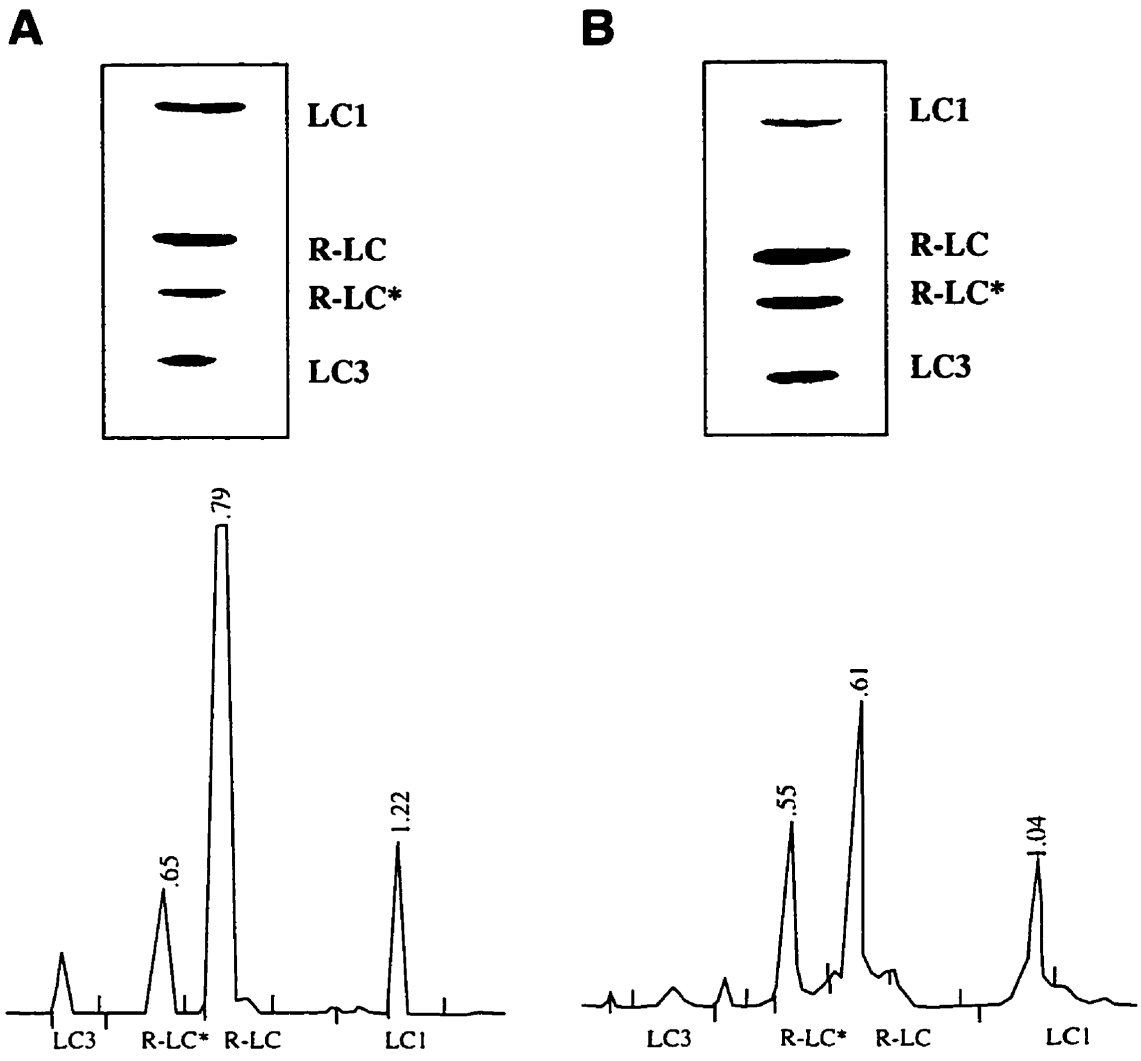

Figure 14. Pyrophosphate-polyacrylamide isoeletric focussing gel illustrating the separation and identification of the three myosin light chains of the rat gastrocnemius muscle. The gels were scanned by a Hewlett Packard Scan Jet IIc. Diagram A represents the resting conditions and diagram B represents the post-stimulated condition (10 s of 10 Hz stimulation). Each lane shows the light chain 1 (LC1), the non-phosphorylated form of the regulatory light chain (R-LC), the phosphorylated form of the regulatory light chain (R-LC*), and the light chain 3 (LC3). In the same figure, the laser densitometer scan of the corresponding gel is shown. The area under the peak representing the R-LC* was used to calculate the phosphate content, expressed as percent of the total (R-LC + R-LC*) regulatory light chains present in the sample. The number over each peak represents the relative position of the band in the specific lane analized on the gel.

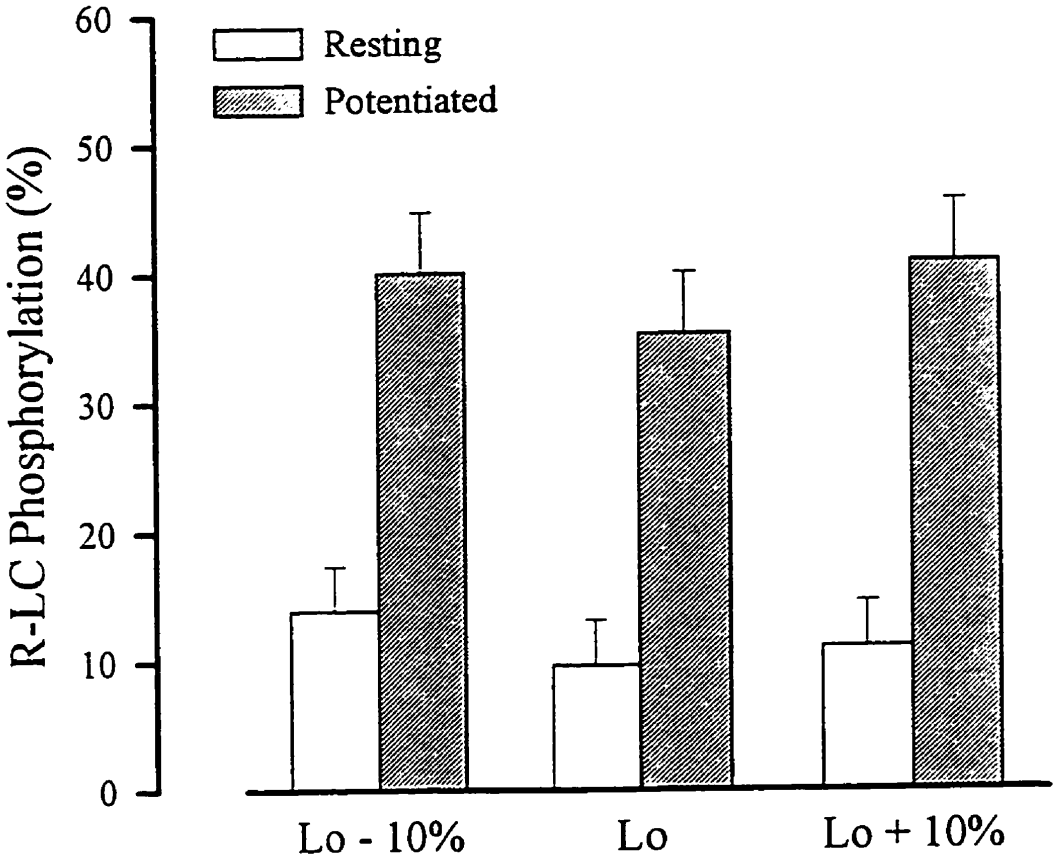

Figure 15. Myosin regulatory light chain phosphorylation at three muscle length conditions, for rested and potentiated (10 s of 10 Hz repetitive stimulation) muscles. Each vertical bar on top of the column represents SEM.

DISCUSSION

As in previous studies (Lopez et al. 1981; Moore and Persechini, 1990; Wallinga-de Jonge, 1979), it was observed that changes in muscle length affected potentiation. Further, the degree of staircase potentiation was inversely related to the muscle length, similar to previous observations (Roszek et al. 1994; Wallinga-de Jonge, 1979; Chapter 4). An increase in phosphate content of the R-LC during repetitive stimulation was also observed in our results, consistent with previous investigations (MacIntosh et al. 1993; Moore and Stull, 1984; Palmer and Moore, 1989), suggesting that potentiation is associated with R-LC phosphorylation. However, this increase was essentially independent of muscle length, suggesting that R-LC phosphorylation does not directly modulate the length-dependence of staircase. This observation does not permit resolution of the apparently contradictory results between the degree of potentiation during staircase (Wallinga-de Jonge, 1979) and during posttetanic potentiation (Moore and Persechini, 1990) at various lengths.

Our results seem to indicate that either factors in addition to R-LC phosphorylation contribute to staircase potentiation or the effects of R-LC phosphorylation on potentiation of a twitch contraction are changed at different lengths. This latter effect could be modulated by the interfilament distance (Yang et al. 1992), or changes in processes involving Ca^{2+} activation in the myoplasm (Palmer and Moore, 1989).

Stretching the muscle causes a decrease in interfilament space (Martyn and Gordon, 1988). This decrease may be responsible for an approximation between actin and myosin (Adhikari and Fajer, 1996), and may result in altered force generation in muscle during submaximal activation, as suggested by Fuchs and Wang (1991). It is possible that this decrease in interfilament space as the muscle is stretched also changes the effect of R-LC phosphorylation in staircase potentiation. If potentiation via R-LC phosphorylation is caused by an increase in the average cross-bridge position away from the thick filament, as suggested by Sweeney et al. (1993), the impact of this effect could be diminished at

longer muscle lengths. This hypothesis would be consistent with our results, i.e., a given level of R-LC phosphorylation had a greater effect on the force enhancement at short lengths, where the interfilament space is greater. Also, this would agree with observations of Yang et al. (1992), who observed an absence of R-LC phosphorylation-induced potentiation at a sarcomere length of 3.2 μm, at submaximal Ca^{2+} concentrations.

It is known that R-LC phosphorylation affects the degree of potentiation more effectively at conditions of low Ca^{2+} concentration (Sweeney and Stull, 1986; Persechini et al. 1985; Metzger et al. 1989; Sweeney and Stull, 1990) or during attenuated sarcoplasmic reticulum Ca^{2+} release (Palmer and Moore, 1989). It has been observed in frog skeletal muscles that the amplitude but not likely the duration of Ca^{2+} transient in response to a single stimulation is diminished at short lengths, and activation induced by Ca^{2+} release increases in muscle elongated beyond optimal length (Lopez et al. 1981). If this is true for mammalian skeletal muscle, this would result in a greater effect of R-LC phosphorylation on force enhancement at shortened muscle length, as we observed at L_o - 10%. Conversely, a decreased effect of R-LC phosphorylation on potentiation of a twitch contraction would be expected at L_o + 10%. This is also consistent with our observations.

The changes observed in $dP \bullet C_t^{-1}$ follow the same pattern as changes observed for DT: at L_o - 10% the increase in $dP \bullet C_t^{-1}$ was greater than at L_o and L_o + 10%. Conversely, although the C_t is abbreviated at short lengths and increased in stretched muscles during a single twitch contraction, it does not change significantly during repetitive stimulation regardless of muscle length. These findings together suggest that potentiation at all lengths studied was probably not related to changes in duration of activation, but rather to an increased intensity of activation during repetitive stimulation. This is consistent with the augmentation of R-LC phosphorylation during 10 Hz stimulation observed at all length conditions. Since it is expected that R-LC phosphorylation increases Ca^{2+} sensitivity of force generation (Sweeney and Stull, 1986) and rate of tension development

(Vandenboom et al. 1995), these effects would increase DT without affecting contraction time.

However, it is striking that R-LC phosphorylation was similar at all lengths studied; a decreased level of Ca^{2+} liberated from the sarcoplasmic reticulum during a twitch contraction would be anticipated to reduce the quantity of the Ca^{2+}•calmodulin complex available to bind with MLCK. In this case, if Ca^{2+} release is diminished at short lengths, then activation of MLCK would be reduced at L_o - 10%, resulting in a smaller increment in phosphorylation of R-LC. Perhaps changes in the magnitude of the Ca^{2+} transient during repetitive stimulation could explain our observations. For example, if the Ca^{2+} transient associated with the first stimulus was of relatively small amplitude (integral), but successive Ca^{2+} transients were augmented, then after 10 s, the same degree of R-LC phosphorylation could be achieved. This pattern of change in Ca^{2+} transients would also explain the greater enhancement of the twitch response during the repetitive stimulation at the short length. It should be cautioned however, that at the present time it is not known whether or not the amplitude and/or time course of these Ca^{2+} transients change during repetitive stimulation. One indication that the Ca^{2+} transients do not change is the fact that C_t is not altered during the repetitive stimulation. It is known that caffeine, which enhances the twitch response by enhancing Ca^{2+} release, also causes C_t to be increased (MacIntosh and Gardiner, 1987).

It is recognized that the change in force over time for stimulated contractions must represent the combined effects of several factors which may enhance or diminish the force response. The net effect of these factors is expressed in the measured response (in this case, DT) at a given time. Therefore, it is conceivable that the observation of a greater potentiation at short lengths than at long lengths could be the result of greater fatigue (negative influence) at the longer lengths. This, however, is in contrast with several reports which have shown greater fatigue at short lengths than at long lengths (Sacco et al. 1994; Aljure and Borrero, 1968; Gauthier et al. 1995; Gauthier et al. 1993). These observations do not confirm that stimulation at 10 Hz for 10 s will result in less

fatigue at a given length, but they suggest that differences in length dependence of fatigue will not likely contribute to the observed differences in twitch potentiation at a given level of R-LC phosphorylation.

Previous investigation showed that the increased Ca^{2+} release from the sarcoplasmic reticulum observed at stretched muscle lengths was achieved without length-dependent changes in Ca^{2+} uptake or Ca^{2+} binding properties (Ridgway and Gordon, 1975). With more Ca^{2+} in the myoplasm at elongated muscle lengths and no length-dependent changes in Ca^{2+} uptake, it may be expected that $½R_t$ would be increased. Prolongation of relaxation at a long muscle length was in fact observed in this study. Mechanical factors related to passive stiffness may also contribute to slowing of relaxation at long sarcomere lengths.

This study has shown that length-dependent staircase potentiation in mammalian skeletal muscle is not directly modulated by R-LC phosphorylation. Interaction of R-LC phosphorylation with Ca^{2+} release and/or myofilament lattice spacing may be responsible for the length-dependence of staircase potentiation, and these possibilities need to be investigated in further studies.

SUMMARY

Changes in muscle length affect the degree of staircase potentiation in skeletal muscle, but the mechanism by which this occurs is unknown. In this study we tested the hypothesis that length-dependent change in staircase is modulated by phosphorylation of the myosin regulatory light chains (R-LC), since this is believed to be the main mechanism of potentiation. *In situ* isometric contractile responses of rat gastrocnemius muscle during 10 s of repetitive stimulation at 10 Hz were analyzed at optimal length (L_o), L_o - 10% and L_o + 10%. The degree of enhancement of developed tension during 10 s of repetitive stimulation was observed to be length-dependent, with an increase of 118.5 ± 7.8%, 63.1 ± 3.9% and 45.6 ± 4.1% (mean ± SEM), at L_o - 10%, L_o and

L_o + 10% respectively. Staircase was accompanied by an increase in the average rate of force development of 105.6 ± 7.7%, 55.6 ± 4.1% and 37.2 ± 4.4%, for L_o - 10%, L_o and L_o + 10%, respectively. R-LC phosphorylation after 10 s of 10 Hz stimulation was higher than under resting conditions, but not different among L_o - 10% (40 ± 3.5%), L_o (35 ± 3.5%) and L_o + 10% (41 ± 3.5%). This study shows that there is a length-dependence of staircase potentiation in mammalian skeletal muscle, that may not be directly modulated by R-LC phosphorylation. Interaction of R-LC phosphorylation with length-dependent changes in Ca^{2+} release and intermyofilament spacing may explain these observations.

Chapter 6

Length-dependence of muscle activation and twitch potentiation in skeletal muscle

INTRODUCTION

Muscle length affects force production in skeletal muscle. The developed tension (DT) of tetanic contractions is maximal when muscle length permits optimal overlap of myofilaments, decreasing at shorter or longer lengths. This property of muscle gives a well defined force-length relationship (Gordon et al. 1966a; Gordon et al. 1966b; ter Keurs et al. 1978). Furthermore, it has been shown that the force-length relationship is altered during submaximal activation. Close (1972a) and Rack & Westbury (1969) showed that the force-length relationship for twitch contractions reaches a peak at a muscle length longer than the optimal degree of filament overlap. This characteristic of the force-length relationship has been recognized as length-dependence of muscle activation.

Length-dependence of activation is likely effected by changes in Ca^{2+} sensitivity. Studies with skinned fiber preparations have shown that there is a leftward shift in the force-pCa^{2+} relationship when the muscle is evaluated at a long length (Endo, 1973; Endo, 1972; Martyn and Gordon, 1988; Stephenson and Williams, 1982), giving a greater force at a given Ca^{2+} concentration. A series of studies have shown that the increased Ca^{2+} sensitivity associated with a long length is a function of the lattice spacing (Godt and Maughan, 1981; Fuchs and Wang, 1991; Wang and Fuchs, 1994; Wang and Fuchs,

1995) . When the muscle is at a long length, the filaments are probably closer together than when the muscle length is short, and this may increase the probability of myosin/actin interaction.

Caffeine has been a valuable tool in the study of muscle activation and Ca^{2+} sensitivity. Lopez et al. (1981) have shown that when intact single fibers from the frog are exposed to 1 mM caffeine, the degree of force enhancement induced by caffeine is length dependent: a greater potentiation is observed at short muscle lengths than at longer lengths. In the presence of caffeine the optimum length for twitch force was the same length as the one at which maximum tetanic force was obtained. Therefore, caffeine abolishes the length-dependence of activation. Furthermore, studies with skinned fiber preparations have shown that caffeine depresses the length-dependence of activation by increasing Ca^{2+} sensitivity to force at short sarcomere lengths (Wendt and Stephenson, 1983; de Beer et al. 1988a).

Muscle length also affects the degree of activity-dependent potentiation. When fast-twitch skeletal muscle at 37°C undergoes a period of low-frequency stimulation, the force of the twitch contraction increases during the first seconds of stimulation (MacIntosh, 1991; Krarup, 1981b; MacIntosh and Kupsh, 1987). This increase in DT is known as staircase potentiation, and studies have shown that muscles stimulated at short lengths give a greater degree of staircase potentiation than muscles stimulated at long lengths (Close, 1972a; Wallinga-de Jonge, 1979; Roszek et al. 1994, Chapters 4 and 5).

The mechanism responsible for staircase potentiation appears to be an increased Ca^{2+} sensitivity of the myofilaments. Repetitive stimulation results in increased phosphorylation of the regulatory light chains (R-LC) of myosin, and this increase is proportional to the degree of staircase potentiation (Manning and Stull, 1982; Moore et al. 1985). Studies with skinned fiber preparations have shown that with R-LC phosphorylation, there is a leftward shift in the force-Ca^{2+} relationship (Persechini et al. 1985). Since length-dependence of muscle activation and length-dependence of

potentiation seem to operate via an increased Ca^{2+} sensitivity of the myofilaments, and caffeine abolishes length-dependent activation, we decided to test the hypothesis that caffeine also depresses the length-dependence of twitch potentiation.

METHODS

In this study, female Sprague-Dawley rats weighing approximately 220 grams were used. These animals were housed in a room with a 12:12h light:dark cycle, and standard rat chow and water were provided *ad libitum*. All procedures of this study were approved by a committee for the ethical use of animals for research.

***In situ* muscle preparation.** The animals were deeply anaesthetized with intramuscular injections of ketamine-xylazine (ketamine 100 $mg \bullet ml^{-1}$, xylazine 100 $mg \bullet ml^{-1}$, mixed 85:15 IM; given 0.11 $ml \bullet 100g^{-1}$). A deep level of anaesthesia was maintained throughout the procedures with supplemental injections when needed. A cannula (polyethylene 60 tubing) was placed into the lumen of a jugular vein of the animals for administration of caffeine during the experiments.

The procedures for isolation of the gastrocnemius muscle have been described (MacIntosh and Gardiner, 1987; MacIntosh and Kupsh, 1987). Briefly, the triceps surae were exposed and the gastrocnemius muscle was cleared from connective tissue. The insertions of the soleus and plantaris muscles were detached from the Achilles tendon and partially separated from the gastrocnemius. The calcaneus was cut and the Achilles tendon was connected to an isometric force transducer (Grass FT10) via a thin stainless steel wire. The sciatic nerve was severed close to the spine, and its distal stump was carefully placed across a pair of electrodes for indirect stimulation of the gastrocnemius muscle.

The hindlimb was immobilized with two pins, one placed perpendicularly into the femur and the other axially in the severed tibia. The loosened skin from the hindlimb was

pulled up around the muscle to form a pocket which was filled with mineral oil. Rectal and oil bath temperatures were monitored and regulated at approximately 37°C throughout the experiment using radiant heat. At the end of the experiment, the rats were killed with an intravenous injection of saturated KCl.

Muscle length and force measurements during experiments. The optimal length (L_o) for DT was determined with a series of double pulse contractions (5 ms delay). The reason for using double-pulse contractions was based on observations of Lopez et al. (1981). Length-dependence of activation is absent with double-pulse stimulation, resulting in an estimation of L_o which is not different from that obtained with longer tetanic stimulation. This L_o was the reference (0 mm) for the other six lengths used during the study, ranging from -3 mm to +3 mm relative to L_o. The force transducer, mounted on a rack and pinion device, allowed precise adjustments in the position of the transducer (4 mm per rotation), and therefore in muscle length.

The animals were divided into three experimental groups, based on the different doses of caffeine (40 $mg \bullet ml^{-1}$ in saline; 0.85, 1.70 and 2.55 $ml \bullet Kg^{-1}$) that were injected during the experiments. With these injections, and assuming distribution in 70% of body weight, caffeine concentrations would be approximately 0.25mM (group 1, n=8), 0.5 mM (group 2, n=10) and 0.75 mM (group 3, n=7). These concentrations have been shown to have minimal and transient effects on the heart (MacIntosh et al. 1992), and only 25% inhibition of phosphodiesterase activity would be expected with 1 mM caffeine (Butcher and Sutherland. 1962). With the exception of the amount of caffeine injected, all groups followed the same procedures during the experiments.

The test sequence of stimulation in each experiment was as follows: four twitch contractions at 20 s intervals; one twitch contraction at each of seven different lengths (2 s intervals); 10 Hz for 10 s; and one twitch contraction at each of seven different lengths. The first four twitch contractions were elicited at L_o, and were considered control contractions. To determine the force-length relation, one twitch was obtained at

each length from -3 mm to + 3 mm, in 1 mm increments. The order of length changes (increasing or decreasing) was randomized. Stimulation at 10 Hz for 10 s at L_o was anticipated to result in staircase potentiation (MacIntosh, 1991; Krarup, 1981b; MacIntosh and Kupsh, 1987). Immediately following this, the force-length relation was determined again, to quantify the impact of the potentiating stimulation on the twitch response at different lengths. The first twitch contraction recorded was ~3 s after the repetitive stimulation, and the interval between contractions was kept short (2 s) to minimize the effects of elapsed time on the measured response.

A ten minute rest period was permitted, then four twitch contractions were obtained at L_o to assess return to control (prestaircase) conditions. After these twitch contractions, caffeine was injected. Following the injection of caffeine, the stimulation protocol was repeated: four twitch contractions at L_o , seven twitch contractions at different lengths, 10 s of 10 Hz stimulation, and seven twitch contractions at different lengths. The first four twitch contractions (at L_o) in this sequence was used to evaluate the impact of caffeine on the twitch. The seven twitches at different lengths served to allow assessment of the impact of caffeine on the (twitch) force-length relationship and also served as the prestaircase assessment of the force-length relation.

Three other groups were formed for the purpose of evaluating the impact of the stimulation and length changes sequence on the measured response, and to control for volume (vehicle) injection. In all cases there were two series of 10 Hz stimulation with twitch measurements before and after, similar to Groups 1-3. Group 4 (n = 4) did not receive any injections of caffeine. Immediately after the 10 Hz stimulation, 7 twitches were elicited without changing the length (at L_o), to evaluate if potentiation would change during the time of data collection. The same procedure (no change in length) was repeated after the second 10 Hz stimulation.

In Group 5 (n = 6) twitches elicited after 10 Hz were measured at L_o for the first, third, fifth and seventh contraction. After each of these contractions, muscle length was either

increased or decreased by 1 mm for one contraction, and then returned to L_o. This was done to check the possible effects of changing the length *per se* on DT and potentiation. In this series of experiments, there were 6 twitch contractions obtained at L_o after either increasing or decreasing muscle length (3 after each episode of 10 Hz stimulation). These contractions were grouped accordingly (after increasing length to L_o or decreasing length to L_o) and compared with the first contraction after 10 Hz for which there was no length change. Finally, group 6 ($n = 4$) was used as sham treatment, in which saline without caffeine was injected prior to the last repetitive stimulation procedure.

Stimulation (Grass Model S88 stimulator) was done with supramaximal square pulses, 50 μs in duration (1-5 V). Digitized (4000 Hz) contractions were collected during the experiments and analyzed by computer. The DT of contractions was measured as the amplitude difference between the passive tension and the peak of a twitch contraction.

Estimation of the peak of the force-length relation. The twitch DT collected at the seven different lengths was used to define the force-length relationship in our experiments. For every series of 7 twitch contractions at different lengths, non-linear regression was done to find appropriate values for the constants a, b and c for the general equation: $y = a + bX + cX^2$). This equation was then differentiated with respect to the muscle length (X), and set equal to zero ($dy/dX = b + 2cX = 0$), and then solved for X to give the length at which maximal force was obtained.

Statistical analysis. One-way analysis of variance with repeated measures was used to compare twitch contractions recorded in the control experiments at different times, and to compare the effects of caffeine on the DT. Two-way analysis of variance (time, condition) with repeated measures was used to compare the DT before and after injections of caffeine during the repetitive stimulation (10 Hz). Two-way analysis of variance with repeated measures was also used to compare the DT before and after injections of either saline or caffeine. A one-way analysis of variance with repeated measures was used to compare the length at which maximal force was obtained before

and after 0.5 mM and 0.75 mM caffeine, after obtaining these values from the procedures described above. A-priori multiple comparisons were done with Newman-Keul's test when appropriate. The relationship between muscle length and potentiation for the different groups was compared by evaluating the slopes of the length-potentiation relationship. A value of $p < 0.05$ was used to establish statistical significance in all comparisons.

RESULTS

Twitch contractions recorded at L_o before the two periods of repetitive stimulation reveal that there was no muscle fatigue as a result of the protocol followed in this study ($p=0.25$). The DT recorded before the second staircase was 98 $\pm$ 2 % of the value of the twitch recorded before the first staircase.

In situ preparations similar to the one used in this study have been used extensively to investigate twitch potentiation (MacIntosh, 1991; MacIntosh and Kupsh, 1987; Roszek et al. 1994). However, it was necessary to determine that the procedures chosen for the evaluation of the effects of muscle length on potentiation did not have some unexpected effect on the measurement. More specifically, we wanted to know if: (1) the twitch contractions recorded during the data collection immediately after 10 s stimulation at 10 Hz would still be potentiated to the same extent as during the peak of the staircase, and (2) the DT of twitch contractions recorded during this period would not be affected by changes in length *per se*. Using the results of Group 4, we compared the degree of potentiation at the end of the staircase (last contraction recorded during 10 s of 10 Hz), the potentiation at the first twitch recorded following the 10 Hz stimulation and the potentiation from the last of the seven twitch contractions recorded at L_o after the staircase. In the first series of repetitive stimulation, the degree of potentiation was 51 $\pm$ 1%, 54 $\pm$ 7 % and 49 $\pm$ 8 % for the twitch at the end of 10 Hz stimulation, and the first and 7th poststaircase twitch, respectively ($p = 0.92$). In the second series of repetitive stimulation, the degree of potentiation was 50 $\pm$ 7 %, 53 $\pm$ 7 % and 47 $\pm$

7 % for the three twitches, respectively ($p = 0.85$).

The same approach was used with Group 5, comparing the twitches recorded at L_o after the muscle had been stretched or shortened, as explained in the Methods. Since there was no effect of time on the degree of potentiation (see above), comparison of all twitch contractions preceded by shortening with all contractions preceded by stretching without concern for when in the series the twitch was obtained seems justified. The degree of potentiation did not change significantly, with force increases of 57 $\pm$ 11 % (first post-staircase twitches), 60 $\pm$ 5 % (after shortening) and 44 $\pm$ 5 % (after stretching). The results of these two groups together indicate that the protocol we chose to use did not result in consistent change in DT related to the pattern of length change or the time over which contractions were collected.

When comparing the DT of the sham and caffeine treated animals, a two-way analysis of variance showed a significant group (sham and caffeine) by time (before and after injections) interaction, for all doses of caffeine used. Before injections, the DT was similar in the groups, but DT was greater after caffeine injections than after saline. Therefore, the results obtained in the experiments that caffeine was used were a consequence of the action of this drug, and not due to any responses of the animal to the injection itself.

Caffeine and repetitive stimulation. Figure 16A shows superimposed twitch contractions recorded at L_o before and after administration of $\sim$0.75 mM caffeine during an experiment. The apparent increase in DT was associated with an increase in the contraction time, without changing the rate at which force is developed, as observed previously (MacIntosh and Kupsh, 1987). All doses of caffeine increased twitch DT, as shown in Table 2, and this increase was proportional to the caffeine dose.

Superimposed twitch contractions recorded at 0, 5 and 10 s of repetitive stimulation (10 Hz), before (Figure 16B) and after (Figure 16C) $\sim$0.75 mM of caffeine show the typical

TABLE 2

Effects of different dosages of caffeine on twitch developed tension (N). Values are mean ± SEM.

Caffeine	Before	After	% increase
0.25 mM	2.65 ± 0.46	2.78 ± 0.48	4.7 ± 0.6 *
0.50 mM	2.94 ± 0.32	3.18 ± 0.36	8.0 ± 2.0 *
0.75 mM	2.87 ± 0.32	3.24 ± 0.37	12.4 ± 1.6 *

asterisk (*) depicts significant increase ($p < 0.05$)

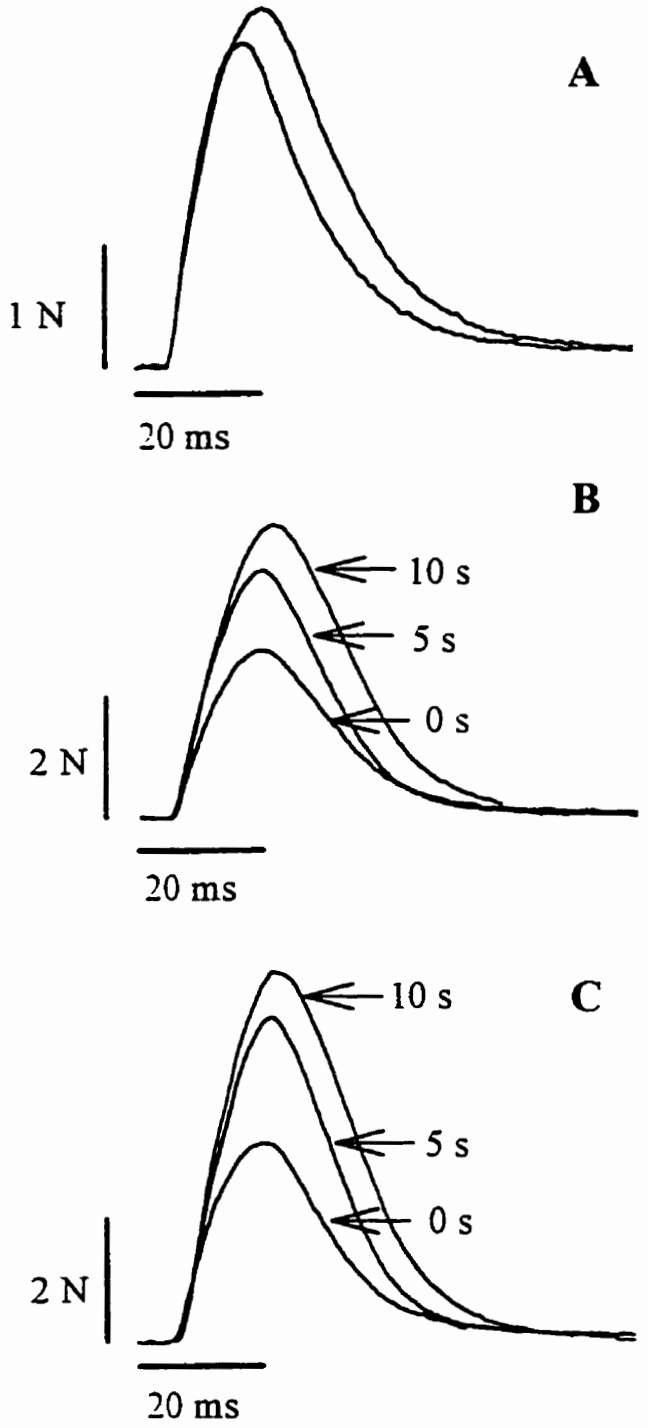

Figure 16. Twitch contractions recorded during one experiment. (A) Superimposed twitch contractions before and after ~0.75 mM caffeine. Note that the increase in force is associated with an increase in contraction time. (B) Superimposed twitch contractions recorded at 0, 5 and 10 s of 10 Hz stimulation before caffeine administration. The increase in force is not associated with an increase in contraction time. (C) Superimposed twitch contractions recorded during 10 s of 10 Hz stimulation after ~0.75 mM caffeine administration. Note the similarity with the staircase recorded before caffeine.

staircase potentiation. The twitch DT increased during stimulation without a change in the baseline. Note that, although the first twitch contraction is bigger after caffeine administration than before, the relative increase in DT during the repetitive stimulation is similar in the two conditions. It is clear that the increase in DT during staircase is not associated with a large increase in contraction time. Figure 17 shows the mean values for the three experimental groups, showing a very similar pattern during the staircase. In all cases, a two-way analysis of variance shows no significant condition by time interaction. However, main effects are significant for both condition (caffeine) and time for all three groups.

Effects of caffeine on the force-length relation of twitch contractions. Figure 18 shows the force-length relationship for twitches recorded before and after caffeine administration. Figures 18A and 18B show the effects of ~0.5 mM and ~0.75 mM caffeine, respectively. The peak of the force-length relationship before caffeine (0.47 ± 0.32 mm and 0.64 ± 0.36 mm, for the two groups, respectively) is shifted slightly to the right when compared to L_o determined using double-pulse stimulation. As stated in the introduction, this is recognized as length-dependence of activation. When caffeine concentration was ~0.25 mM, this relation did not change (results not shown). Caffeine at ~0.5 and ~0.75 mM changed the shape of the force-length relationship such that the length for maximal force was now at - 0.09 ± 0.12 mm and - 0.06 ± 0.32 mm, respectively. These values were statistically different from those values obtained before caffeine administration. Figure 18 also shows that the degree of caffeine-induced potentiation is a function of muscle length, where relative force enhancement is less when the muscle length is longer (lower panels), and these effects are more pronounced with the higher dose of caffeine (~0.75 mM). These changes induced by caffeine eliminate the length dependence of muscle activation.

Figure 19 shows superimposed twitch contractions recorded before and after 10 s of 10 Hz stimulation. These traces were recorded at muscle lengths of -3 mm (left) and +3 mm (right).

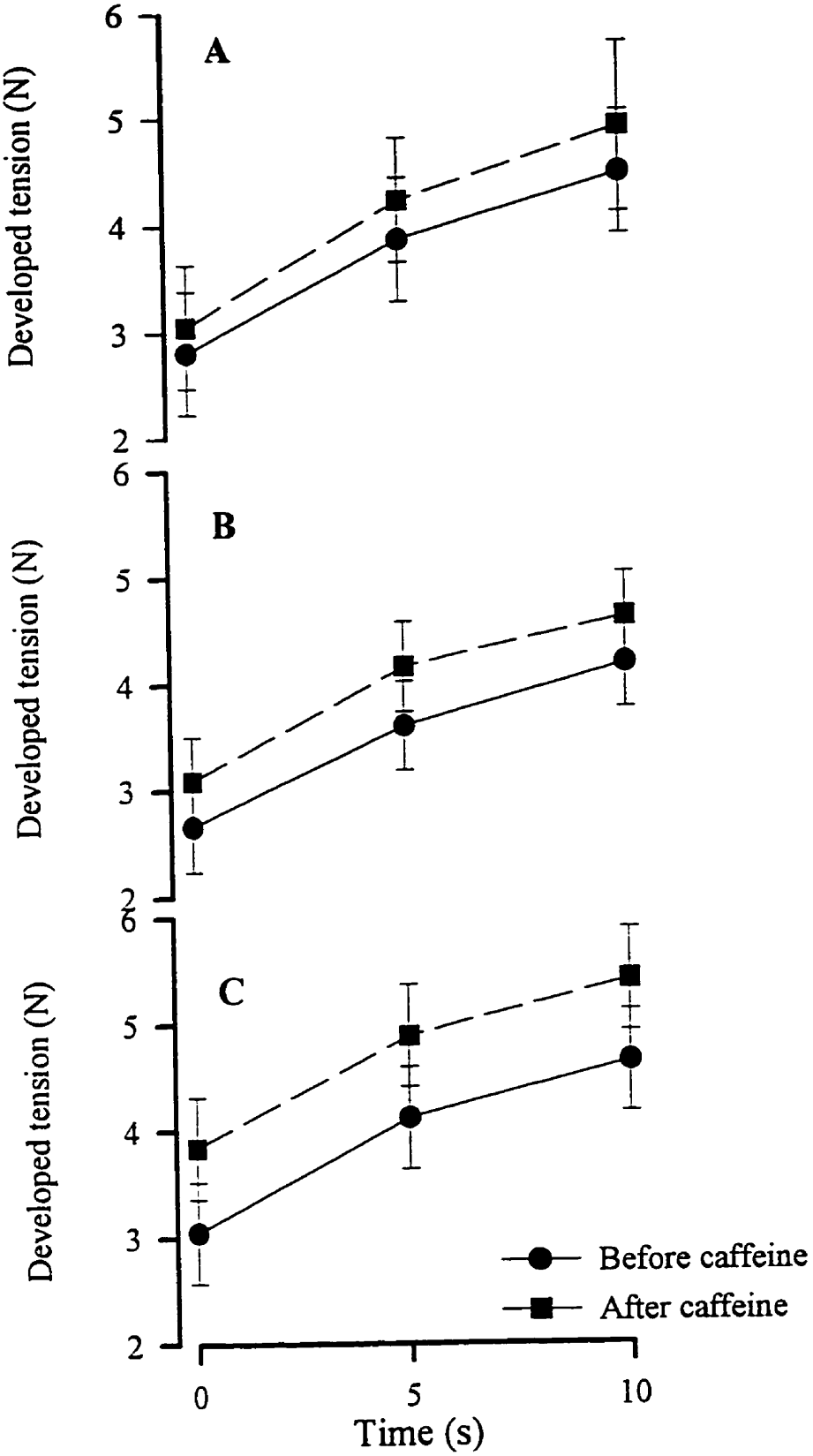

Figure 17. Developed tension (DT) recorded during repetitive stimulation for the three experimental groups, that received ~0.25 mM (A), ~0.5 mM (B) and ~0.75 mM (C) caffeine. All groups showed a positive staircase response before and after caffeine treatment.

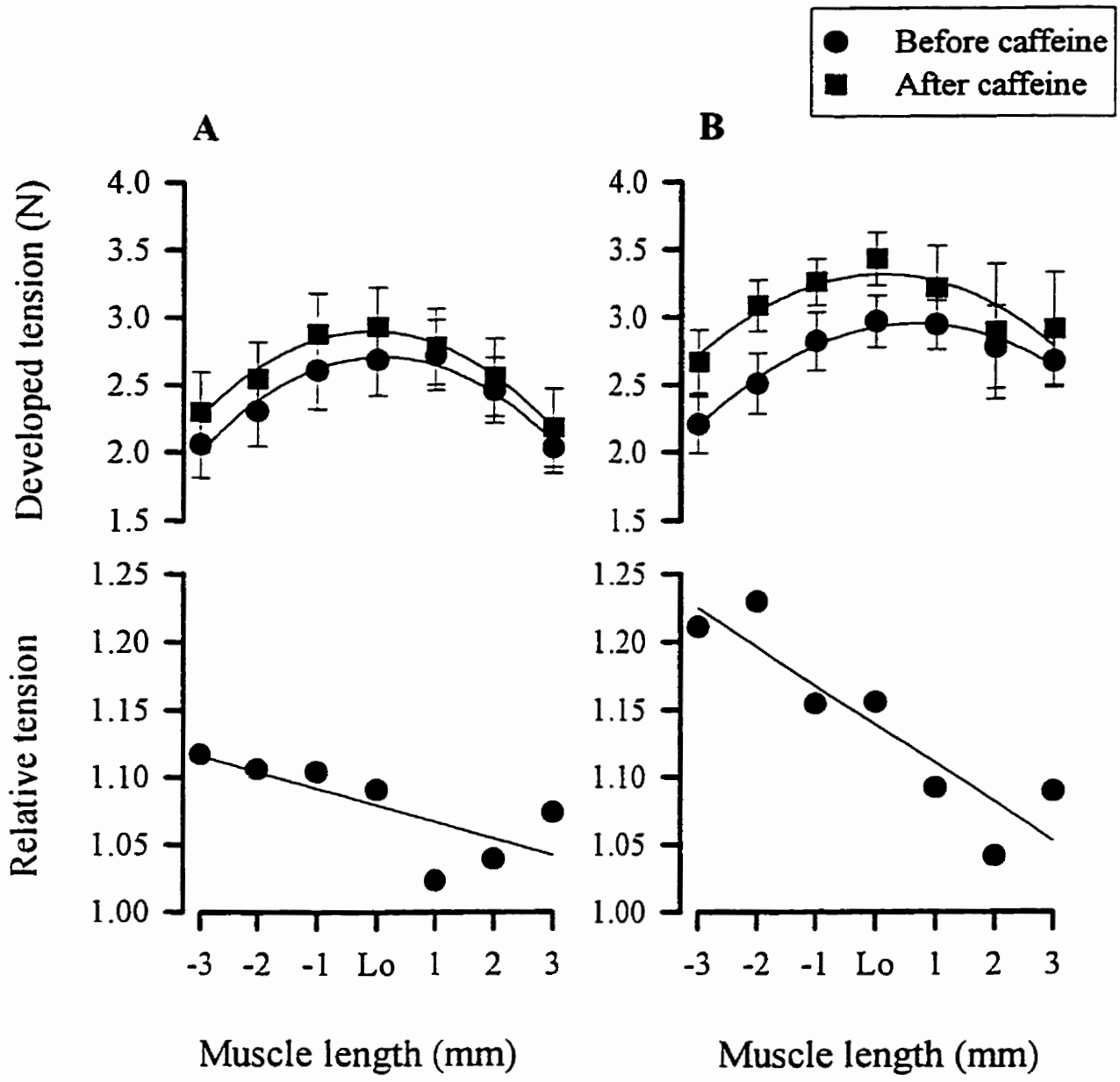

Figure 18. The top panels show the force-length relationship for twitch contractions recorded before and after (A) ~0.5 mM and (B) ~0.75 mM caffeine administration. Optimal length (L_o) was defined using double-pulse contractions. The lines fitted to these curves represent a 2 order polynomial regression. Before caffeine, the force-length relation for twitches has a peak force that is located to the right of L_o. This feature represents the length-dependence of muscle activation. In the presence of caffeine, the length-dependence of staircase is depressed, in such a way that the peak of the force-length relationship is at L_o. The bottom panels show the relationship between muscle length and the degree of force enhancement after caffeine administration. Note the length-dependence of caffeine-induced potentiation.

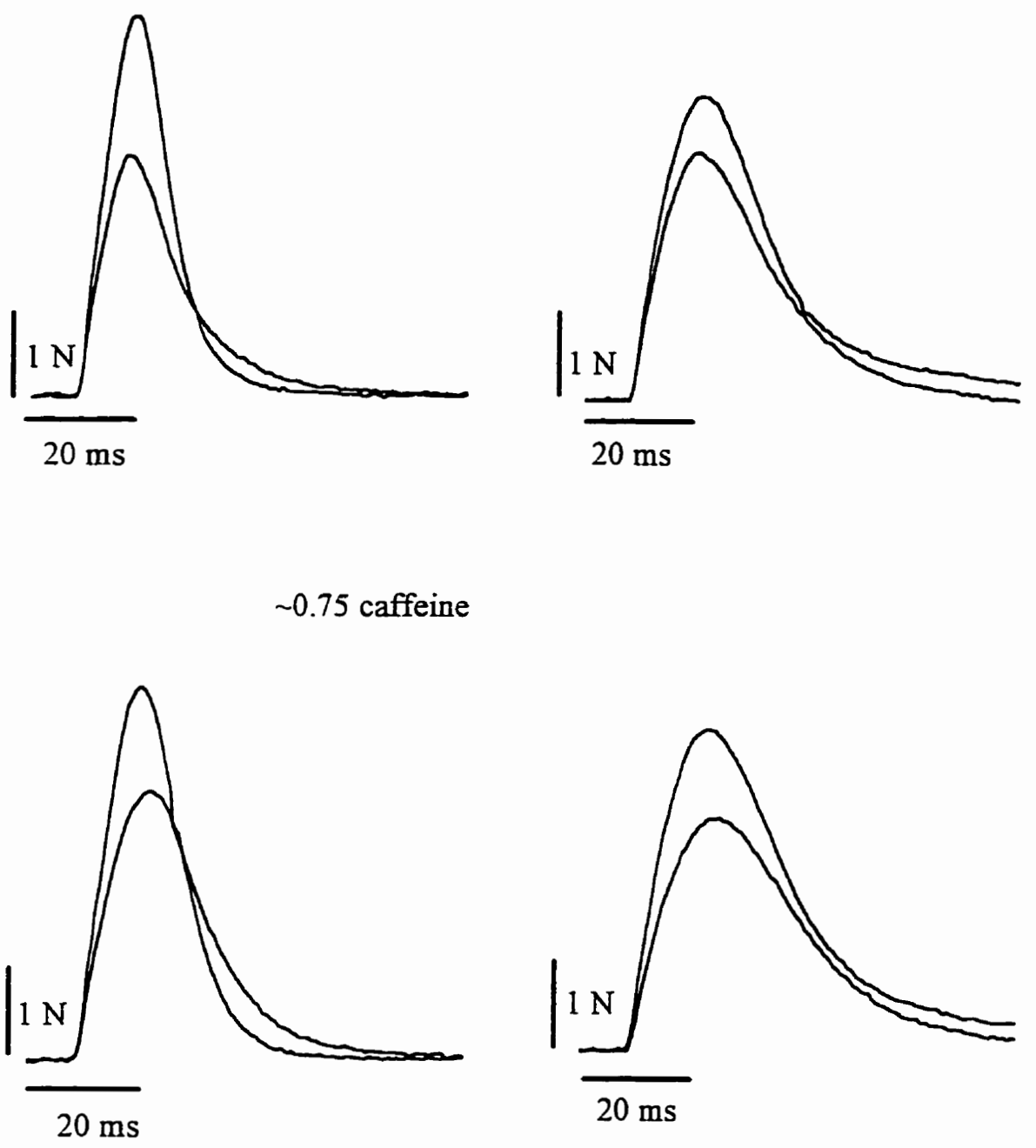

Figure 19. Superimposed twitches recorded before and after ~0.75 mM caffeine at muscle lengths of -3 mm (left panels) and +3 mm (right panels). These twitches were recorded before and after 10 s of 10 Hz stimulation. Before caffeine, the degree of potentiation depends on the muscle length at which the response is measured. After caffeine, this length-dependence is suppressed, as the force enhancement is similar at both lengths.

Before caffeine administration, there is clearly a length-dependence of staircase, where the increase in DT at - 3 mm is much greater than the increase observed at +3 mm. This length dependence is highly depressed after caffeine administration, as seen in the lower traces after ~0.75 mM caffeine was administered. The relative increase in twitch DT no longer depends on the muscle length. When a concentration of ~0.5 mM was used, the difference in potentiation was intermediate between these two examples shown in the figure.

Figure 20 shows the force-length relation for twitch contractions recorded before and after 10 s of 10 Hz repetitive stimulation, with and without caffeine (~0.75 mM) treatment. In the absence of caffeine, staircase potentiation was more evident at short than long lengths. This length-dependence of potentiation results in a shift in the apparent peak of the force-length relation, similar to that described above for caffeine potentiation (and evident here in Figure 20). The length at which maximal force was obtained after staircase was 0.40 $\pm$ 0.16 mm (before caffeine) and 0.11 $\pm$ 0.2 mm (after caffeine).

Figure 21 shows the relationship between muscle length and relative activity-dependent potentiation for two groups (control and ~0.75 mM). Without caffeine there is a linear decrease in the relative potentiation as muscle length is increased (DT = 1.47 - 0.05•ML, r^2 = 0.95). At a concentration of ~0.25 mM caffeine did not affect the length-dependence of potentiation (not shown). The negative slope of the potentiation-muscle length relationship was diminished after ~0.5 mM of caffeine (DT = 1.51 - 0.02•ML, r^2 = 0.32), and the slope was not significantly different from zero after ~0.75 mM of caffeine (DT = 1.53 - 0.009•ML, r^2 = 0.43). Therefore, with the highest dose of caffeine used in this study, there is no effect of length on the degree of staircase potentiation.

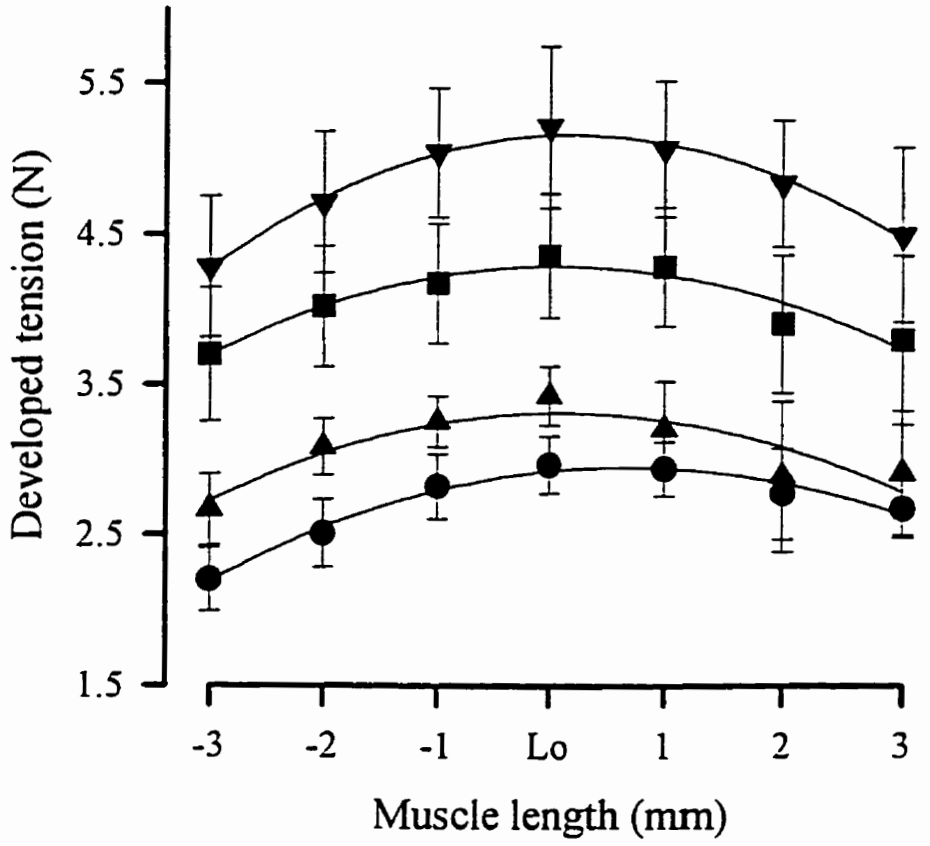

Figure 20. The force-length relationship for twitch contractions recorded before 10 s of 10 Hz stimulation, before and after ~0.75 caffeine treatment (circles and triangles), respectively. Also, the figure shows twitch contractions recorded after 10 s of 10 Hz of repetitive stimulation, before and after ~0.75 caffeine treatment (squares and inverted triangles, respectively). Although there is a length-dependence of activation before caffeine, the shape of the force-length relationship after caffeine intervention is similar to that recorded in potentiated twitches.

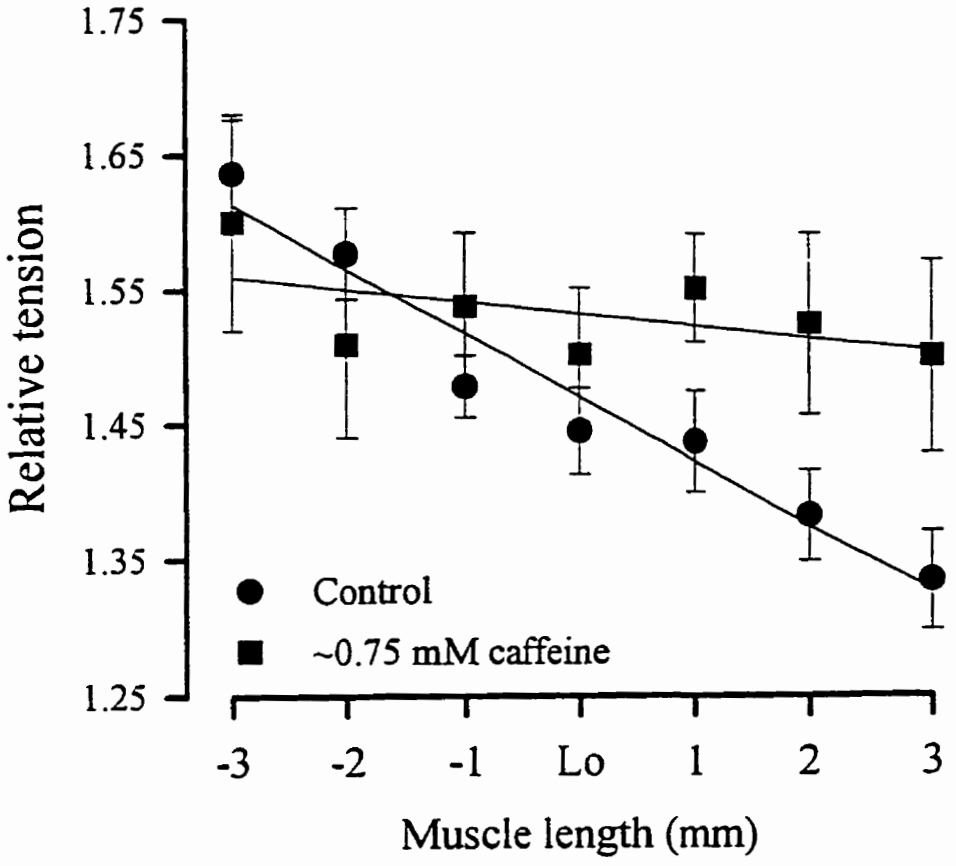

Figure 21. The relationship between activity-dependent staircase potentiation and muscle length, before and after ~0.75 mM caffeine. Before caffeine, the degree of potentiation is inversely proportional to muscle length. The slope of the relationship after caffeine is not different from zero, showing that length no longer affects the degree of potentiation.

DISCUSSION

The main observation of this paper is that caffeine abolished the length-dependence of twitch potentiation when the concentration was ~0.75 mM. This was accomplished together with the depression of length-dependence of muscle activation. Although Lopez et al. (1981) have shown previously that caffeine depresses length-dependence of activation in intact single cells from the frog, this is the first study to show this to be the case in intact mammalian muscle, and the first to demonstrate depression of length-dependence of activation with caffeine concentration less than 1 mM.

The force-length relationship for the twitch contractions showed a peak beyond the optimal length for force development, determined with double pulse contractions. Since double pulse contractions likely give optimal length similar to that represented by tetanic contractions, this finding confirms that there is a length dependence of muscle activation, as reported previously in other mammalian muscles (Close, 1972a; Rack and Westbury, 1969; Chapter 4).

As reported previously, activity-dependent staircase potentiation presented an inverse relationship with muscle length. The degree of force enhancement was greater at short muscle lengths than at longer lengths. Studies with skinned fiber preparations have shown that when the R-LC of myosin are phosphorylated there is a leftward shift in the force Ca^{2+} relationship, i.e. there is a greater force for a given Ca^{2+} concentration (Persechini et al. 1985; Persechini and Stull, 1984). Furthermore, observations with x-ray diffraction in skeletal (Levine et al. 1996) and limulus (Levine et al. 1991) muscle have suggested that R-LC phosphorylation increases Ca^{2+} sensitivity by permitting the cross-bridges to swing away from the myosin backbone, closer to actin filaments. If this is the case in intact muscles, and based on the assumption that stretching the muscle puts the filaments closer together (Godt and Maughan, 1981), then the impact of R-LC phosphorylation could be diminished at longer lengths, where the myosin cross-bridge is already closer to the actin binding sites. At short muscle lengths the filaments would

be further apart, and the effects of R-LC phosphorylation would be maximized.

We also confirmed previous observations that caffeine potentiated the twitch contraction in a dose-dependent fashion. Potentiation induced by caffeine has been associated with an increase in Ca^{2+} release from the sarcoplasmic reticulum (Allen and Westerblad, 1995). This is consistent with the fact that caffeine increases the contraction time of a twitch contraction (MacIntosh and Gardiner, 1987). After caffeine, there would be more Ca^{2+} available to bind to troponin, and subsequently there would be a greater degree of muscle activation with a single pulse of stimulation. Caffeine also increases Ca^{2+} sensitivity to force production. de Beer et al. (1988a) have shown that when 10 mM caffeine is used in skinned fiber preparations, there is a leftward shift in the force-pCa^{2+} relationship at short sarcomere lengths. Wahr and Metzger (1998) have suggested that caffeine increases Ca^{2+} sensitivity by slowing the rate of cross-bridges detachment. This increased Ca^{2+} sensitivity after caffeine observed by de Beer et al. (1988a) is absent at a long sarcomere length, showing that this caffeine-induced increase in Ca^{2+} sensitivity is length-dependent. This is consistent with the length-dependence of caffeine potentiation illustrated in Figure 18.

Caffeine changed the length at which maximal force was obtained, in a way that the force-length relationship became similar to that obtained using double pulse contractions. This finding is in agreement with Lopez et al. (1981), who showed that a variety of potentiating agents [(caffeine, 1mM), Zn^{2+}, NO_3^-, and tetraethylammonium] affect the shape of the twitch force-length relationship. It becomes similar to the tetanic force-length relationship. However, the increase in Ca^{2+} release induced by caffeine was not length-dependent in the study by Lopez et al. (1981). Therefore, it seems that changes in length-dependence of activation are related to Ca^{2+} sensitivity of the myofilaments. It is interesting to notice that a lower concentration of caffeine, as we have used, is enough to affect length-dependent activation.

The main finding of this paper was that, associated with depression of length-dependence

of muscle activation, caffeine depressed the length-dependence of staircase potentiation. The shape of the force-length relationship after caffeine was the same as that for a potentiated twitch. As a result, the relative increase in DT during repetitive stimulation was not dependent on muscle length. It is interesting to note that even after this leftward shift in the peak of the force-length relationship there is still considerable activity-dependent potentiation, as shown in Figure 20.

The magnitude of staircase potentiation (both absolute and relative) at a long length is greater after administration of caffeine than before. This finding is different from what we had anticipated: that the degree of potentiation after caffeine at a longer length would be lower than before caffeine. This was based on the idea that the effects of increasing length and using caffeine would be additive, both increasing Ca^{2+} sensitivity. If that was the case, there would be less likelihood of further change in Ca^{2+} sensitivity during staircase potentiation, and the linear relationship after $\sim$0.75 mM caffeine as shown in Figure 21 would be situated lower on the Y (relative tension) axis. The intersection point with the data obtained before caffeine would be at approximately L_o + 3 mm.

The mechanism responsible for this higher potentiation at a long length after caffeine is not known, and it deserves further investigation. It may be that caffeine alters the degree of R-LC phosphorylation. Since R-LC phosphorylation is a Ca^{2+} dependent process [for a review see Sweeney et al. (1993)], and caffeine enhances Ca^{2+} release from the sarcoplasmic reticulum, R-LC phosphorylation is likely greater after caffeine.

Therefore, there may be a dual effect of caffeine in the regulation of length-dependence of potentiation: depressing the length-dependence of activation, and increasing the degree of staircase potentiation, independent of muscle length. This is consistent with the idea that the mechanism of length-dependence of activation is related to the mechanism of length-dependence of twitch potentiation.

SUMMARY

There is a length-dependence of muscle activation, effected by an increased Ca^{2+} sensitivity of the myofilaments when muscle is at a long length. Caffeine is known to abolish the length-dependence of muscle activation. Muscle length (ML) affects the degree of staircase potentiation as well. Since twitch potentiation is also correlated with an increased Ca^{2+} sensitivity of the filaments, we tested the hypothesis that caffeine depresses the length-dependence of staircase potentiation. *In situ* isometric twitch contractions of rat gastrocnemius muscle before and after 10 s of 10 Hz stimulation were analyzed at seven different lengths. Contractions were analysed before and after different doses of caffeine. Before caffeine, length dependence of activation was observed, and the degree of potentiation after 10 Hz stimulation showed a linear decrease with increased length (DT = 1.47 - 0.05•ML, r^2 = 0.95, where DT is developed tension). At a concentration of ~0.25 mM, caffeine did not affect the length-dependence of activation. Length-dependence of activation was decreased by caffeine at concentrations of ~0.5 and ~0.75 mM. Furthermore, the negative slope of the potentiation-muscle length relationship was diminished after ~0.5 mM of caffeine, and the slope was not significantly different from zero after ~0.75 mM of caffeine (DT = 1.53 - 0.009•ML, r^2 = 0.43). This study showed that length-dependence of activation in intact skeletal muscle is diminished by caffeine. Caffeine also suppressed the length-dependence of staircase potentiation, suggesting that its mechanism may be closely related to the mechanism for length-dependence of activation.

Chapter 7

Length-dependence of staircase potentiation: Interactions with caffeine and dantrolene sodium

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INTRODUCTION

When fast-twitch skeletal muscle at 37°C is stimulated at a low-frequency, the force of the twitch contraction increases during the first seconds of stimulation. This increase in developed tension is known as staircase potentiation, and its primary mechanism appears to be phosphorylation of the regulatory light chains of myosin (R-LC) (Moore et al. 1985; Moore and Stull, 1984). Studies with skinned fiber preparations have shown a leftward shift in the force-Ca^{2+} relationship when the R-LC are phosphorylated (Persechini et al. 1985). This suggests that R-LC phosphorylation increases the sensitivity of the myofilaments to Ca^{2+}. It is believed that R-LC phosphorylation acts by moving the cross-bridge away from the myosin filament, positioning it closer to the actin filament (Levine et al. 1996).

The magnitude of twitch enhancement during staircase depends on the muscle length at which the measurement is made (Wallinga-de Jonge, 1979; Roszek et al. 1994). Studies have shown that stimulation of muscles at short lengths results in a greater degree of staircase potentiation than stimulation at long lengths (Wallinga-de Jonge, 1979; Chapters 4, 5 and 6). However, the mechanism that permits this length-dependence of twitch potentiation is not clear. In a previous study, we have shown that R-LC phosphorylation is not different when staircase is conducted at different lengths, indicating that R-LC

phosphorylation cannot account for the length-dependence of staircase potentiation (Chapter 5). It has also been demonstrated that caffeine (< 1.0 mM) abolishes the length-dependence of staircase potentiation (Chapter 6).

It is recognized that developed tension of tetanic contractions is maximal when muscle length permits optimal overlap of filaments (ter Keurs et al. 1978). In contrast, Close (1972a) showed that the force-length relationship for twitch contractions shows a plateau at a muscle length longer than the optimal degree of filament overlap. This suggests that the apparent level of activation during a single stimulus (twitch contraction) is increased with increasing muscle length. This has been referred to as length-dependence of muscle activation. Studies with skinned fibers have shown a leftward shift in the force-Ca^{2+} relationship at long muscle lengths (Endo, 1973; Endo, 1972; Martyn and Gordon, 1988; Stephenson and Williams, 1982) similar to that seen with increased R-LC phosphorylation. Furthermore, Balnave and Allen (1996) have observed in intact single fibers from the mouse that the increased level of activation is not due to differences in free Ca^{2+} concentration.

The mechanism which has been proposed to explain this length-dependence of activation is that increasing length brings the myofilaments closer together, thereby enhancing the probability of cross-bridge interactions. This would increase force at sub-maximal Ca^{2+} concentration. In fact, compression of the myofilament lattice at a fixed length in skinned fibers has the same effect (Godt and Maughan, 1981). Therefore, the mechanisms for both length-dependence of activation and force enhancement due to R-LC phosphorylation appear to be the similar.

Studies with skinned fiber preparations have shown that caffeine increases Ca^{2+} sensitivity and depresses the length-dependence of activation (Wendt and Stephenson, 1983; de Beer et al. 1988a). If the mechanism of length-dependence of twitch potentiation is related to the mechanism for length-dependence of activation, then caffeine should abolish length-dependence of staircase potentiation. This was demonstrated in a

previous study (Chapter 6). Caffeine also increases release of Ca^{2+} from the SR (Kovacs and Szucs, 1983; Allen and Westerblad, 1995), which complicates the possible interpretation of such a manipulation. Dantrolene, a drug that causes a reduction of Ca^{2+} release from the terminal cisternae with each stimulation pulse (Hainaut and Desmedt, 1974; Desmedt and Hainaut, 1977; Leslie and Part, 1981), could be used to oppose this effect of caffeine. Dantrolene should reverse the effects of caffeine on Ca^{2+} release but not the length-dependence of Ca^{2+} sensitivity. Dantrolene alone should not affect the length-dependence of potentiation.

In this paper we considered the hypothesis that the abolition of the length-dependence of staircase potentiation by caffeine persists in the presence of dantrolene. We have tested this hypothesis by comparing twitch potentiation at different lengths, with and without caffeine and dantrolene sodium.

METHODS

In this study, female Sprague-Dawley rats weighing 180-220 grams were used. These animals were housed in a room with a 12:12h light:dark cycle, and standard rat chow and water were provided *ad libitum*. All procedures of this study were approved by a committee for the ethical use of animals for research. Care and treatment of the animals was according to the Canadian Council on Animal Care.

This study used this *in situ* preparation as opposed to an *in vitro* model because potentiation is much greater at physiological temperatures that at a room temperatures (Moore et al. 1990), and previous investigations have shown difficulty in maintaining viability of isolated muscle fibers from mammalian skeletal muscle viable during experiments conducted *in vitro* at 37°C (Lannergren and Westerblad, 1987).

Animals were randomly separated into two groups based on the sequence of treatments. In both cases, contractile responses were assessed under three treatment conditions. The

first group (n=7) was given the following treatment sequence: (a) no treatment, (b) caffeine (40 $mg \bullet ml^{-1}$ in saline) and (c) dantrolene sodium (40 $mg \bullet ml^{-1}$ in propylene glycol). This sequence was done because we wanted to check which effects of caffeine were reversible after attenuation of Ca^{2+} release by dantrolene. As stated in the introduction, it was expected that caffeine would eliminate length-dependence of potentiation, and this effect would not be reversed by dantrolene. The second group was given: (a) no treatment, (b) propylene glycol and (c) dantrolene sodium. In this case, we wanted to determine whether or not decreasing Ca^{2+} release would have an effect on length-dependence of potentiation. Propylene glycol was used to assess the possible effect of the solvent, since dantrolene was dissolved in propylene glycol.

Sequential doses of caffeine, dantrolene or propylene glycol (0.04 mL at a time) were injected during the experiments, when appropriate. Approximately 15 injections were performed during each treatment for all muscles studied. The cumulative doses of caffeine, assuming total body water distribution (60% of body weight) would give approximately 1.0 mM caffeine concentration. This concentration has been shown to have minimal and only transient effects on the heart (MacIntosh et al. 1992).

***In situ* muscle preparation.** The animals were deeply anaesthetized with intramuscular injections of ketamine-xylazine (ketamine 100 $mg \bullet ml^{-1}$, xylazine 100 $mg \bullet ml^{-1}$, mixed 85:15 IM; dose: $0.11ml \bullet 100g^{-1}$). A deep level of anaesthesia was maintained throughout the procedures with supplemental injections when needed. A cannula (polyethylene 60 tubing) was carefully placed into the lumen of a common jugular vein of the animals for administration of caffeine, propylene glycol or dantrolene sodium during the experiments.

The triceps surae were exposed and the gastrocnemius muscle was cleared from connective tissue. The insertions of the soleus and plantaris muscles were then detached from the Achilles tendon and partially separated from the gastrocnemius muscle. An *in vivo* measurement of the muscle length was done with a vernier scale (calipers model Measy 2000, Switzerland, error < 0.03 mm). The muscle length was defined as the

distance between the most proximal and the most distal visible ends of the muscle fibers. This *in vivo* measurement was used to calculate 10% of the muscle length, to permit adjustment of the muscle length during the experiments. This subsequent adjustment was made relative to an experimentally determined optimal length.

The calcaneus was cut and the Achilles tendon with a small piece of the calcaneus still attached was connected to an isometric force transducer (Grass FT10) via a thin stainless steel wire. The sciatic nerve was severed close to the spine. The distal stump of the cut sciatic nerve was placed across a pair of electrodes for indirect stimulation of the gastrocnemius muscle. The hindlimb was immobilized with two pins, one placed perpendicularly into the femur and the other axially in the severed tibia. The loosened skin from the hindlimb was stretched and anchored to form a pocket which was filled with mineral oil. Rectal and oil bath temperatures were monitored and regulated at approximately 37°C throughout the experiment using radiant heat. At the end of the experiment, the rats were sacrificed with an intravenous injection of KCl.

Muscle length adjustments during the experiments. Prior to each sequence of repetitive stimulation, L_o was determined with a series of double pulse contractions (5 ms delay), which has been shown to correspond to the same L_o as tetanic contractions (MacIntosh and Kupsh, unpublished). It is known that single pulses of stimulation (twitch contraction) likely result in an overestimation of the optimal length (Close, 1972a; Lopez et al. 1981), so twitches were not used in determination of L_o. This experimentally determined optimal length was used as a reference length for calculation of the changes in muscle length (10%) when needed. It can be estimated, based on measurements by Zuurbier and Huijing (1991), that $\pm$10% of muscle length would result in $\pm$20% fibre length changes. If it is assumed that optimal sarcomere length was 2.4 μm then $\pm$20% changes would give sarcomere lengths of 1.92 μm and 2.84 μm. The force transducer, mounted on a rack and pinion device, allowed precise adjustments in the position of the transducer (4 mm per rotation), and thus in muscle length.

Force measurements during the experiments. During each of the three main treatment conditions in each group (no treatment, caffeine or propylene glycol and dantrolene sodium) the following stimulation protocol was given: (1) three twitch contractions (3 s intervals) elicited at L_o - 10%, L_o and L_o + 10 (random order), (2) repetitive stimulation at L_o with 5Hz for 20 s which was the conditioning stimulus, where we expected to induce staircase potentiation and (3) three twitch contractions (3 s intervals) elicited at L_o - 10%, L_o and L_o + 10 (random order), initiated 20 s after the repetitive stimulation. Application of the conditioning stimulus at a single length (experimentally determined optimal length), with subsequent assessment of the magnitude of potentiation at three lengths, should assure that any chemical potentiating (or fatiguing) influences would be common among these length conditions. This is essentially the same approach used by Moore and Persechini (1990) when they studied length-dependence of posttetanic potentiation.

Stimulation (Grass Model S88 stimulator) was done with supramaximal square pulses, 50 μs in duration (1-5 V). Digitized (3000 Hz) contractions were collected during the experiments and analyzed by computer. The developed tension (DT) was measured as the amplitude difference between the resting tension and the peak of a twitch contraction.

Statistical analysis. Analysis of variance for repeated measures was used for statistical comparisons. When appropriate, post-hoc comparisons were done with Student Newman-Keuls test. A value of $p < 0.05$ was used to establish statistical significance in all comparisons.

RESULTS

Single twitch contraction. Twitch DT was assessed five minutes after each conditioning stimulation, and this was compared with the twitch amplitude prior to the repetitive stimulation, to confirm that the twitch contraction returned to the same amplitude after each sequence of repetitive stimulation. Under each treatment condition, twitch DT

returned to the pre-conditioning stimulus value (overall mean difference = -4.01 ± 1.56%), showing no significant difference ($p > 0.4$ for all treatments). This suggests that intra-animal differences in force magnitude of twitch contractions were not apparently due to fatigue during the experiments.

Values of force during a twitch contraction at the three lengths for the muscles with different treatments are shown in Tables 3 and 4. In the control condition, the decline of DT with length changes was not symmetrical around L_o. The decrease at L_o - 10% was greater ($p < 0.05$) whereas at L_o + 10% the change was not significant. This is consistent with length-dependence of activation. As shown in Table 3, propylene glycol did not change the DT, but dantrolene treatment decreased DT without changing the apparent length-dependence of activation. Table 4 shows that injections of caffeine potentiated the twitch contraction at L_o. Caffeine depressed the length-dependence of activation such that in the control situation, the DT measured at L_o + 10% was significantly less than at L_o. Dantrolene injections administered after caffeine decreased DT to levels of about 50% of control (no treatment) condition (Table 4). However, it did not reverse the effects of caffeine on the length-dependence of activation. DT remained significantly different among all lengths.

Examples of twitch contractions after the different chemical treatments are illustrated in Figure 22. In this case, all contractions were obtained between sequential injections of caffeine (Figure 22A), dantrolene sodium (Figure 22B) or propylene glycol (Figure 22C). It is clear from this figure that injections of caffeine increased the DT of twitch contractions, accompanied by a small increase in the time to peak of contraction. This observation (prolonged contraction time) is also consistent with the idea that much of the caffeine-induced potentiation is associated with an increase in Ca^{2+} release from the SR (Sandow and Brust, 1966). The decrease in DT following injection of dantrolene sodium is comparable with previous results from the literature (MacIntosh, 1991; Krarup, 1981a), and it can be seen that this decrease was accompanied by a decrease in the time to peak of the contraction.

TABLE 3

Developed tension (N) of twitch contractions at different lengths (mean ± SEM) for muscles treated with propylene + dantrolene.

	L_0 - 10%	L_0	L_0 + 10%
Control	1.59 ± 0.36 *	2.26 ± 0.27	1.92 ± 0.11
Propylene	1.46 ± 0.21 *	2.38 ± 0.31	1.93 ± 0.13
Propylene +Dantrolene	0.61 ± 0.16 *	1.31 ± 0.15	1.15 ± 0.06

* Significantly different from L_0 ($p < 0.05$) for that condition

TABLE 4

Developed tension (N) of twitch contractions at different lengths (mean ± SEM) for muscles treated with caffeine + dantrolene.

	L_0 - 10%	L_0	L_0 + 10%
Control	1.34 ± 0.14 *	2.19 ± 0.19	1.99 ± 0.13
Caffeine	1.66 ± 0.18 *	2.44 ± 0.14	1.97 ± 0.21 *
Caffeine + Dantrolene	0.62 ± 0.11 *	1.22 ± 0.17	0.89 ± 0.14 *

* Significantly different from L_0 ($p < 0.05$) for that condition

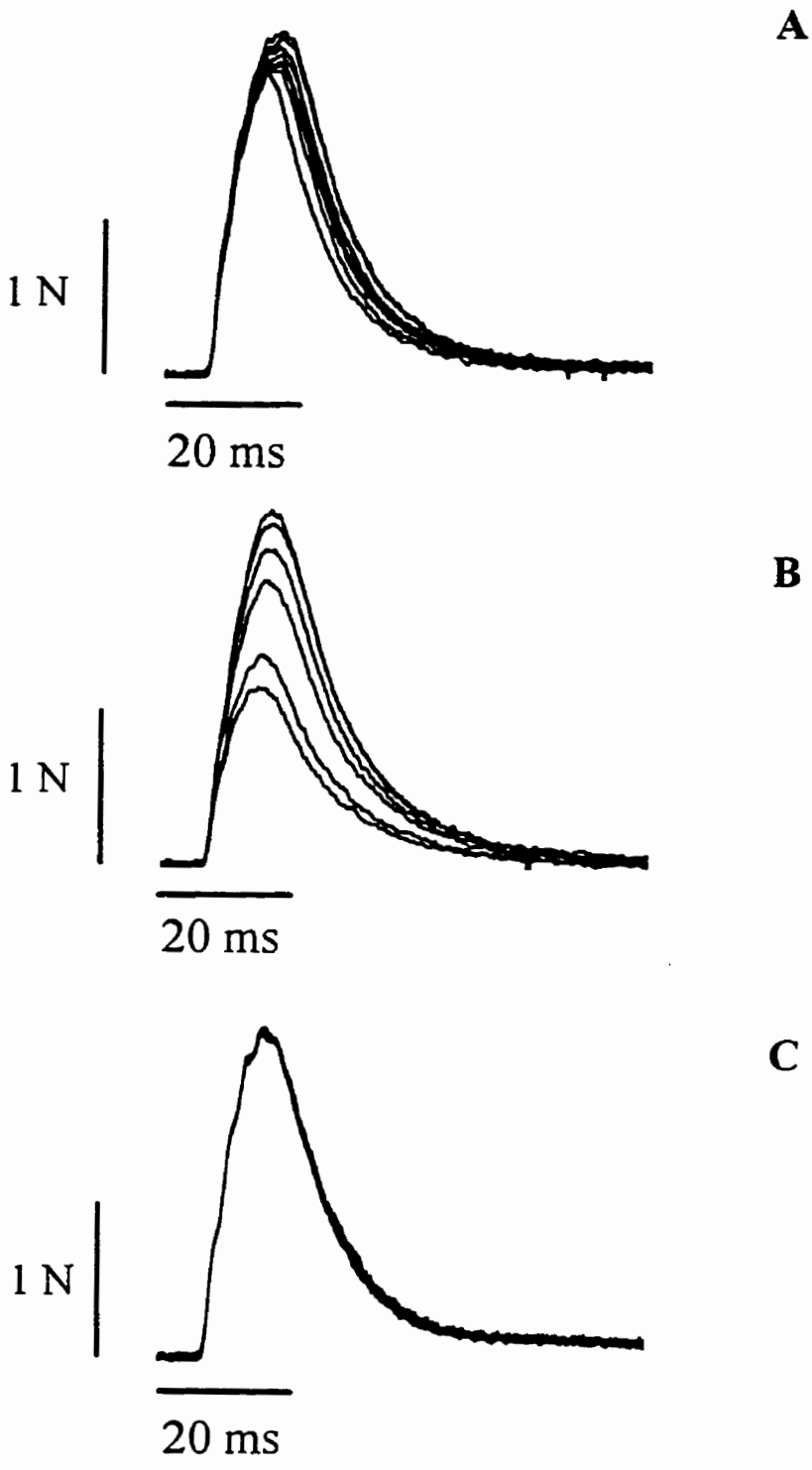

Figure 22. Twitch contractions recorded during consecutive injections of caffeine (A), dantrolene sodium (B) and propylene glycol (C) during typical experiments. Note the increase in DT during consecutive injections of caffeine, and the decrease in DT caused by consecutive injections of dantrolene sodium.

It was observed (example in Figure 22C) that injections of propylene glycol did not change the force development or time course of a twitch contraction. Since dantrolene sodium was dissolved in propylene glycol, and the vehicle alone had no effect, the differences found after dantrolene must be related to the known effects of this drug, e.g., inhibition of calcium release from the SR.

Repetitive stimulation. Stimulation at 5 Hz for 20 s at L_o (conditioning stimuli) resulted in a progressive increase in force development for the five conditions observed in this study. When the peak force recorded during the staircase (usually at 20 s) is expressed relative to the first twitch contraction, a significant increase in DT was observed during the repetitive stimulation for control (61.8 $\pm$ 7%), propylene (57.9 $\pm$ 7%), propylene + dantrolene (43.9 $\pm$ 6%), caffeine (58.1 $\pm$ 6%) and caffeine + dantrolene (60.3 $\pm$ 11%). These values were not statistically different among the five conditions ($p = 0.59$).

There was a length-dependence of staircase potentiation, confirming previous reports (Wallinga-de Jonge, 1979; Roszek et al. 1994; Chapters 4, 5 and 6). In the control situation, the degree of potentiation was inversely related to the muscle length; the magnitude of enhancement of the DT was greater at L_o - 10% than L_o and L_o + 10%. The results of a typical experiment are presented in Figure 23, showing superimposed traces of twitch contractions recorded at the three different lengths before and after 20 s of 5 Hz stimulation . The DT of the twitch contraction recorded at L_o + 10% is greater than the DT recorded at L_o - 10%, probably due to a length-dependence of muscle activation, as stated above. The length-dependence of potentiation is clear from these traces, where muscles at L_o - 10% present an increase in twitch DT greater than that seen at the other lengths.

The magnitude of potentiation resulting from 20 s of 5 Hz stimulation would be expected to reach a maximal value within a few seconds of stopping the repetitive stimulation, then begin to decline with a slow time constant.

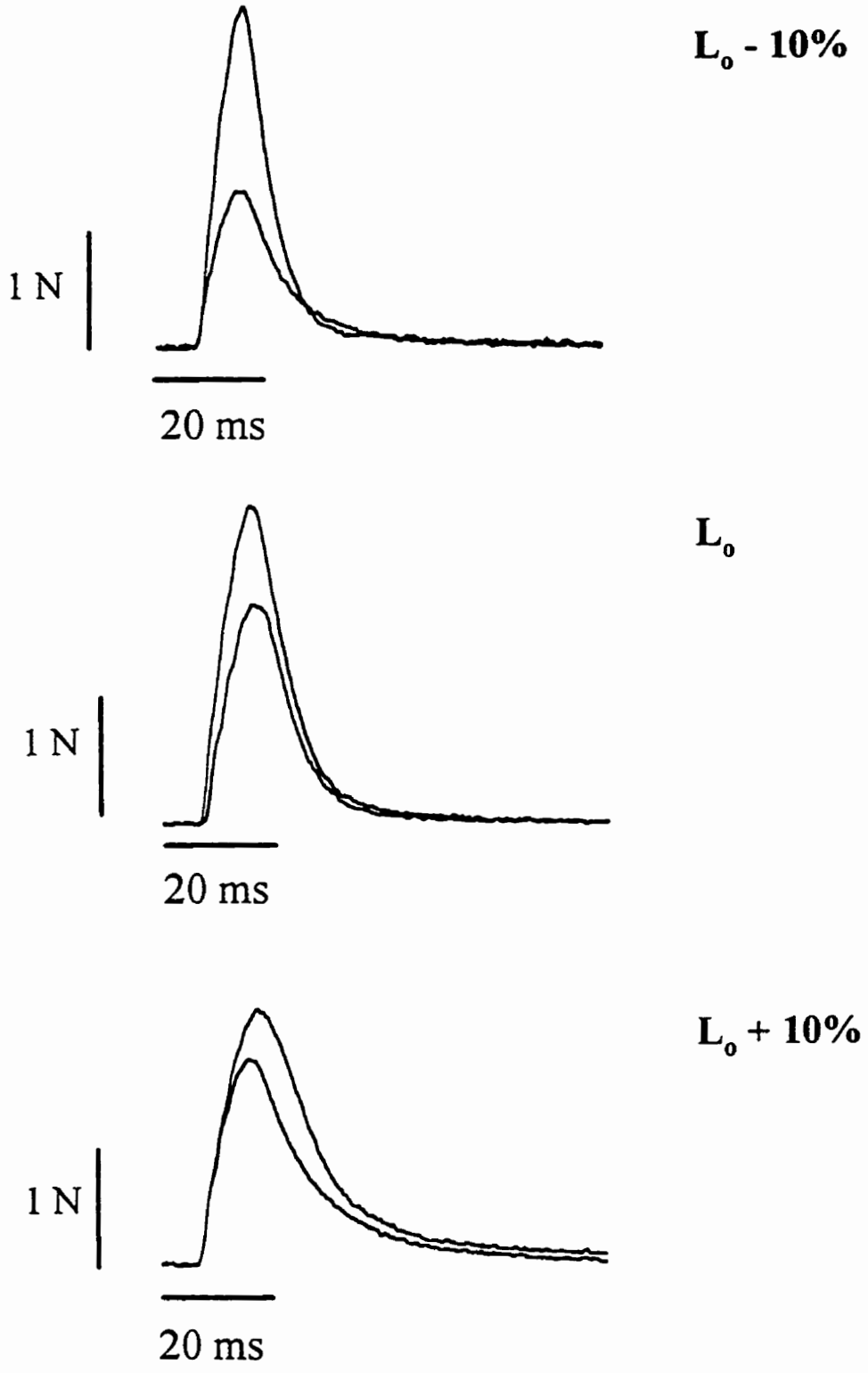

Figure 23. Twitch contractions recorded in control condition before (smaller twitch) and after (larger twitch) 20 s of repetitive stimulation (20 s of 5 Hz) at L_o - 10% (A), L_o (B) and L_o + 10% (C). The degree of potentiation is inversely related to the muscle length.

We chose to permit a delay between the end of the conditioning stimulus and the assessment of potentiation in order to permit resetting the stimulator and data collecting program. Therefore our results could have been affected by the time course of decay of potentiation after 20 s of 5 Hz stimulation. The impact of this effect would have been minimized by our randomized selection of test lengths. However, to check this possibility, the results obtained at L_o during the post-staircase twitches were divided into three subgroups, i.e., when twitches were recorded at 20 s (n = 15), 23 s (n = 10) or 26 s (n = 9) after the period of repetitive stimulation. The relative differences between these twitches and the twitches recorded at the peak of the staircase (20 s of 5 Hz stimulation) were then compared. Values were 82.7 $\pm$ 2.2%, 87.6 $\pm$ 2.5% and 77.9 $\pm$ 4.2% for the twitches recorded at 20 s, 23 s or 26 s respectively, showing that the potentiation was unchanged (p = 0.11) during the time of data collection.

The same procedure was used to check the effects of changing lengths just before the assessment of a potentiated twitch contraction. We grouped the results obtained at 20 s at L_o during the poststaircase twitches in three subgroups: muscles that were not changed previous to the contraction (n = 15), muscles that were shortened (n = 10) and muscles that were stretched (n = 9) before the contraction. When expressed relative to the peak of staircase, values were 82.7 $\pm$ 2.2%, 81.1 $\pm$ 3.9% and 85.2 $\pm$ 3.4% for unchanged, shortened and stretched muscles, respectively, showing no statistical significance (p = 0.68).

Figure 24 shows the mean values representing the increase in DT observed after 5 Hz repetitive stimulation relative to the preconditioning stimulus twitch, at all conditions and muscle lengths. As expected, propylene glycol did not affect the length-dependence of staircase (Figure 24A). Furthermore, it was observed that administration of dantrolene sodium after propylene glycol also did not change the influence of length in the degree of twitch potentiation. In other words, reducing Ca^{2+} release did not provide conditions for an enhancement of twitch potentiation at long lengths. Caffeine treatment eliminated the length-dependence of staircase potentiation, as shown in Figure 24b.

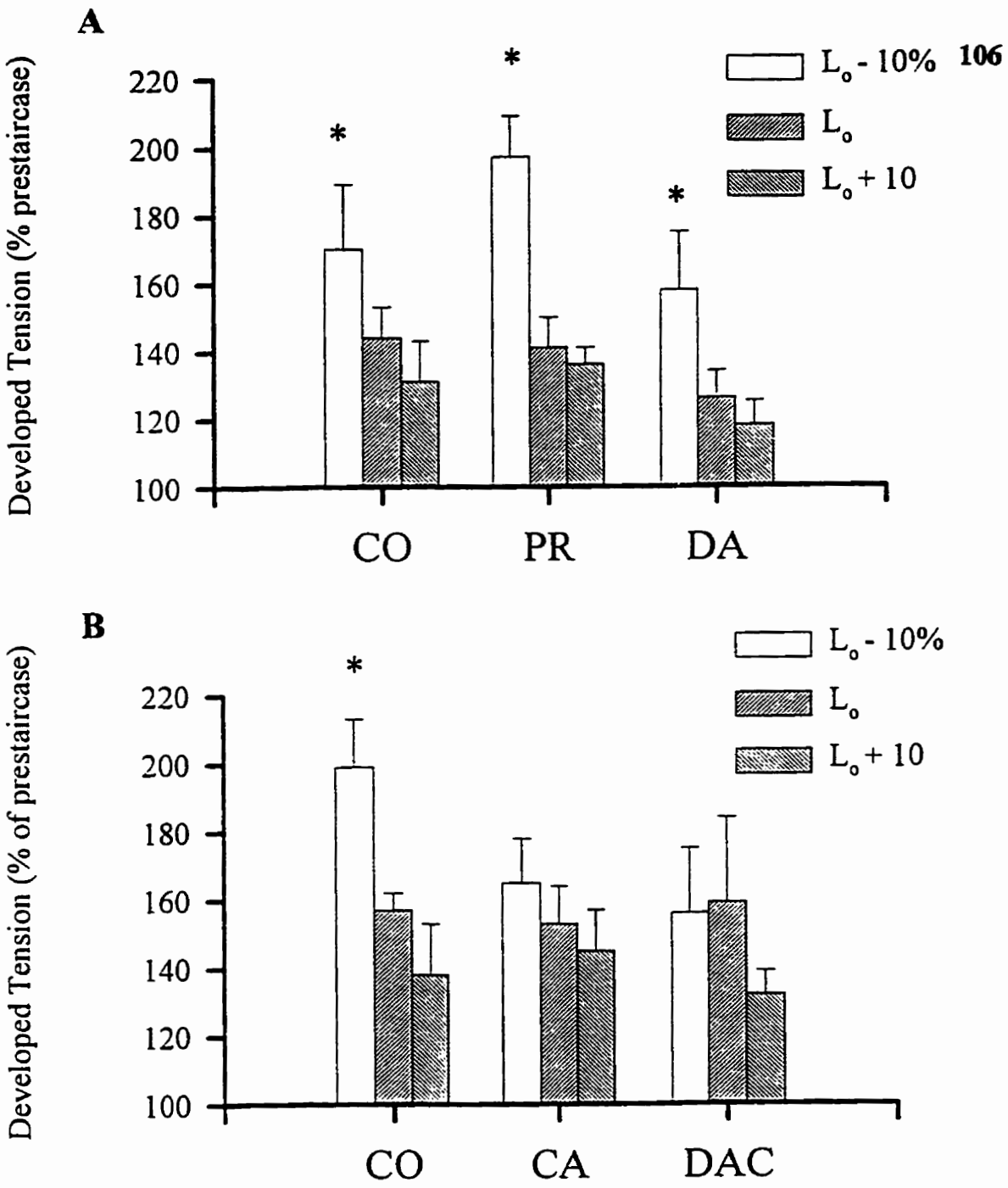

Figure 24. Relative increase in developed tension (% of pre-conditioning stimulus contraction at same length) after staircase potentiation at different treatment conditions. Each vertical bar on top of the column represents SEM, and asterisks (*) represent a significant difference ($p < 0.05$) from the other lengths at a given treatment. (A) muscles treated with propylene glycol and propylene + dantrolene; (B) muscles treated with caffeine and caffeine + dantrolene.

This figure also shows that dantrolene sodium did not reverse the effects of caffeine in eliminating the length-dependence of staircase. As in the case with caffeine, no statistical difference among the three muscle lengths was observed for the caffeine+dantrolene condition (Figure 24B).

Typical twitch contractions recorded prior to and after 20 s of 5 Hz stimulation following caffeine + dantrolene treatment are presented in Figure 25. As mentioned above, caffeine diminished the length-dependence of activation, since the twitch contraction recorded at L_o + 10% was significantly smaller than the twitch recorded at L_o, and this effect was not reversed by dantrolene. The traces show that the increase in force observed after 20 s of 5 Hz repetitive stimulation is basically the same, regardless of the length at which the measurement was made.

DISCUSSION

The main observations in this paper include: (1) caffeine abolished length-dependence of muscle activation in situ, as has been shown previously in skinned fiber preparations (Wendt and Stephenson, 1983; de Beer et al. 1988a), frog single fiber (Lopez et al. 1981) and the whole rat gastrocnemius muscle (Chapter 6); (2) caffeine abolished the length-dependence of staircase potentiation; and (3) dantrolene did not affect the length-dependence of potentiation, whether or not caffeine was present. Together, these observations confirm the hypothesis that the mechanism by which caffeine abolishes length-dependence of activation and length-dependence of potentiation during staircase is by its effects on sensitivity to Ca^{2+}, not its effect on Ca^{2+} release. Since administration of dantrolene without caffeine failed to overcome the depression of potentiation which occurs at a long length, Ca^{2+} availability seems not be responsible for length-dependence of staircase potentiation.

In this study, muscles at a short length had a higher magnitude of potentiation than muscles at L_o and L_o + 10%.

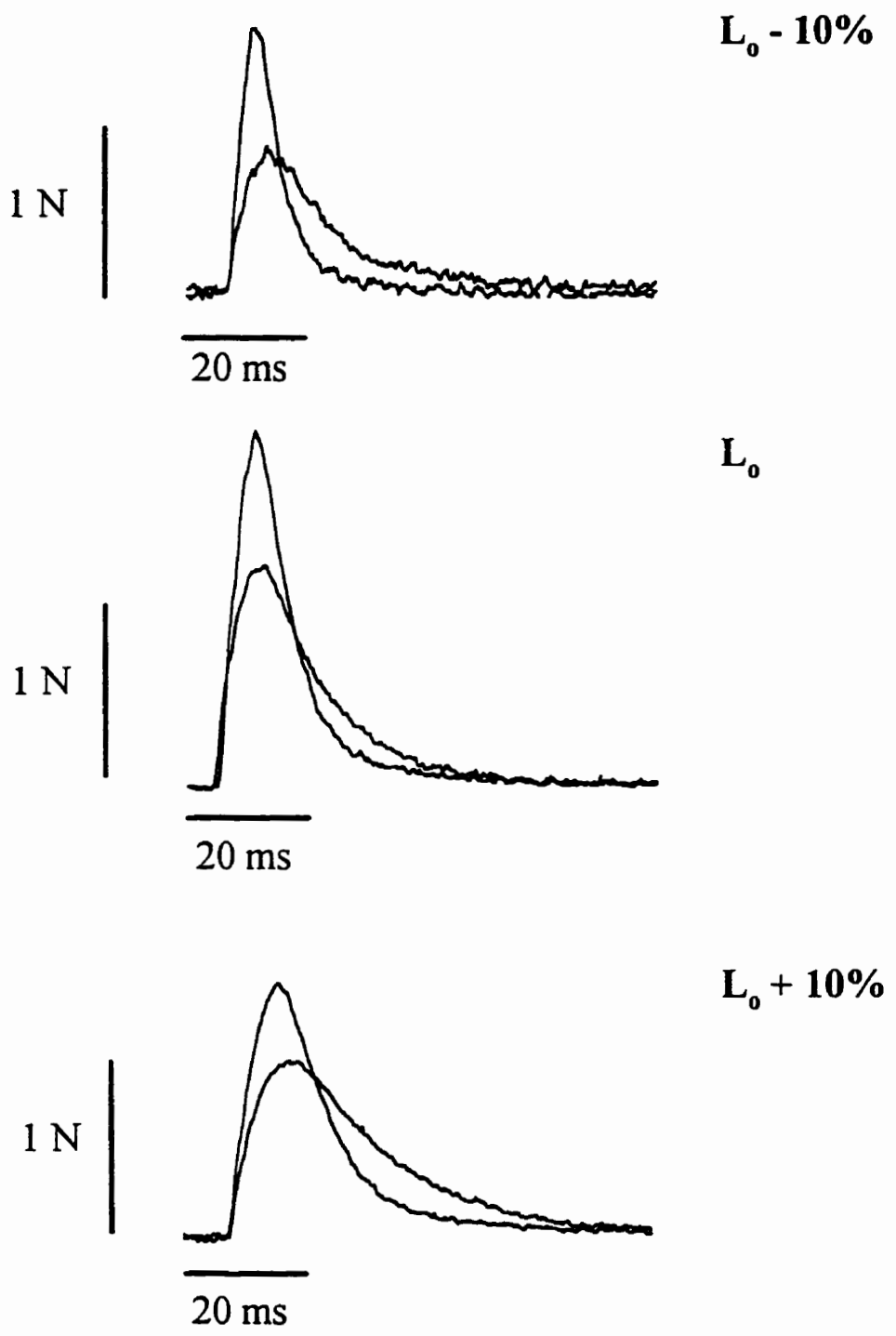

Figure 25. Twitch contractions recorded before (smaller twitch) and after (larger twitch) 20 s of repetitive stimulation (20 s of 5 Hz) at L_0 - 10% (A), L_0 (B) and L_0 + 10% (C) for muscles treated with caffeine and caffeine + dantrolene. Note the absence of length-dependence of twitch potentiation.

This result was similar to previous investigations (Wallinga-de Jonge, 1979; Chapter 5). However, contrary to those studies, the stimulation condition (20s of 5 Hz) in the present study was always performed at L_o. In this way, possible biochemical and/or mechanical changes which contribute to potentiation during repetitive stimulation could not account for the length dependence of potentiation. For example, if repetitive stimulation at different muscle lengths results in different degrees of fatigue (Sacco et al. 1994; Aljure and Borrero, 1968; Gauthier et al. 1993), or if the Ca^{2+} transients during repetitive stimulation changed in different ways at various lengths, the results would be difficult to interpret. By performing the repetitive stimulation at a common length, these problems are avoided. Also, it is important to note that the magnitude of staircase was virtually the same under the different treatment conditions. This is consistent with findings of Krarup (1981a), who observed only a slightly smaller amplitude of staircase (20 s of 5 Hz) with muscles treated with dantrolene.

Close (1972a) reported that the plateau of the force-length relationship is shifted to longer lengths for twitch contractions in comparison with tetanic contractions. This is consistent with the fact that the decrease in DT from optimal to long lengths observed in this study was not as great as the decrease at the short lengths. A decrease in force that was smaller at long muscle lengths than at short lengths has been reported previously for this preparation (Chapter 5), in studies that measured the sarcomere length of frog muscle fibers (Granzier and Pollack, 1990; ter Keurs et al. 1978) or fiber bundles of the mouse extensor digitorum longus (Chapter 3). This supports the idea that there is a length-dependence of activation during twitch contractions in skeletal muscle, permitting an increase of twitch amplitude as length is extended beyond optimal overlap (Close, 1972a; Stephenson and Wendt, 1984).

The rationale for the approach used in this study, was that caffeine has been shown to abolish length-dependence of activation (Lopez et al. 1981; Chapter 6) (as measured by the response of the muscle to electrical stimulation). In fact, after caffeine administration, the DT at long length was different from DT at optimal length in our

study, which is consistent with a shift back to the left in the twitch force-length relationship (see Table 1), supporting the idea that caffeine depresses the length-dependence of activation in intact skeletal muscle. Also, it is interesting to note that the degree of caffeine-induced force increase was inversely related to the muscle length. If there is a diminished degree of activation at short lengths, caffeine would influence force to a greater extent than when the muscles are stretched, and this was the case in this study. This length-dependence of potentiation of force with chemical potentiating agents has been observed before with single muscle fibers from the frog (Lopez et al. 1981).

The main new finding of this paper was that caffeine depressed the length dependence of staircase, but dantrolene, a drug that decreases the amount of Ca^{2+} released by the SR with each stimulating pulse (Hainaut and Desmedt, 1974; Desmedt and Hainaut, 1977; Leslie and Part, 1981), with no effect on Ca^{2+} uptake (Desmedt and Hainaut, 1977) did not restore the length dependence of potentiation after caffeine treatment. This suggests that a mechanism responsible for length-dependence of activation, not related to Ca^{2+} availability in the myoplasm, may be responsible for the length-dependence of staircase potentiation. This finding is also in agreement with observations that intracellular Ca^{2+} transients are independent of muscle length in mammalian single fibers (Balnave and Allen, 1996), i.e., the apparent difference in muscle activation at different muscle lengths is likely not caused by varying levels of intracellular Ca^{2+}.

Previous studies with skinned (Stephenson and Williams, 1982) and intact (Balnave and Allen, 1996) mammalian skeletal muscle have reported an increased sensitivity of the contractile protein to Ca^{2+} as muscle is stretched. Balnave and Allen (1996) observed that this effect is greater when the muscle is stimulated at low frequency, and probably this is also the case during twitch contractions. Therefore, this increased sensitivity as muscle is stretched may explain the length-dependence of activation. This is probably achieved by changes in intermyofilament spacing (Wang and Fuchs, 1994).

Consistent with our findings that caffeine changed length-dependence of activation,

caffeine has been shown to change the length-dependence of Ca^{2+} sensitivity in skinned fibers (Wendt and Stephenson, 1983; de Beer et al. 1988a). The mechanism by which caffeine changes the sensitivity of the myofilaments to Ca^{2+} ions is unclear. Effects in the myosin-actin matrix have been proposed (de Beer et al. 1988a), but more research is needed in this area. Whatever the mechanisms are, it seems logical to argue that Ca^{2+} sensitivity or interaction with length-dependent activation is a potential mechanism by which staircase potentiation is length-dependent.

In a previous investigation we have observed that phosphorylation of the regulatory light chain of myosin (R-LC) after 10 s of 10 Hz stimulation is not length-dependent (Chapter 5). Since R-LC phosphorylation represents a potential mechanism for twitch potentiation (Moore et al. 1985; MacIntosh et al. 1993; Palmer and Moore, 1989; Moore and Stull, 1984) by means of an increase in Ca^{2+} sensitivity to force (Sweeney and Stull, 1986; Persechini et al. 1985), it is tempting to speculate that the actual effect of R-LC phosphorylation at different lengths is not the same. It is believed that R-LC phosphorylation acts by moving the cross-bridge away from the myosin filament, positioning it closer to the actin filament. In this case, this effect could be modulated by differences in the intermyofilament distance at different lengths (Yang et al. 1992). This might decrease the expected effect of R-LC phosphorylation at long lengths, where the myofilaments are already close together.

In conclusion, potentiation of twitch contraction induced by repetitive muscle stimulation is greater when the response is measured at a short length, than when the response is measured at a long length. Caffeine, which was observed to depress the length-dependence of muscle activation during twitch contractions, also suppressed the length-dependence of staircase potentiation. This change was not reversed by dantrolene sodium. Since availability of Ca^{2+} in the myoplasm does not appear to be the mechanism responsible for the length-dependence, Ca^{2+} sensitivity and intermyofilament spacing may explain the results observed in this study.

SUMMARY

In skeletal muscle, there is a length-dependence of staircase potentiation for which the mechanism is unclear. In this study we tested the hypothesis that this length-dependence is effected by the same mechanism as length-dependent activation. To test this hypothesis we have used caffeine, which abolishes length-dependence of activation, and dantrolene sodium, which inhibits Ca^{2+} release. *In situ* isometric twitch contractions of rat gastrocnemius muscle before and after 20 s of repetitive stimulation at 5 Hz were analyzed at optimal length (L_o), L_o - 10% and L_o + 10%. Potentiation was observed to be length-dependent, with an increase in developed tension (DT) of 78 ± 12%, 51 ± 5% and 34 ± 9% (mean ± SEM), at L_o - 10%, L_o and L_o + 10% respectively. Caffeine diminished the length-dependence of activation and suppressed the length-dependence of staircase giving increases in DT of 65 ± 13%, 53 ± 11% and 45 ± 12% for L_o - 10%, L_o and L_o + 10%, respectively. Dantrolene administered after caffeine did not reverse this effect. Dantrolene alone depressed the potentiation response, but did not affect the length-dependence of staircase, with increases in DT of 58 ± 17%, 26 ± 8% and 18 ± 7%, respectively. This study confirms that there is a length-dependence of staircase potentiation in mammalian skeletal muscle. Since dantrolene did not alter this length-dependence, it is apparently not directly modulated by Ca^{2+} availability in the myoplasm. Caffeine suppressed the length-dependence of staircase, as well as the length-dependence of activation. Therefore, the mechanism of length-dependence of potentiation is likely the same as the mechanism for length-dependence of activation.

Chapter 8

Summary and Future Directions

SUMMARY

The first purpose of this dissertation was to examine the effects of muscle length on staircase potentiation. As expected, the degree of staircase was found to be inversely related to muscle length, i.e., force enhancement during repetitive stimulation was higher when the response was measured at a short length. This was the case for both preparations used in this dissertation, the *in vitro* approach using the bundle of fibers from the EDL muscle and the *in situ* approach using the whole gastrocnemius muscle. In contrast with expectations, posttetanic potentiation was also found to be inversely proportional to muscle (sarcomere) length. It seems likely that a common mechanism is responsible for this length-dependence of twitch enhancement.

The second purpose of this dissertation was to investigate the mechanisms responsible for the length dependence of twitch potentiation. From the two hypotheses stated in the introduction, the hypothesis (1), that length-dependence differences in R-LC phosphorylation were responsible for length-dependence of staircase potentiation, was rendered as incorrect. The levels of R-LC phosphorylation were independent of muscle length while the degree of staircase inversely proportional to muscle (or sarcomere) length. The conclusion that length-dependence of potentiation is not mediated by R-LC phosphorylation indicates that other factors may influence twitch potentiation in skeletal muscle. Two independent studies in our laboratory (Rassier et al. 1997; Rassier et al. 1998) come to the same conclusion. Mechanisms of twitch potentiation are a complex

issue not totally understood yet.

It was concluded from studies reported herein that length-dependence of staircase potentiation was modulated by mechanisms related to length-dependence of muscle activation. More specifically, activity-dependent potentiation may be intrinsically related to length-dependence of Ca^{2+} sensitivity. When the muscle is stretched, there is an increased Ca^{2+} sensitivity to force at low-frequency stimulation rates, likely caused by an approximation of the myofilaments. This approximation potentiates the twitch contraction, possibly limiting further increases in DT during repetitive stimulation. In this case, the effects of R-LC phosphorylation-induced potentiation would be reduced, since the proposed mechanism by which R-LC phosphorylation acts also involves an approximation of the myosin cross-bridge and actin filament.

Although using intact muscles and fiber bundles has the advantage of studying muscle properties under physiological conditions, it was not possible to test directly length-dependence of Ca^{2+} sensitivity. This was an obvious limitation in confirming the mechanisms of length-dependence of activity-dependent potentiation. For such experiments, we would need a skinned fiber preparation, where it would be possible to change the extracellular Ca^{2+} concentrations and interfilament spacing. This was beyond the scope of this dissertation.

In study 5, we used dantrolene sodium to decrease Ca^{2+} availability in the myoplasm. We did not actually measure the levels intracellular Ca^{2+} during the twitch contractions. However, Ca^{2+} indicators used currently lack a time resolution to measure Ca^{2+} transients during a twitch contraction at 37°C. Further, our conclusion that Ca^{2+} concentrations are not responsible for length-dependence of Ca^{2+} activation and potentiation agree with observations that Ca^{2+} transients are not altered by length in unfused tetanic contractions of single cells dissected from the mouse (Balnave and Allen, 1995).

This dissertation shows a length-dependence of staircase potentiation, where the degree of force enhancement during low frequency repetitive stimulation is greater when the response is measured at a short length than at a long length. Also, our results strongly suggest that the mechanisms of length-dependence of muscle activation (presumably Ca^{2+} sensitivity) is closely related to mechanisms of length-dependence of staircase potentiation. Until further research proves this hypothesis to be incorrect, it should be accepted as the likely explanation for the length-dependence of twitch potentiation.

Future Directions

As suggested previously, the next step in this line of research would be to investigate interactions among force potentiation, Ca^{2+} sensitivity and interfilament spacing. Studies with skinned fibre preparations, in which the interfilament spacing can be changed by osmotic compression can be useful in this regard. This kind of preparation has been used to study potentiation and Ca^{2+} sensitivity. When the force is potentiated by phosphorylating the R-LC (with addition of calmodulin and myosin light chain kinase), there is a leftward shift in the Ca^{2+}-force relationship (Persechini et al. 1985; Sweeney and Stull, 1986). This process is similar to that which occurs when the muscle is stretched (Endo, 1973; Endo, 1972; Martyn and Gordon, 1988; Stephenson and Williams, 1982). Changing the interfilament spacing independent of sarcomere length could test the hypothesis that filament spacing itself is the modulator of length-dependent potentiation.

A potential tool following this line would be the use of individual myofibrils. In this preparation, it is possible to investigate the behaviour of every sarcomere in series. This approach makes it possible to evaluate the force-length relationship of individual sarcomeres, under conditions of filaments compression or not, with and without potentiating agents. These investigations could provide insights into the mechanisms regarding length-dependence of twitch potentiation, and how R-LC phosphorylation acts to potentiate a twitch contraction.

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IMAGE EVALUATION
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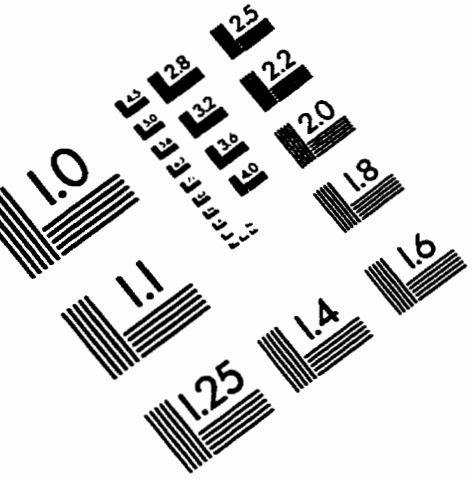

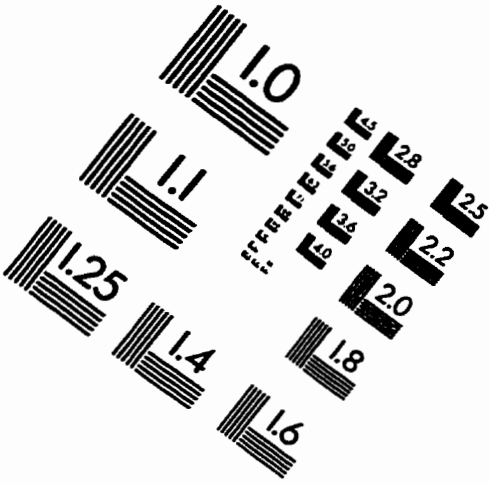

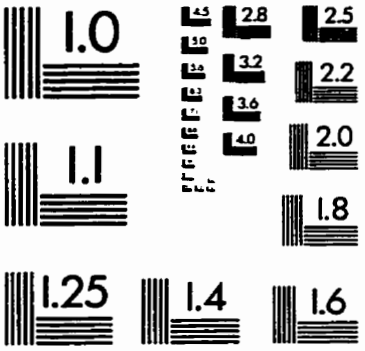

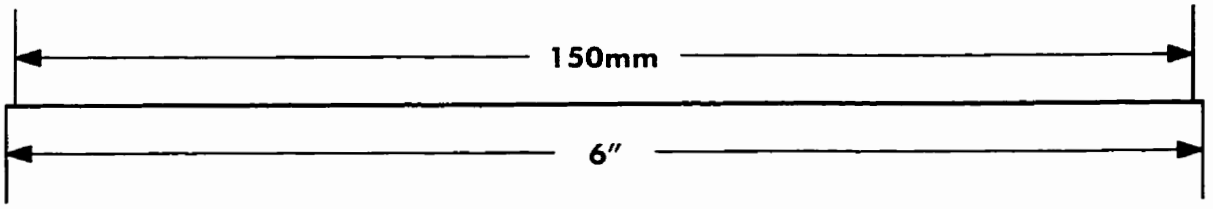

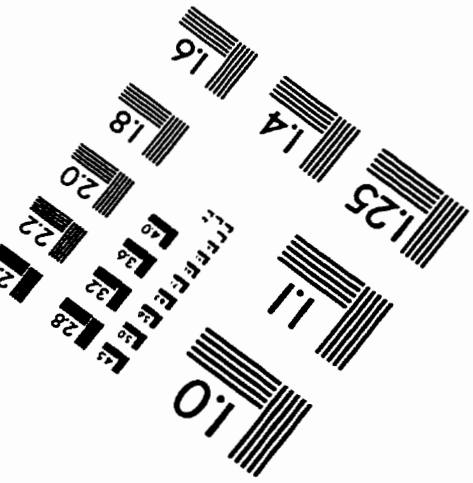

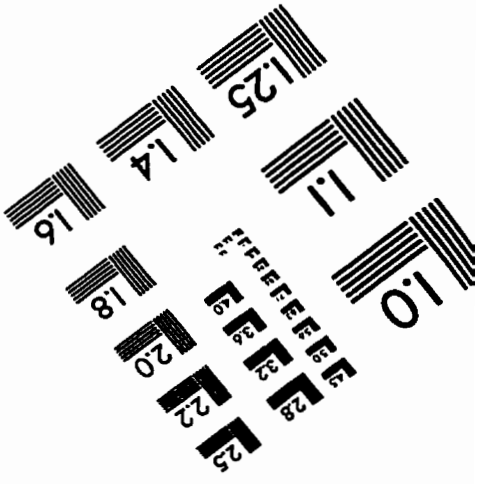

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989