TROPONIN C REGULATION OF LENGTH-DEPENDENT CALCIUM-SENSITIVITY IN RABBIT SKINNED PSOAS MUSCLE FIBRE SEGMENTS

by

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ABSTRACT

The involvement of troponin C (TnC) in regulating the length dependence of calcium sensitivity was studied in rabbit skinned psoas muscle fibre segments. Force-pCa curves were acquired from these isolated segments at resting sarcomere lengths (2.4 μm) and at lengths (3.0 μm) longer than the plateau of the tension-length relationship. Partial extraction of endogenous TnC from fibre segments was performed by bathing the fibre in a solution consisting of 20 mM imidazole and 5 mM ethylenediaminetetraacetate at pH 7.85, while a more complete removal of TnC was facilitated by the addition of 1 mM trifluoperazine to the extraction medium. Treated fibres were then reconstituted with either native rabbit skeletal TnC or a mutant TnC in which the amino acid residue at position 130 of the protein's high-affinity domain was replaced with serine (I130S), glycine (I130G) or recombinant isoleucine (I130). These TnC mutants have been shown previously to possess destabilized alpha-helices in the protein's carboxy-terminal domain resulting in altered ion-affinities of the associated Ca^{2+}/Mg^{2+} binding sites (Trigo-Gonzalez *et al.*, 1993). Protein removal and replacement were assayed by examining changes in the fibre's ability to generate contractile force and subsequently confirmed by silver stained sodium dodecylsulfate polyacrylamide gels of fibre segments. Following fibre reconstitution, force-pCa relations were measured again at both resting and long sarcomere lengths. Results from this study indicate that myofilament calcium sensitivity is neither affected by the method of endogenous TnC extraction nor by mutations to this region of TnC's high-affinity domain.

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To my grandfathers, may the grace of God be upon them.

I have flown too high on your wings, now I must earn a pair of my own.

INTRODUCTION

Calcium-Activated Muscle Contraction

The development of contractile force in striated muscle is preceded by a series of depolarization events, terminating in the release of calcium from the sarcoplasmic reticulum. Transient increases in the cytosolic calcium concentration activate the contractile apparatus, determining the extent of interaction between thick and thin myofilaments. This regulation of the contractile process is dependent primarily on the level of thin filament activation (Zot and Potter, 1987).

In relaxed skeletal muscle, interaction between the contractile myofilaments is inhibited by the actin-associated regulatory proteins, tropomyosin and troponin. Tropomyosin is a 40 nm long regulatory protein that has a molecular weight of approximately 70 kD. It consists of two alpha-helical subunits that wrap about one another to form a coiled-coil structure. Individual tropomyosin molecules aggregate in a head-to-tail fashion to create a continuous tropomyosin strand which then becomes associated with an individual F-actin polymer. In the relaxed state, tropomyosin is positioned peripherally on the actin filament providing steric impedance to the mechanical and biochemical interaction of myosin and actin (Murray and Weber, 1974).

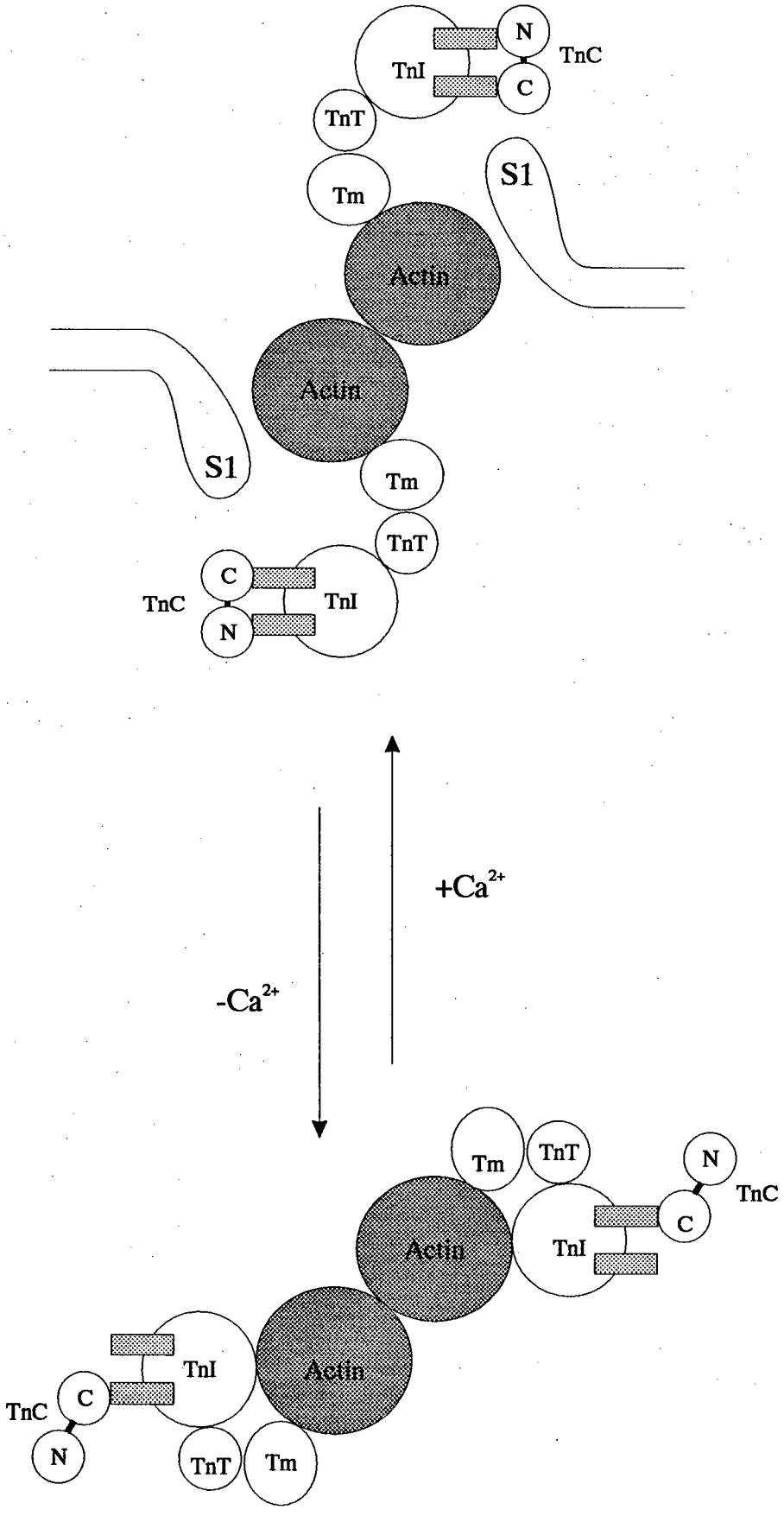
Associated with the tropomyosin filament at a periodicity of 40 nm is the troponin complex. A heterotrimeric regulatory protein, troponin's subunits function to anchor the complex to other thin-filament structures and to translate changes in the sarcoplasmic

calcium concentration to the remainder of the contractile apparatus. The largest of the troponin subunits is troponin T (TnT), which has a molecular weight of approximately 37 kD and a rod-like appearance (Zot and Potter, 1987). Functionally, TnT serves a structural role in binding to tropomyosin and securing the troponin complex onto the thin filament. Troponin I (TnI) is the inhibitory subunit of the troponin complex; its direct binding to the actin filament sterically prevents the activation of myosin Mg^{2+}-ATPase by actin (Greaser and Gergely, 1973). The 24 kD globular protein also binds to TnT and to troponin C, the third member of the troponin complex. Troponin C (TnC) is the smallest of the protein subunits, with a mass of approximately 16 kD. It is an effective calcium-binding protein that responds primarily to transient increases in cytosolic calcium concentration which accompany membrane depolarization. In addition to binding metal ligands, TnC forms strong interactions with the TnI subunit (Ruegg *et al.*, 1989; Van Eyck *et al.*, 1991; Swenson and Fredricksen, 1992; Koboyashi *et al.*, 1994; Ngai *et al.*, 1994;).

The interplay between regulatory and contractile components of the thin filament determines the degree of thin filament activation and, in turn, the extent of interaction between actin and myosin. Binding of calcium to TnC both potentiates and enhances TnC-TnI interactions, resulting in an altered conformation of TnI and a subsequent disinhibition of actomyosin Mg^{2+}-ATPase (Potter and Johnson, 1982; Zot and Potter, 1987). In addition, a reorientation of the troponin complex, in the presence of calcium, causes a concomitant relocation of tropomyosin into the region of the actin groove (Huxley, 1973). As a result, S1 binding sites on the surface of the actin filament become exposed, allowing for mechanical and biochemical interaction between the contractile filaments (Huxley, 1973) (Figure 1).

Figure 1: Cross-Sectional Profile of Thin Filament Structures and Orientation in the Presence and Absence of Calcium

Schematic representation of the thin filament structures in skeletal muscle, with specific reference to the interactions between regulatory and contractile proteins in the presence and absence of calcium. Strengthening of bonds between TnI and TnC in the presence of calcium is associated with a concomitant reorganization of the troponin complex and the release of actomyosin inhibition by TnI. The letters N and C represent the amino and carboxy terminal domains of TnC, respectively. Adapted from Ruegg, 1986.


N
TnC
TnI
C
TnT
Tm
S1
Actin
Actin
S1
Tm
TnT
C
TnC
TnI
N
+Ca2+
-Ca2+
Tm
TnT
N
TnC
C
Actin
TnI
TnI
Actin
TnC
C
N
TnT
Tm

The force-generating events that ensue upon thin filament activation are best described by the sliding filament theory (Huxley and Hanson, 1954; Huxley and Niedegerke, 1954). This hypothesis proposes the relative movement of actin filaments towards the center of the sarcomere, with no change in the length of the filaments, as being the primary tension-generating mechanism in muscle tissue. Once the thin filament is activated, electrostatic forces are likely to guide S1 heads towards newly exposed binding sites on actin, resulting in the formation of tension-generating cross-bridges. Next, actin catalyzes the release of ATP hydrolysis products from S1, initiating a conformational change in the myosin head and the development of a power stroke directed towards the center of the sarcomere. This force producing state is rapidly terminated in the presence of ATP which causes dissociation of the acto-S1 complex (Huxley, 1957; Lymm and Taylor, 1971).

Sarcomere Length-Dependent Properties of Skeletal Muscle

The Tension-Length Relationship

The ability of a muscle fibre to generate maximal force is regulated by many parameters; indeed, both calcium and ATP levels are important modulators of contractile performance. However, even at maximal levels of activation, the tension generating ability of striated muscle is determined by the extent of myofilament overlap. This fundamental relationship of muscle is best described by the so-called tension-length curve (Ramsey and Street, 1940; Gordon *et al.*, 1966; Edman and Reggiani, 1987).

As outlined by Gordon et al (1966), the curve describes the experimentally measured normalized tension developed by individual sarcomeres at various lengths. At fixed myofilament lengths, the degree of overlap between thick and thin filaments is determined exclusively by the length of the sarcomere. The tension-length curve consists of three phases. The ascending limb exists at short sarcomere lengths and produces increasing, yet submaximal, levels of tension. This phenomenon is likely due to the mechanical interference of thin filaments as they move towards the center of the sarcomere during contraction. As sarcomere length is reduced further, tension continues to decrease due to both the interaction of thin filaments with opposing Z-lines and to physical deformation of the myosin filament. The plateau of the tension-length curve is characterized as the region of highest force production. Here, the sarcomere length reflects optimal filament overlap and, thus, maximal cross-bridge formation without thin filament interference. The descending portion of the curve exists at long sarcomere lengths and is associated with a decrease in tension. This result reflects a progressive decrease in filament overlap and a subsequent reduction in force-generating interactions.

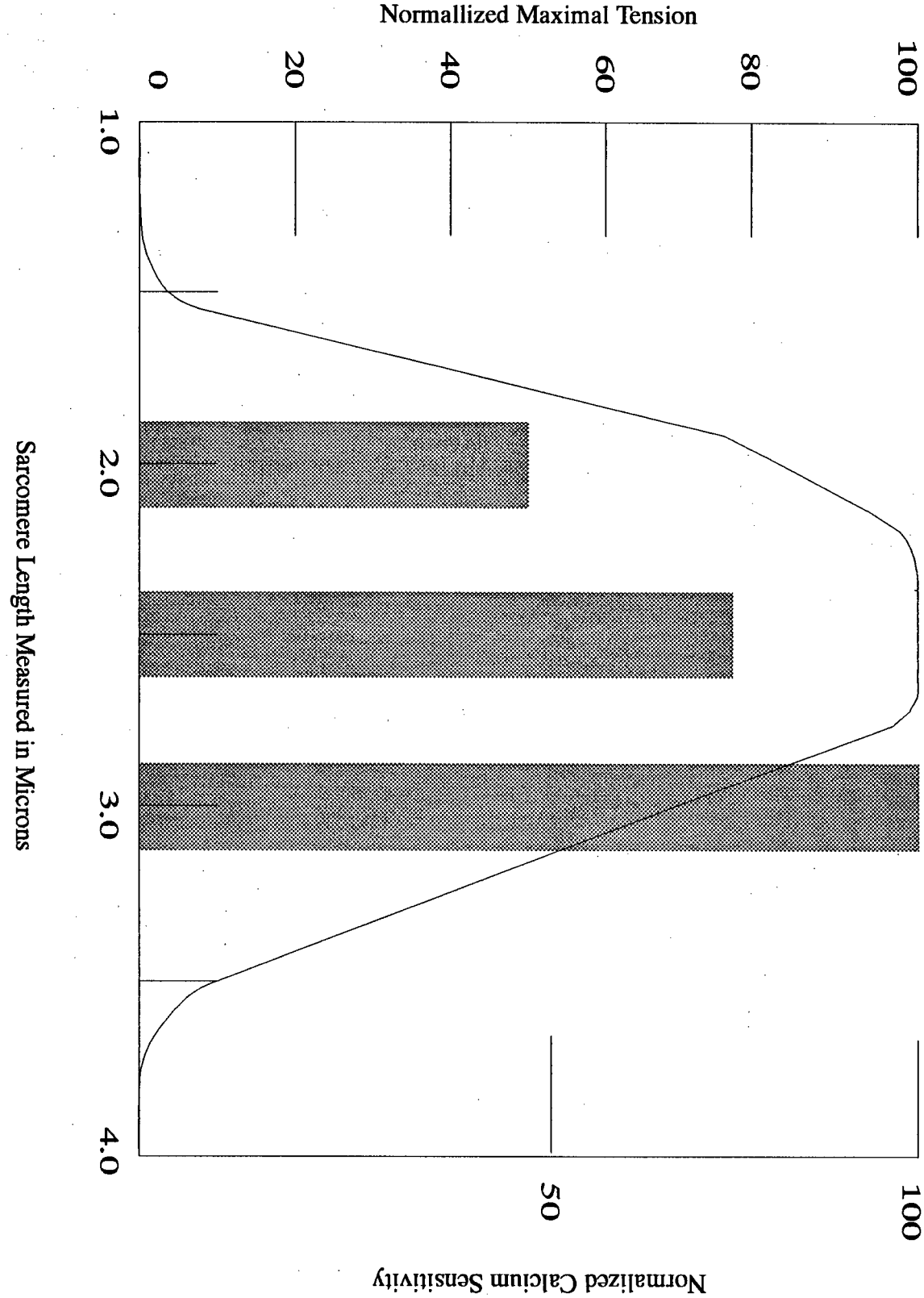
The importance of sarcomere length in influencing contractile performance is clearly indicated not only by its effect on force production, but also by its observed effect on the calcium sensitivity of striated muscle fibres (Babu *et al.*, 1987; Babu *et al.*, 1988; Gulati *et al.*, 1990; Moss *et al.*, 1991; Morimoto and Ohtsuki, 1994) (Figure 2).

Calcium Sensitivity

In 1972, Endo and co-workers reported that muscle fibre lengthening causes both a decrease in the threshold for calcium activated muscle contraction and the development

Figure 2: Sarcomere Length-Dependent Properties of Striated Muscle

Graphical representation of the changes in contractile performance that accompany increased sarcomere length in striated muscle fibres. The left axis shows the tension-length curve for rabbit psoas muscle fibres, indicating a plateau region of maximal force production between sarcomere lengths of 2.4 μm and 2.55 μm. The hatched columns show the normalized sarcomere length dependence of calcium sensitivity in striated muscle fibres. Note that at long sarcomere lengths, i.e. 3.0 μm, maximal tension development is decreasing rapidly, while the calcium sensitivity of these fibres is approaching maximal.


Normallized Maximal Tension
100
80
60
40
20
0
1.0
2.0
3.0
4.0
Sarcomere Length Measured in Microns
Normalized Calcium Sensitivity
100
50

of maximal force. Therefore, in response to fibre stretch and increasing sarcomere lengths, the calcium sensitivity of the contractile filaments increases such that the force-pCa relationship uniformly shifts to the left. "The simplest and most direct interpretation [of this data] is that the apparent affinity of the force regulatory sites for calcium are modified at different lengths of the sarcomere." (Stephenson and Wendt, 1984). These authors suggested that changing the sarcomere length during sub-maximal calcium-activated muscle contraction should result in the formation of local calcium fluxes. Using either obilin or aequorin as an indicator (Stephenson and Wendt, 1984; Gordon and Ridgeway, 1993), investigators showed that calcium ions were removed from the region of the myofibrils in response to shortening a muscle fibre, while calcium returned to this region in response to rapid fibre lengthening. It is not clear, however, why changes in local calcium concentrations, brought about by relatively rapid length steps would result in a sustained calcium sensitivity of the fibre. Work by several investigators has shown that for both cardiac (Fabiato and Fabiato, 1978; Hibberd and Jewel, 1982; Kentish *et al.*, 1988) and skeletal (Endo, 1972; Moss, 1979; Stephenson and Williams, 1982; Martyn and Gordon, 1988) muscle there is a marked increase in calcium sensitivity as fibres are passively lengthened to sarcomere lengths greater than the plateau of the tension-length relation. Similarly, a decrease in calcium sensitivity has been seen in fibres at short sarcomere lengths.

While the existence of altered calcium sensitivity at different lengths of the sarcomere is well known (Bressler and Morishita, 1991; Bressler and Morishita, 1992; Bressler *et al.*, 1994), the mechanisms by which this modulation occurs remain enigmatic.

The concentration of calcium in the sarcoplasm could be an important determinant of fibre performance and sensitivity. For example, "it seems likely that geometrical relations between the sites of calcium release, binding and sequestration may alter with changes in sarcomere length and so alter the diffusion distances involved." (Stephenson and Wendt, 1984). However, it is not likely that spatial changes in filament orientation would alter the ability or efficacy with which calcium can bind to the contractile apparatus because interfilament distances are at least fifty times greater than the calcium cation in its most hydrated form (Martyn and Gordon, 1988). More likely, however, is that a spatial reorientation of the filaments causes changes in the mircoDonnan potential between the filaments and the surrounding sarcoplasm.

The myofilaments carry a predominant negative charge on their surfaces, which is likely to become more equally distributed along the lengths of the filaments in response to fibre stretch (Stephenson and Wendt, 1984). Indeed, it has been shown previously that increasing either the net negative charge or the distribution of charge on the myofilaments by lowering ionic strength and pH causes an increase in calcium sensitivity of the muscle fibre (Martyn and Gordon, 1988; Bressler and Morishita, 1992). This behavior is thought to reflect an increased electrostatic interaction between the negatively-charged filaments and the calcium cations within the sarcoplasm. Therefore, reorientation of myofilamentous structures in response to stretch may result in a distribution of charge density along the lengths of the filaments, thereby increasing the fibre's calcium sensitivity (Martyn and Gordon, 1988). The importance of distributing this charge along the entire

filament length has been supported by other investigators (Allen and Moss, 1987; Kentish *et al.*, 1988; Metzger and Moss, 1988; Gulati and Babu, 1985).

Unpublished studies by members of our group (1992) have shown that decreasing the myofilament lattice spacing with the long chain polymers, PVP and Dextran (Godt and Maughan, 1981), causes an increase in force production at resting sarcomere lengths with a minimal shift in calcium sensitivity (Appendix I, Figure 3a). These fibres, however, show a larger increase in calcium sensitivity upon fibre stretch (Appendix I, Figure 3b). It has been suggested that increased myosin cross-bridge attachment under conditions of osmotic compression directly enhances thin filament cooperativity, causing both the calcium affinity and conformation of TnC to change (Hannon *et al.*, 1992). This behavior of the myosin cross-bridge is supported by the data obtained in the Dextran study (Appendix I, Table 1). This indicates that a highly collapsed myofilament lattice in the presence of both Dextran and long sarcomere lengths results in an increased calcium sensitivity due to the high probability of myosin cross-bridge attachment.

Further experiments were carried out by Bressler and co-workers (1992) to determine the effects of low ionic strength on the calcium sensitivity of striated muscle fibres. Under these conditions, maximal tension development rose significantly, suggesting a higher population of force-generating myosin cross-bridges than was seen in controls. It has already been reported that the effects of increased force production and myosin cross-bridge density are an increase in the calcium sensitivity of the muscle fibre (Fuchs and Wang, 1991; Wang and Fuchs, 1994). Both the increased force (data not shown) and the calcium sensitivity (Appendix II, Figures 4a and 4b) (Appendix II, Table

2) observed under low ionic strength conditions reflect more bound cross-bridges (Martyn and Gordon, 1988). The development of increased force per existing cross-bridge is unlikely, because the maximal velocity of shortening (Vmax) does not decrease in low ionic strength (Julian and Moss, 1981) and because both stiffness and thin filament mass increase (Brenner *et al.*, 1984).

Therefore, while electrostatic forces, charge distribution and myosin cross-bridge attachment seem to be involved in determining the calcium sensitivity of a fibre at a given sarcomere length, it has become increasingly apparent that these parameters regulate calcium sensitivity by their effects on TnC affinity and conformation. Thus, many researchers have extended their investigation of length-dependent calcium sensitivity to include the regulatory proteins that are part of the signal transduction pathway leading from calcium release to force production (Gulati *et al.*, 1990; Moss *et al.*, 1991). Changing sarcomere length results in transient intracellular calcium fluxes in the regions of the myofilaments (Kentish *et al.*, 1988; Gordon and Ridgeway, 1993) and, thus, many investigators have suggested that the mechanism of length-sensitive force generation is controlled by calcium-regulatory processes. Since troponin C is the binding protein for calcium in striated muscle fibres, it is likely to participate in any transient changes in calcium sensitivity. Furthermore, length-dependent changes in the lattice of the myofilaments have also been postulated to increase the ability and efficacy with which the troponin complex can bind calcium (Kentish *et al.*, 1988).

A role for TnC as the 'length sensor' in cardiac muscle has been proposed by Gulati et al (1990), and this lab has recently confirmed that TnC is an important regulator

of tension and calcium sensitivity in skeletal muscle (Bressler *et al.*, 1994). Extraction of endogenous TnC from striated muscle tissue results in a significant decrease of both force and length sensitivity of calcium (Moss *et al.*, 1985; Babu *et al.*, 1988; Bressler et al, 1994). Similarly, this depression of fibre calcium sensitivity is reversed after the reintroduction of TnC into the fibre.

Architecture of Troponin C

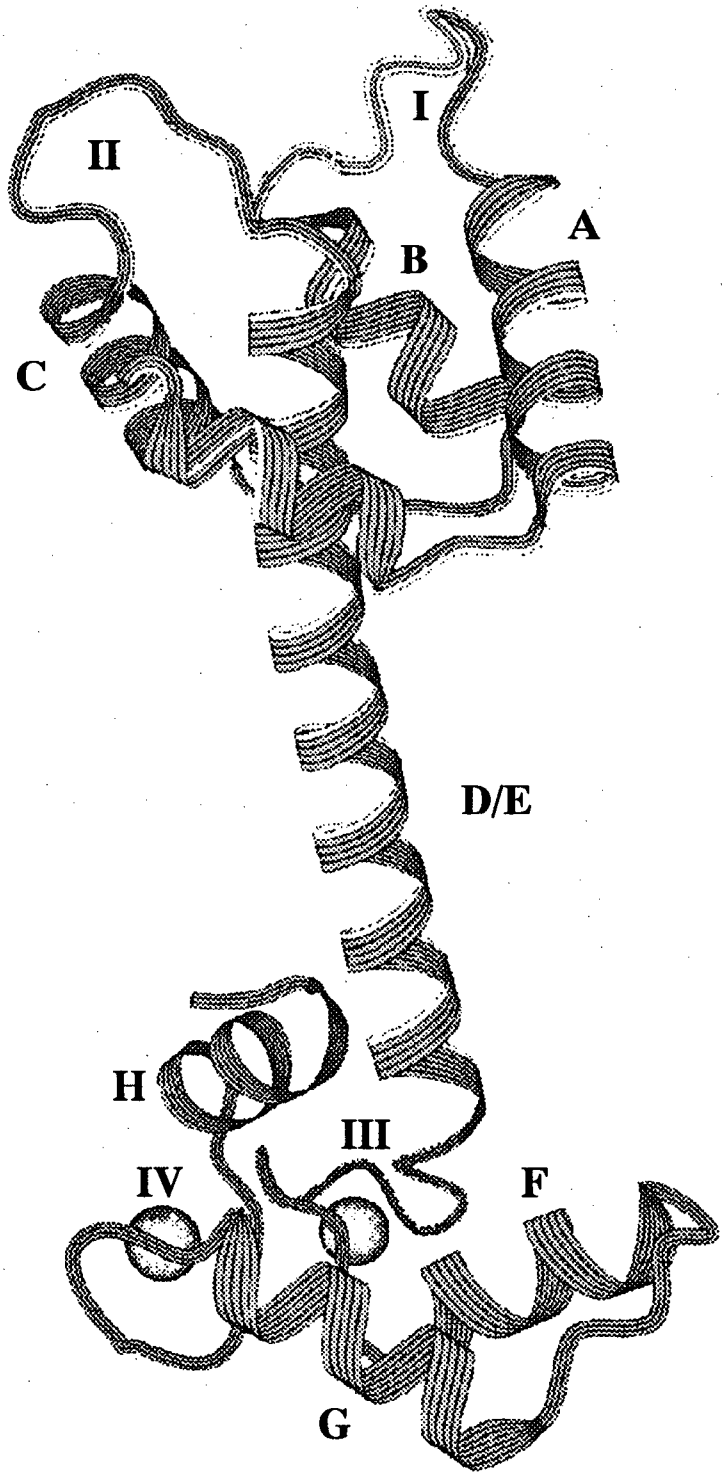
Protein Structure

Troponin C belongs to a large family of calcium-binding proteins that includes calmodulin, calbindin and parvalbumin. These EF-hand binding proteins are characterized by a continuous 30 amino acid calcium-binding region that adopts a helix-loop-helix conformation. Binding of calcium occurs in the central loop region which consists of 12 amino acids (Collins *et al.*, 1977). The residue specificity in this region is likely to determine the binding properties of the protein, thus accounting for the variable range of calcium affinities demonstrated among members of this family (Shaw *et al.*, 1991). Whereas, most of the proteins within the class have an ubiquitous distribution in the body, the highly conserved TnC gene is expressed exclusively in striated muscle tissue (Parmaceck *et al.*, 1991).

The dumbbell-shaped troponin C molecule is approximately 750 nm long and consists of 159 amino acid residues which are organized into distinct amino and carboxy terminal domains. The protein consists of eight functional alpha helices that are arranged into four calcium-binding sites and a domain-connecting helical linker (Figure 5). This nine-turn linker region is replete with amino acids having a specific propensity for

Figure 5: Ribbon Structure of Native Chicken Skeletal Muscle Troponin C

Ribbon structure of chicken skeletal muscle troponin C indicating helical segments and calcium binding loops. The amino terminal domain of the protein, is characterized by helices A through D and the Ca^{2+} specific binding sites I and II. The carboxy terminal domain consists of helices E through H and Ca^{2+}/Mg^{2+} sites III and IV. Note that these high affinity bindings sites are occupied under all physiological calcium concentrations. The interdomain helical linker (D/E) is presented in its extended form as determined from the crystal structure of TnC. Adapted from Trigo-Gonzalez *et al.*, 1993.


I
II
A
B
C
D/E
H
III
IV
F
G

helix formation, with the exception of the glycine residue at position 92 which is thought to impart flexibility to the protein (Herzberg and James, 1985). The strategic positioning of this residue, at the center of the protein, suggests that under certain conditions, such as muscle contraction, TnC may fold over to allow communication between the N and C terminal domains (Herzberg and James, 1985). This hypothesis is further supported by the indication that the TnC molecule may adopt a different structural conformation *in vivo* from that of the elongated conformation determined with crystal studies (Heidorn and Trewhella, 1988; Mehler *et al.*, 1991; Parmaceck *et al.*, 1991).

The amino terminal domain of TnC consists of the protein's first 85 amino acid residues; this in turn, corresponds to the location of four helices (A to D) and two calcium-specific binding sites (Sites I and II) located at residues 27 to 38 and 63 to 74 respectively (Collins *et al.*, 1977). The relatively low affinity for calcium at these sites suggests that they are unoccupied under conditions were muscle is relaxed. Thus, they mediate TnC's actions in response to increased calcium (Potter and Gergely, 1975). The amino terminal region of TnC is physiologically important in initiating the cascade of events leading to contractile disinhibition; as such it is referred to as the protein's regulatory domain. The relatively low affinity for calcium at sites I and II is thought to reflect both the amino acid composition of the binding loops in the N-domain and the inter-helical angles in this region (Herzberg and James, 1985). Furthermore, the regulatory domain possesses two salt bridges which have stabilizing effects on the apo state of the protein that also contribute to the low calcium affinities at sites I and II.

The carboxy terminal domain of TnC consists of amino acids 86 through 159, which form helices E to H. The two high affinity cation-binding loops in this region are located at residues 103 to 114 and 139 to 150, forming sites III and IV respectively. The binding properties of this region are significantly different from those exhibited in the regulatory domain. The high affinity with which sites III and IV bind divalent cations ensures that these sites are permanently occupied at resting physiological pH's and calcium and magnesium concentrations. Furthermore, this region does not demonstrate an absolute specificity for calcium and thus it may bind other divalent cations, such as magnesium. While the Ca-specific binding sites of TnC directly mediate the contractile response, the protein's non-specific binding sites have primarily structural implications (Zot and Potter, 1982). Indeed, anchoring of the protein to both TnI and the thin filament requires the occupation of sites III and IV (Moss, 1992; Negele *et al.*, 1992). This binding causes dramatic changes in protein conformation and helicity which stabilizes both Tn subunit interactions (Trigo-Gonzalez *et al.*, 1992; Li *et al.*, 1994) and the entire troponin-tropomyosin complex (Zot and Potter, 1982).

Troponin C and Troponin I Interactions

Ligand binding to both the high and low affinity sites of TnC causes significant changes in the protein's secondary and tertiary structure which in turn regulate the integrity of TnC-TnI interactions (Herzberg *et al.*, 1986). The physiologically relevant interactions between TnC and TnI appear to exist in the region of TnC's C-terminal domain; both the inhibitory and regulatory peptides of TnI bind to this region with great

affinity (Van Eyck *et al.*, 1991; Ngai *et al.*, 1994). The inhibitory peptide of TnI (TnIp) represents the minimum sequence necessary to inhibit the formation of the acto-S1 complex (Ruegg *et al.*, 1989). This peptide segment which extends between TnI residues 96-116 binds both to actin and to the C-terminal domain of TnC, switching between the two in a calcium-dependent manner (Talbot and Hodges, 1981; Van Eyck and Hodges, 1988). Therefore, the effect of calcium binding to the regulatory domain appears to be translated through the high affinity domain of TnC, resulting in the transfer of TnIp from actin to TnC and the disinhibition of the contractile machinery.

Joint communication between the N and C terminal domains of TnC has been further supported by the determination that TnIp binds to TnC's regulatory domain in a calcium-dependent manner (Swenson and Fredricksen, 1992). This suggests that TnC may undergo significant conformational changes in the presence of high calcium concentrations, allowing both of its domains to interact, together forming a single binding site for TnIp. Directly interpreting this result, many investigators have suggested that calcium binding to sites I and II results in protein folding via the D/E helical linker of TnC (Grabarek *et al.*, 1986; Strang and Potter, 1992). In fact, removing a portion of the linker region to generate a more compact protein has been shown to result in a more efficient release of actomyosin inhibition (Xu and Hitchcock-deGregori, 1988).

Tissue Specific TnC Proteins

Both cardiac and skeletal muscle express different isoforms of the TnC protein, although both of these isoforms subserve the same function in their respective tissues.

Cardiac muscle and slow skeletal muscles are characterized by a TnC isoform that possesses only three active calcium-binding sites. Thus, the primary structural difference between sTnC and cTnC are modifications in the amino acid sequences at the low affinity calcium-binding site I which render this site inactive and unable to bind calcium. As a result, the functional role of the amino terminal domain in cTnC is performed solely through calcium binding at site II. Thus, since only one calcium ion is required for activation of contraction in these tissues, cardiac muscle has the potential of being more easily excitable than fast skeletal muscle in which binding of two calcium ions is required to facilitate contraction (Parmaceck and Leiden, 1991).

Many studies have exploited this difference in structure between TnC isoforms to examine the specific effects of calcium binding to TnC's regulatory domain. Experiments focusing on the exchange of TnC between cardiac and skeletal muscle have clearly demonstrated that the contractile properties of both tissue types can be significantly modified in the presence of a foreign TnC isoform (Babu *et al.*, 1988; Gulati *et al.*, 1990; Morimoto and Ohtsuki, 1994). Cardiac muscle displays a higher degree of calcium sensitivity than that exhibited in skeletal muscle. Furthermore, under normal physiological conditions, cardiac myocytes perform at levels of sub-maximal force generation, but tension may increase in response to both increased cardiac volume and stretch. It has been suggested that these features of cardiac muscle are mediated by the presence of cTnC, and that skeletal muscle may not be as effective in this manner due to its slightly modified TnC structure. In fact, this hypothesis has been confirmed by experiments in which endogenous cTnC was removed from cardiac myocytes and replaced with the fast or

skeletal isoform of TnC (Gulati *et al.*, 1990). Results of these studies showed conclusively that the presence of sTnC caused a decrease in the calcium sensitivity of cardiac muscle which was reversed upon reintroduction of the native TnC isoform. Similarly, the analogous set of experiments in which native skeletal TnC was replaced by the cardiac isoform of the protein caused an increase in the calcium-dependent length sensitivity of skeletal muscle fibres (Babu *et al.*, 1988).

A second line of evidence has been provided by mutagenesis studies in which cardiac TnC was modified to possess an active calcium-binding site I. In these studies, the calcium sensitivity of cardiac muscle was also depressed resulting in a shift of the force-pCa relationship to a point midway between that of untreated cardiac and skeletal muscle (Gulati *et al.*, 1989; Gulati *et al.*, 1990). The smaller decrease in calcium sensitivity demonstrated in this study is likely to reflect a less pronounced perturbation between TnC and the cardiac isoform of TnI.

Indeed, extensive mutagenesis studies utilizing both fast and slow TnC have supported these conclusions, confirming that structural manipulation of TnC's amino terminal domain significantly modifies the contractile properties of both cardiac and skeletal muscle tissues (Gulati *et al.*, 1990; Putkey *et al.*, 1991). This class of experiments has elegantly assessed the binding of calcium to site I of the regulatory domain as an important suppressive mechanism on calcium sensitivity while demonstrating that site II functions primarily in the absolute coordination of contractile events. In light of this data, the differential length-sensitive capabilities of cardiac and skeletal muscle have been elucidated. While the low affinity sites obviously have an important role in regulating

length-dependent calcium sensitivity, the action of the high affinity calcium-binding sites in these processes have yet to be established.

The Skeletal Muscle Length Sensor

As both the binding protein for calcium and the mediator of the contractile response, troponin C is the ideal protein with which to begin an examination of calcium-dependent processes in skeletal muscle. Indeed, much evidence has been collected to support the conclusion that TnC plays a central role in the regulation of calcium sensitivity at different lengths of the sarcomere. While removal of endogenous TnC from skeletal muscle causes an absolute depression in the force-pCa relationship of treated fibres, it similarly diminishes the observed increase in calcium sensitivity of the fibre in response to stretch (Babu *et al.*, 1986; Moss *et al.*, 1986; Bressler *et al.*, 1994). Mutagenesis studies have also shown that altering TnC structure and conformation may modulate the contractile response of skeletal muscle, either increasing or decreasing a fibre's length-dependent calcium sensitivity depending on the nature of the mutation and on the extraction protocol utilized (Gulati *et al.*, 1990; Moss *et al.*, 1991).

These results, however, must not be interpreted in isolation from the remaining troponin subunits. TnC has been shown to possess strong and physiologically relevant interactions with other members of the troponin complex. These interactions facilitate transmission of the calcium signal to the contractile filaments and, therefore, represent another potential location for contractile modulation (Morimoto and Ohtsuki, 1994). For example, the significance of subunit interaction in governing calcium sensitivity has been

demonstrated by the differential reduction in calcium sensitivity as seen between the introduction of a site I cTnC mutant into cardiac muscle versus the introduction of sTnC (Gulati *et al.*, 1990). In the latter case, an inappropriate coordination of sTnC with cTnI resulted in a more dramatic depression of calcium sensitivity at different sarcomere lengths. This finding has been supported by Morimoto & Ohtsuki (1994) who have shown that the Ca^{2+} sensitivity and cooperativity of striated muscle fibres requires the interaction of TnC with other members of the troponin complex.

As mentioned previously, the functionally relevant associations between TnI and TnC exist in the region of the protein's high affinity domain. While binding of calcium to sites I and II of TnC acts as the trigger event for muscle contraction, this response must be translated through the protein's carboxy terminal domain. Therefore, the integrity of TnC-TnI interactions in this region of the protein are paramount to successful transduction of the calcium signal from TnC to the contractile filaments. Eitehr structural modulation or attenuation of this domain may result in altered subunit interactions leading to diminished contractile performance of the muscle fibre.

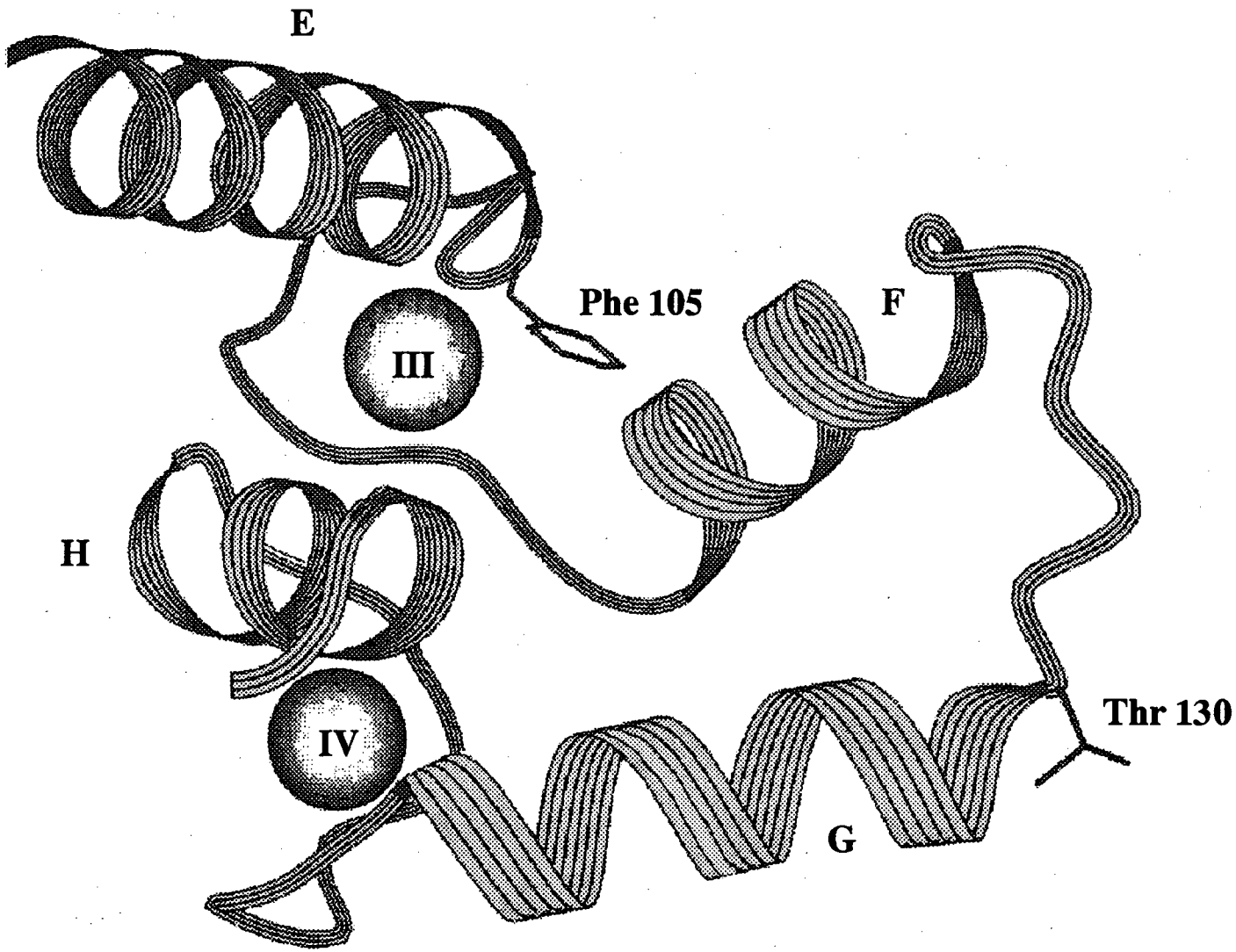
Hence, this study undertakes an extensive analysis of the effects of C-domain attenuation on the length dependence of calcium sensitivity by utilizing specific TnC variants, each containing a serial replacement of the amino acid at position 130 of the high affinity domain.

Troponin C Mutants

Position 130 of chicken skeletal muscle TnC lies in the N-cap of the G-helix within the protein's high affinity domain (Figure 6), i.e. at a position between the F and G

Figure 6: Ribbon Structure of Chicken Skeletal Muscle TnC's High Affinity Domain

The locations of amino acid residues where mutagenesis was performed are specifically highlighted. The naturally occurring Thr 130 residue was substituted by serine (I130S), glycine (I130G) and recombinant isoleucine (I130) to produce "high-affinity mutants of TnC with tailored calcium affinities". The Phe residue at position 105 was substituted by tryptophan (F105W), producing a reporter region capable of measuring calcium binding to sites III and IV by tryptophan fluorescence. Adapted from Trigo-Gonzalez *et al.*, 1993.


E
Phe 105
F
III
H
IV
Thr 130
G

helices. Mutations at this site have the potential to alter both behavior and binding affinities at sites III and IV (Trigo-Gonzalez *et al.*, 1993). Furthermore, mutation of the first amino acid residue of an alpha-helix (i.e. the N-Cap) has significant consequences both on the stability of the affected helix and on the ion-affinity of the associated binding sites. Indeed, mutations at the 130 position resulted in a maximal four-fold decrease in calcium affinity at both sites III and IV as measured by tryptophan fluorescence (Trigo-Gonzalez *et al.*, 1993).

Substitution of the threonine residue, which occurs naturally at this position, significantly disrupts side chain interactions with adjacent helical residues, thus destabilizing the entire helix. Accordingly, substitutions "that were capable of forming a hydrogen bond with the main chain (Ser) conferred higher affinities on the variant proteins than residues unable to form such bonds (Gly and Ile)" (Trigo-Gonzalez *et al.*, 1993). While mutations at position 130 had clear implications on the stability and affinity of the Ca^{2+}/Mg^{2+} sites, they appeared to have no effect on the cooperativity of binding in this region as demonstrated by their values of the Hill coefficient (Trigo-Gonzalez *et al.*, 1993).

Therefore, this series of mutant proteins is likely to be an effective tool in studying length-dependent changes in calcium sensitivity because they possess destabilized high-affinity binding sites with altered ion affinities and because their mutation exists in a region of the protein which is salient for modulating TnC-TnI interactions.

To that end, this study has employed three of these specific TnC variants whose effects on protein destabilization range from minimal (I130S) to moderate (I130G) and

maximal (I130). Extraction of endogenous TnC from skinned rabbit psoas skeletal muscle fibres was followed by stoichiometric replacement of the thin filament with high affinity TnC mutants. Subsequently, the calcium sensitivity and cooperativity of these treated fibres was monitored at both short and long sarcomere lengths to determine the physiological consequences of high-affinity domain destabilization. Furthermore, this analysis has been extended to assess whether such effects are the direct result of protein modification or, rather, a result of disrupted TnC-TnI interactions in this region of the protein.

This study also addresses the controversy of TnC extraction techniques that exists in the literature by examining the effects of partial (50% or less) or nearly complete removal of endogenous TnC on the force generation, cooperativity, and calcium sensitivity of treated muscle fibres. Silver-stained polyacrylamide gels of both extracted and reconstituted skeletal muscle fibres provides both qualitative and quantitative support for the conclusions presented here.

Overview

The specific objective of this study was to determine the effect of high-affinity domain destabilization of TnC on the length-dependence of calcium sensitivity in rabbit skinned single psoas muscle fibres. Established experimental techniques were used to extract endogenous TnC from fibre segments and, subsequently, reconstitution of these fibres was performed with high affinity TnC variants. The force-calcium relationship of

these treated fibres was measured at short and long sarcomere lengths to detect any changes in the length-dependence of calcium sensitivity.

MATERIALS AND METHODS

Fibre Preparation

Mature white male New Zealand rabbits were pre-injected with the anaesthetic Innovar and then killed by a lethal dose of Sodium Pentobarbitol. Whole right and left psoas muscles were dissected from these animals and immediately placed in cold relaxing solution on ice (*solution A,* Table 3). Muscles were subsequently rinsed in relaxing solution and left in the cold (5°C) for an additional two hours. Excised muscles appeared to have contracted upon removal and, therefore, this solution facilitated tissue relaxation. Fibre bundles were subsequently prepared by mechanically stripping the muscle tissue into bundles of approximately 50 fibres and tying them at their ends to wooden sticks by surgical silk. Fibres were initially stored for 15 hours at 4°C in skinning solution (*solution B,* Table 3), and approximately 12 - 15 hours later, the fibre bundles were placed in fresh solution. Fibres were then stored at -20°C and used within three months. The high concentration of glycerol in the skinning solution (50% v/v) disrupted both the sarcolemma and intracellular membranes (Weber and Tortzekl, 1954; Moss, 1992). Perforation of the sarcolemmae by glycerol ensured unimpeded access to the contractile filaments within each fibre during the experimental protocol, whereas similar treatment of the sarcoplasmic reticulum ensured that all intracellular calcium stores had been depleted, allowing for precise control of calcium levels during experimentation (Moss, 1992). Subsequently, all fibres treated in this manner are referred to as having been 'skinned'.

Force Measurement

Experiments were carried out on single muscle fibre segments ranging in length from 1mm to 5 mm. Once isolated, fibres were mounted in an experimental chamber between a force transducer (Akers model AE801; Horten, Norway) and a servomotor (Cambridge Technology model 300S). The servomotor held the muscle fibre isometrically during contraction, while the transducer translated contractile force into a voltage signal which was displayed on a digital oscilloscope (Tektronix model TDS 420 or Nicolet model 3091). Experimental traces were printed out on either a laser printer (Everex model Laserscript LS) or an xy-plotter (HP model 7036), and force measurements were subsequently made by hand, directly from these records.

The experimental chamber consisted of a series of wells, each with a capacity for 0.2 ml of solution. The temperature within each of these wells was set to 15ºC by a cold water pump that continuously perfused the outside walls of the experimental chamber. Fibres were mounted within the muscle chamber by wrapping their ends around stainless steel wire hooks that had been pretreated with nitric acid to roughen their surfaces and to prevent fibre slippage during activation. Both the transducer and the motor were mounted on a ratchet device that could be raised or lowered to facilitate the transfer of fibres between individual wells of the experimental chamber.

Prior to beginning an experiment, isolated fibres were bathed in a relaxing solution containing 0.5% Triton-X 100 for 5 minutes, to ensure the complete destruction of all membranous elements. Fibres were then briefly activated in pCa 4.8 (i.e. $-\log[Ca^{2+}] = 4.8$)

until tension reached half maximal to ensure that fibres were firmly wrapped around the wire hooks.

Stimulation of these fibres was achieved by immersing them in a series of activating solutions (*solution C,* Table 3), each with an increasing concentration of calcium. The strength of activating solutions ranged from pCa 6.6 to pCa 4.8, by increments of 0.2 pCa units. Fibre segments were allowed to reach steady-state tension (F) at each pCa before being transferred into a solution with a higher calcium concentration. Once maximum tension was achieved, the fibres were immediately returned to the original relaxing solution (*solution A,* Table 3). Subsequently, tension development (F) at each pCa was calculated as a percent of the maximum tension developed (Fmax). This method of force normalization was necessary to facilitate a statistical comparison between individual fibres.

Sarcomere Length Assessment

The sarcomere lengths of individual fibres were monitored throughout the experimental protocol and were determined either by laser diffraction (Rudel and Zite-Ferenczy, 1980) or by video-image analysis (Slawnych *et al.*, 1995). A 10 mW helium-neon laser (Hughes model PC) mounted on a bracket below the experimental chamber transilluminated the fibre and the resultant diffraction pattern was projected onto a calibrated grid placed a fixed distance (6.5 cm) above the fibre. The entire fibre segment could be translated through the laser beam to assess sarcomere lengths at the two ends and along the middle of the fibre. In another series of experiments, the experimental chamber

was mounted on the stage of an inverted optical microscope (Nikon model DIAPHOT 300) and video images of the fibre were subsequently recorded during the experimental protocol. Fourier transformation performed on these video images provided an alternative method of measuring sarcomere length (Slawnych *et al.*, 1995) and was used in assessing sarcomere length homogeneity of the fibre segment (Figure 7). Specifically, fibre segments were considered as periodic sarcomeric patterns with spatial frequencies in the range of 0.26 to 0.71 μm^{-1} corresponding to sarcomere lengths in the range of 1.4 to 3.8 μm. As such, fibre images could be represented in terms of a Fourier series. This transformation was efficiently carried out on video images of the fibre using the Fast Fourier Transform (FFT) (Cooley and Tukey, 1965). Once mounted between the force transducer and servomotor, the fibres could either be passively shortened or lengthened by micromanipulators to achieve the desired sarcomere length. The stage of the microscope was also fitted with guide rails to facilitate the transfer of a fibre between individual wells of the experimental chamber.

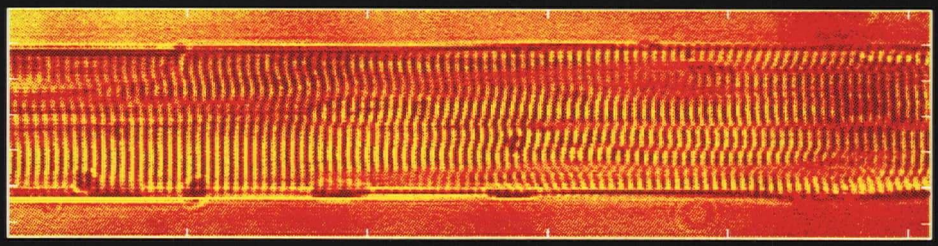
Mutant Troponin C Preparation

Fusion variants of recombinant chicken troponin C were prepared in the Laboratory of Dr. Thor Borgford at Simon Fraser University as described previously (Trigo-Gonzalez *et al.*, 1993). Briefly, troponin C genes containing the amino acid substitutions serine, glycine and isoleucine at position 130 in the high-affinity domain were expressed in *E. Coli* strain QY13. Variant proteins were purified to homogeneity by a two-step anion exchange chromatography

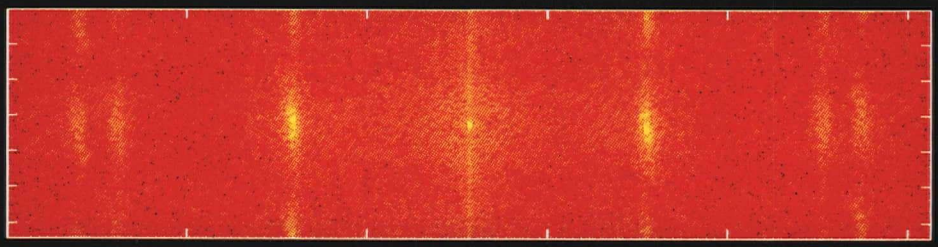
Figure 7: Fourier Image Analysis of Skinned Single Skeletal Muscle Fibre Segment

A video image of a relaxed skeletal muscle fibre is shown in the upper panel. The whole, half and quarter Fourier spectra of the fibre segment are presented in the lower panels of the figure.

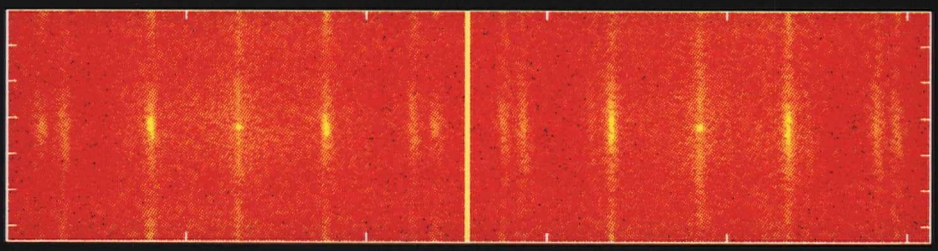
Original Image



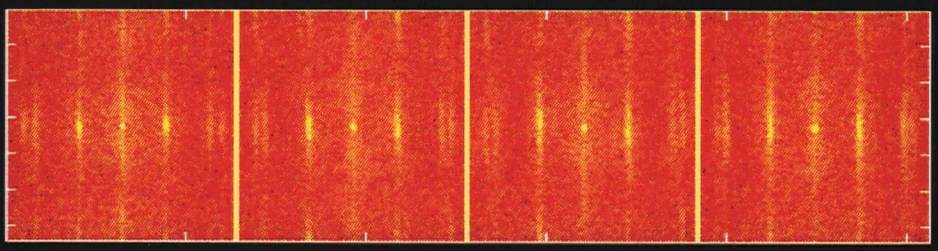
Full Spectrum



Half Spectra



Quarter Spectra



method (Trigo-Gonzalez *et al.*, 1993). Purified proteins were lyophilized and stored at -20°C until required for fibre reconstitution. Comparative studies have demonstrated that TnC retains a high degree of homology among species, justifying the utilization of chicken skeletal troponin C in this study.

Endogenous Troponin C Removal

Fibres at resting sarcomere lengths were treated with a standard TnC extraction solution (*solution D,* Table 3) for 10 minutes and subsequently activated in pCa 4.8 until maximal tension was achieved (Cox *et al.*, 1981; Moss, 1992). A reduction in maximal force between treated and control fibres was used to gauge the amount of TnC removed from fibre segments (Moss *et al.*, 1985). Fibres not reaching a minimum of 50% reduction in force were reimmersed in extraction solution for another 10 minutes. The presence of the calcium chelator, EDTA, in the extraction medium facilitated the removal of divalent cations from the high-affinity binding sites of TnC resulting in dissociation of troponin complexes from the thin filaments (Moss, 1992).

In another series of experiments, a more complete extraction of TnC was performed by bathing the fibre segments for 5 minutes in a trifluoperazine (TFP) containing solution (*solution E,* Table 3). Upon removal from the extraction medium, fibres were washed three times in relaxing solution to ensure the removal of residual TFP, and they were then activated in pCa 4.8 (Moss, 1992). Only those fibres exhibiting a minimum 75% reduction in maximal force were utilized in this study.

Troponin C Reconstitution

Troponin C extracted fibres were reconstituted with either native rabbit skeletal muscle TnC or with a specific TnC mutant by bathing the fibre in relaxing solution containing 1 mg/ml TnC for four intervals of ten seconds each (Moss *et al.*, 1985). The force-pCa relationships of these fibres were subsequently measured at the initial sarcomere lengths and at longer sarcomere lengths. Criteria for the successful reconstitution of all fibres was set at 75% recovery of the maximal force measured in control fibres.

Polyacrylamide Gel Electrophoresis

The removal and reconstitution protocols were confirmed both qualitatively and quantitatively by examination of silver-stained SDS polyacrylamide gels (SDS-PAGE) of fibre segments at various stages of the experimental protocol. Specifically, this was performed by scanning silver-stained gels with a desktop scanner (HP model Scanjet IIC), and then analyzing the optical density profile of the resultant image with computer software (NIH Image version 1.57). By this method, individual bands on SDS gels were represented as intensity peaks; the area under each peak was assumed to be proportional to the quantity of protein in the respective band. Intensity profiles from 30 untreated fibre segments showed that the area of the TnC band was 24.9 ± 1.2% of the TnI band area. Thus, comparison of TnC:TnI ratios after removal and reconstitution protocols provided a quantitative indication of the percent change in the fibre's TnC content (Moss, 1985).

Comparing absolute TnC protein load with the resultant band intensity produces a biphasic relationship with a linear component in the physiological range of TnC content

(Figure 8). From this calibration curve, it was determined that untreated 3-mm skinned fibre segments contain approximately 21 ng of TnC (Yates and Greaser, 1983) which is in agreement with previously published values (Moss, 1985). By these quantitative measures of TnC content (i.e. TnC:TnI intensity ratios and TnC calibration), one can make absolute conclusions about the amount of TnC in individual fibres at each stage of the experimental protocol and, furthermore, one can correlate TnC content to force production.

To prevent protein degradation, fibre segments and TnC proteins were both dissolved in a sample buffer (*solution F,* Table 4) (Reiser, personal communication). They were subsequently stored at -20°C until time of use. Subsequently, electrophoretic examination of these samples was performed on a minigel apparatus (BioRad model Mini-PROTEAN II Cell) at an approximate current of 38 mA through the 4% stacking gel (*solution G,* Table 4) and 48 mA through the 15% separating gel (*solution H,* Table 4) (Laemmli, 1970). Composition and pH of the running buffer (*solution I*) (Rieser, personal communication) is described in Table 4. After complete progression of the current through the separating gel, the power source was disconnected and gels were chemically fixed (*solution J,* Table 4) for three intervals of one hour each to inhibit diffusion of protein bands.

Prior to commencing the silver staining protocol, gels were first washed at room temperature in ultrapure MilliQ distilled water for two 15-minute intervals and then re-fixed in a 10% gluteraldehyde solution for approximately 15 hours at room temperature (*solution K,* Table 5).

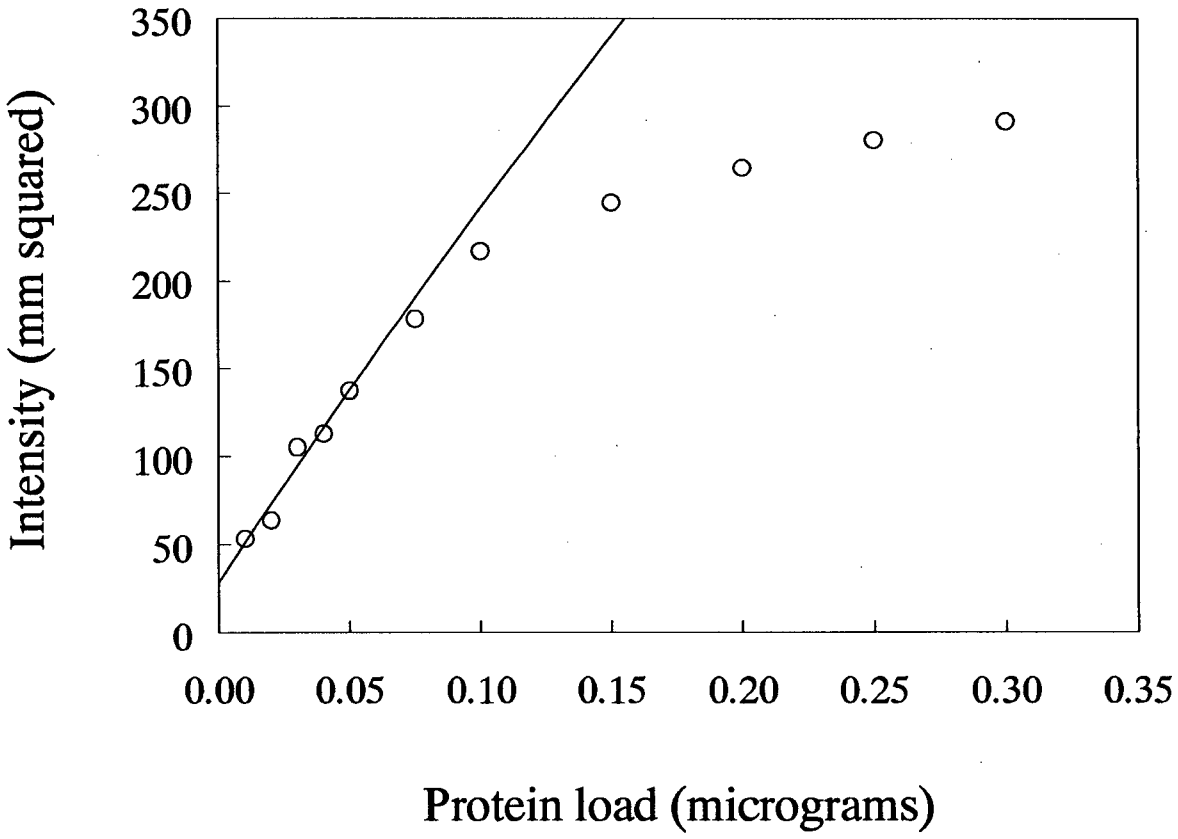


Figure 8: TnC Calibration Curve

Experimentally-derived calibration curve for TnC utilizing silver-stained SDS polyacrylamide gels. The non-linearity of silver stain was minimized by pretreatment of gels with an aqueous 10% glutaraldehyde fixation (Guilian *et al.*, 1983). The linear portion of the curve represents the physiologicaly relevant concentration range for TnC. It was determined by this relationship that skinned single fibre segments contained approximately 7 ng of TnC per mm of fibre, assuming that TnC accounts for 0.156% of total fibre weight (Yates and Greaser, 1983).

This fixation technique has been shown to normalize the non-linear staining properties of silver (Guilian *et al.*, 1983), thus allowing comparison of individual gel products. Protein band visualization, in this study, utilized silver rather than Commassie Blue because of the higher degree of sensitivity conferred by the silver staining technique. The silver staining method (Tsai and Frasch, 1982) was preceded by the washing of gels in ultrapure MilliQ distilled water for three intervals of 15 minutes each. Gels were then immersed in a silver solution (*solution L,* Table 5) for 10 minutes and re-washed in distilled water. Staining of these gels (*solution M,* Table 5) (Tsai and Frasch, 1982) occurred rapidly and, development was allowed to proceed only until the TnC band became visible. Gels were then immediately transferred to a stop solution (*solution N,* Table 5) for ten minutes to terminate the developing process and to prevent non-selective staining of the entire gel.

Gels were subsequently dried at room temperature between two sheets of taught cellophane membrane backing (BioRad model 543 Gel Dryer) for 48 hours.

Statistical Analysis

For the purpose of this study, variance of results has been expressed as standard error. These values are indicated as vertical bars on all graphical data where the number of experiments performed exceeds four.

All force-pCa responses were fit to the Hill Equation by a non-linear least squares method.

$$F = Fmax/\ 1 + 10^{(pCa - pK)(n)}$$

Statistical comparisons of pK (the calcium concentration at which tension was half maximal) and n (related to the slope of the curve) for each curve were carried out using a two-tailed Student's t-test with a 95% confidence limit. The results of these analyses are reported on Tables, which summarize the data obtained under various experimental conditions.

Table 3: Composition of Solutions for Fibre Preparation and Activation (in mM)

	Relaxing Solution	Skinning Solution	Activating Solution	Extraction Solution I	Extraction Solution II
	Solution A	*Solution B*	*Solution C**	*Solution D*	*Solution E*
KCl	100.00	100.00	-	-	-
$MgCl_2$	9.00	9.00	-	-	-
Na_2ATP	4.00	4.00	3.29	-	-
MOPS	10.00	10.00	121.20	-	-
Na_2EGTA	5.00	5.00	15.00	-	-
Glycerol	-	50% (v/v)	-	-	-
$Ca(Prop)_2$	-	-	5.82	-	-
$Mg(Prop)_2$	-	-	5.14	-	-
KProp	-	-	5.57	-	-
Na_2CP	-	-	15.60	-	-
KOH	-	-	59.00	-	-
DTT	-	-	0.50	-	-
Na_2EDTA	-	-	-	5.00	5.00
Imidazole	-	-	-	20.00	20.00
TFP	-	-	-	-	1.00
pH	7.0	7.0	7.0	7.9	7.2
Temperature	4°C	-20°C	15°C	15°C	15°C

* Concentrations indicated are for activating solution at pCa 9.0 and 200 mM ionic strength. The strength of this solution was altered by changing the concentration of $Ca(Prop)_2$ and appropriately adjusting the concentrations of the remaining reagents to reflect a total ionic strength of 200 mM.

Table 4: Composition of Solutions for SDS Polyacrylamide Gel Electrophoresis* (in %)

	Sample Buffer	Stacking Gel	Seperating Gel	Running Buffer	Fixation Solution I
	Solution F	*Solution G*	*Solution H*	*Solution I*	*Solution J*
Urea	48.000	-	-	-	-
Thiourea	15.200	-	-	-	-
DTT	1.156	-	-	-	-
Trizma Base	0.604	1.430	5.676	0.300	-
SDS	3.000	0.106	0.131	0.100	-
Bromophenol	0.004	-	-	-	-
Glycine	-	-	-	1.440	-
Acrylamide	-	3.867	14.525	-	-
Bisacrylamide	-	0.105	0.395	-	-
APS	-	0.149	0.047	-	-
Temed	-	0.199	0.062	-	-
Ethanol	-	-	-	-	40.000
Acetic Acid	-	-	-	-	5.000
pH	7.0	-	-	8.3	-
Temperature	-20°C	RT	RT	RT	RT

* All solutions are expressed as a percentage weight per volume (w/v)

Table 5: Composition of Solutions for Silver Stained SDS Polyacrylamide Gel Electrophoresis* (in %)

	Fixation Solution II	Silver Stain	Developer	Stop Solution
	Solution K	*Solution L*	*Solution M*	*Solution N*
NaOH	-	2.000	-	-
NH_4OH	-	0.400	-	-
$AgNO_3$	-	0.700	-	-
Acetic acid	-	-	-	7.000
Citric acid	-	-	5.000	-
Formaldehyde	-	-	0.100	-
Glutaraldehyde	10.000	-	-	-

* All solutions are expressed as a percentage weight per volume (w/v)

RESULTS

Sarcomere Length-Dependent Properties of Skeletal Muscle

Figure 9 shows original tension records from a single fibre segment at three sarcomere lengths. A force plateau for these fibres was measured between sarcomere lengths of 2.4 and 2.5 μm. Tension development was markedly reduced as fibres were passively shortened (2.0 μm) or lengthened (3.0 μm) to new sarcomere lengths.

The existence of a length-dependent mechanism for calcium sensitivity was also confirmed by comparing force-pCa relationships obtained from single fibres at short, intermediate and long sarcomere lengths (Figure 10). The pK and n values for each of these curves is shown in Table 6. The significant leftward shift in the pK of these fibres at longer sarcomere lengths clearly supports a length-dependent increase in the calcium sensitivity of the myofilaments.

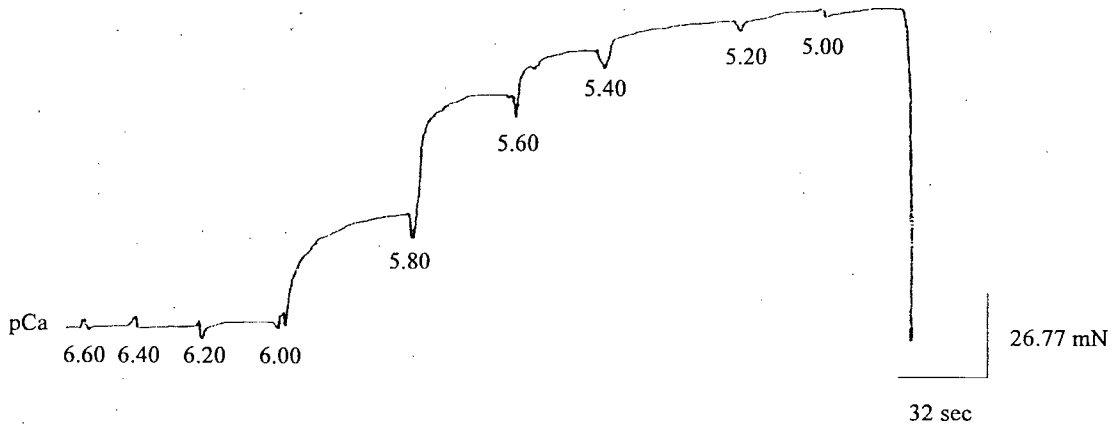
Troponin C Regulation of Calcium Sensitivity

Figure 11a shows tension transients from a single muscle fibre at the plateau of the tension-length curve before extraction of endogenous TnC, after partial extraction of endogenous TnC, and after reconstitution of the fibre with I130S. Activation of the fibre after partial extraction of endogenous TnC was accompanied by a significant reduction in maximal force production. Subsequent reconstitution of the fibre with TnC resulted in the redevelopment of force to near control levels. Therefore, it may be inferred that

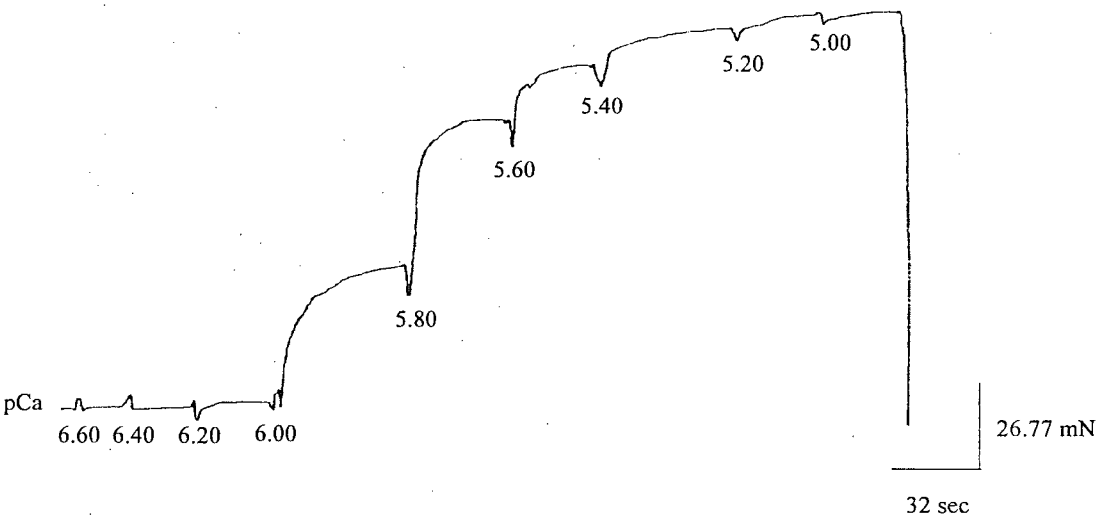
Figure 9: Effects of Sarcomere Length on the Force Development and Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

Original tension records obtained from a single skinned fibre segment at increasing calcium concentrations and sarcomere lengths. The force-pCa trace shown in A was acquired at a sarcomere length of 2.0 μm, while Trace B was taken at 2.5 μm and Trace C at 3.0 μm. Note the change in both maximal force development and calcium sensitivity of the fibre segment as sarcomere length increases.

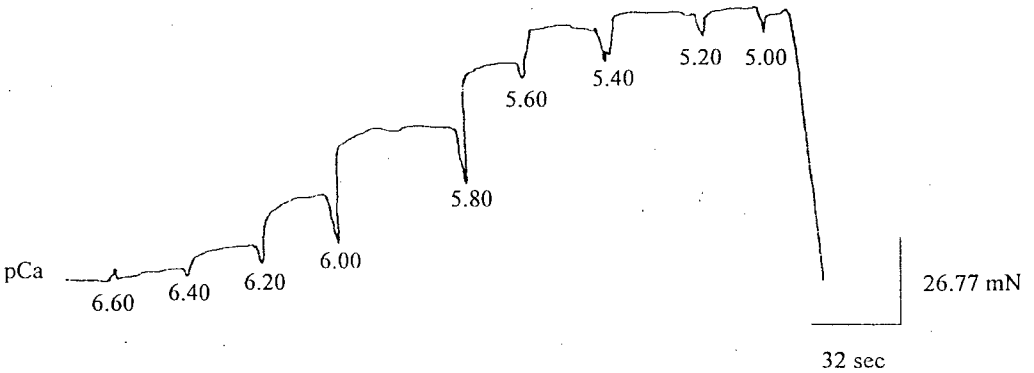
A. Short Sarcomere Length



B. Intermediate Sarcomere Length



C. Long Sarcomere Length



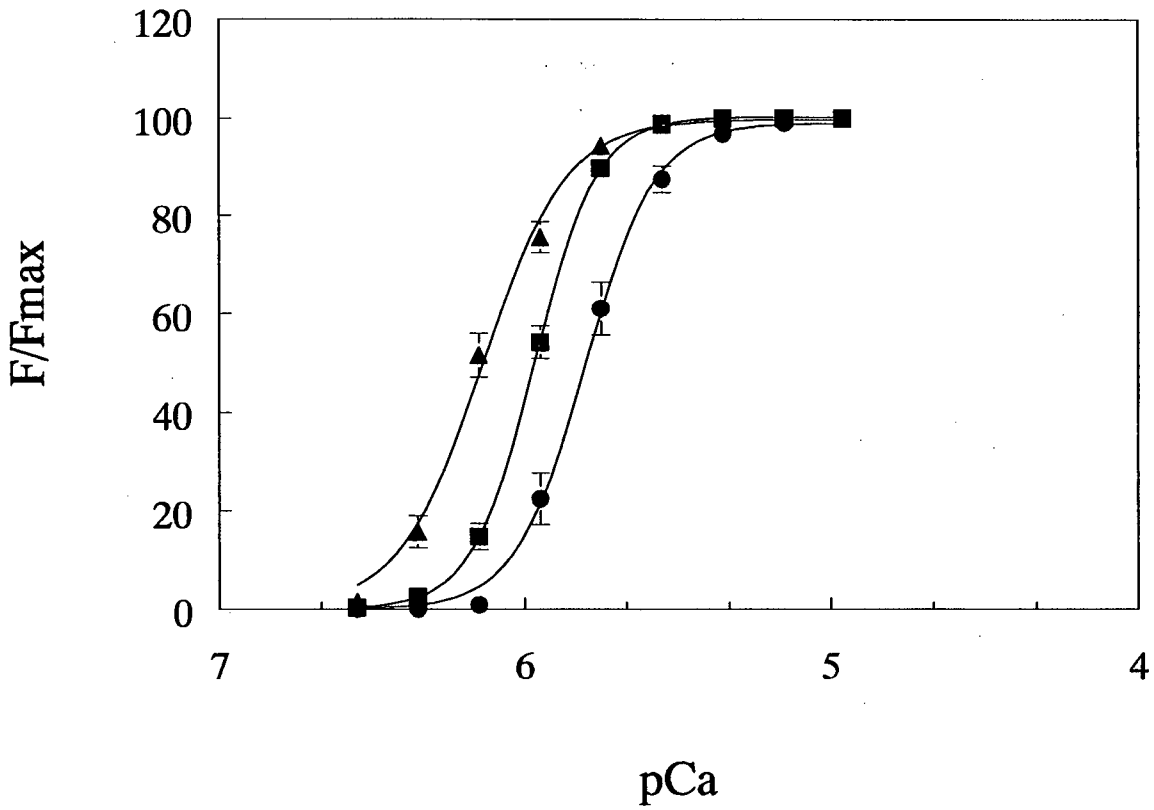


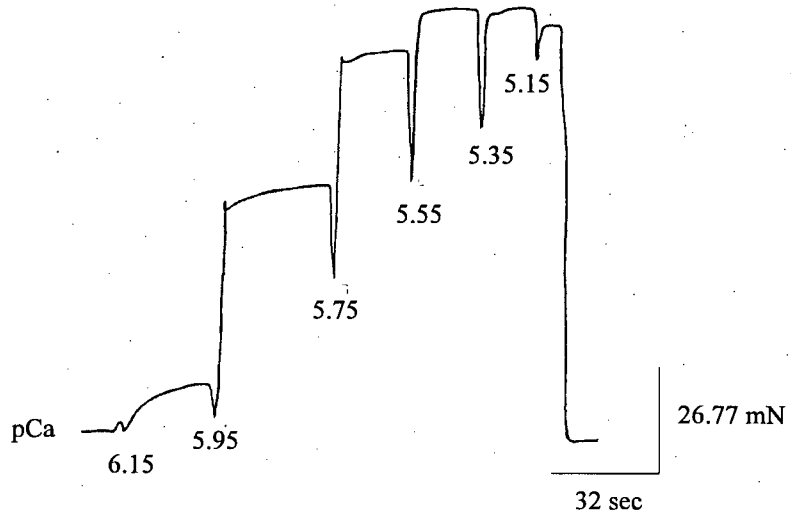
Figure 10: Effect of Increased Sarcomere Length on the Calcium Sensitivity of Skinned Single Muscle Fibre Segments

A comparison of the force-pCa curves for untreated control fibres at three different sarcomere lengths. Parameters of the Hill equation for each trace are given in Table 4. Vertical bars indicate the standard error of each data point. Δ = 3.0 μm, 8 fibres; ■ = 2.5 μm, 11 fibres; ● = 2.0 μm, 6 fibres.

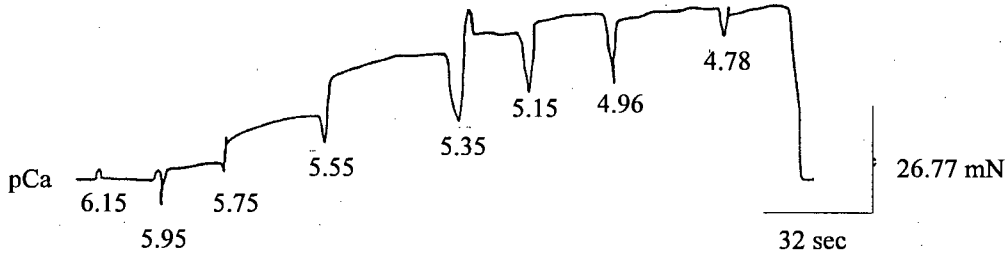
Figure 11a: Effects of TnC Extraction and Reconstitution on the Force Development and Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

Original tension records obtained from a single skinned fibre segment at increasing calcium concentrations before extraction of endogenous TnC (Trace A), after partial extraction of endogenous TnC (Trace B), and after reconstitution with an I130S mutant of TnC (Trace C). There is a marked reduction in both maximal force and calcium sensitivity of the fibre after removal of endogenous TnC (Trace B) which is reversed upon the reintroduction of I130S (Trace C). Note the change in time scale for the third trace.

A. Control Force-pCa Response

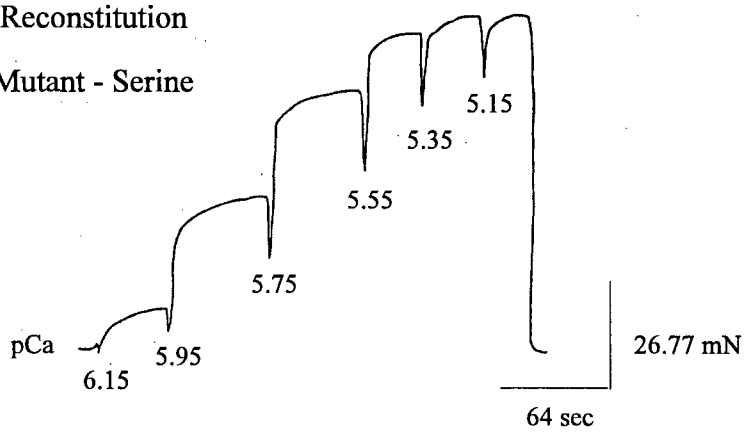


B. Partial Removal of Endogenous TnC



C. After Reconstitution

TnC Mutant - Serine



TnC is successfully reintegrated into the thin filament structure upon fibre reconstitution, retaining both its conformation and subunit interactions.

Plotting the force-pCa relationships for each of the tension traces in Figure 11a, clearly shows that partial extraction of endogenous TnC is also accompanied by a dramatic decrease in the calcium sensitivity and cooperativity of the fibre (Figure 11b). These parameters of contractile performance are restored, however, upon fibre reconstitution (Table 6). Interestingly, changes in cooperativity that accompany partial removal of endogenous TnC are likely to reflect modified interactions between the functional units of the thin filament. This behavior is also reversed upon fibre reconstitution, indicating a high degree of fibre recovery after removal and reconstitution protocols.

To assess the effects of non-selective binding of TnC to thin filament structures during the reconstitution process, unextracted single fibres were treated with I130S pre-soaks of variable time lengths, and subsequently the fibres' force-calcium relationship was analyzed (Figure 12). Suboptimal storage conditions have been shown to cause TnC depletion in skinned fibre segments, thereby diminishing the calcium-sensitive response of the fibre (Moss, 1992). This condition is manifested by an increased calcium sensitivity of the fibre in response to TnC pre-soaks. The results indicate that there was no change in calcium sensitivity of the contractile filaments under these conditions (Table 6), suggesting no interference of filamentous structures in the presence of excess TnC. Furthermore, this result has been used to confirm minimal protein degradation within fibres prior to usage.

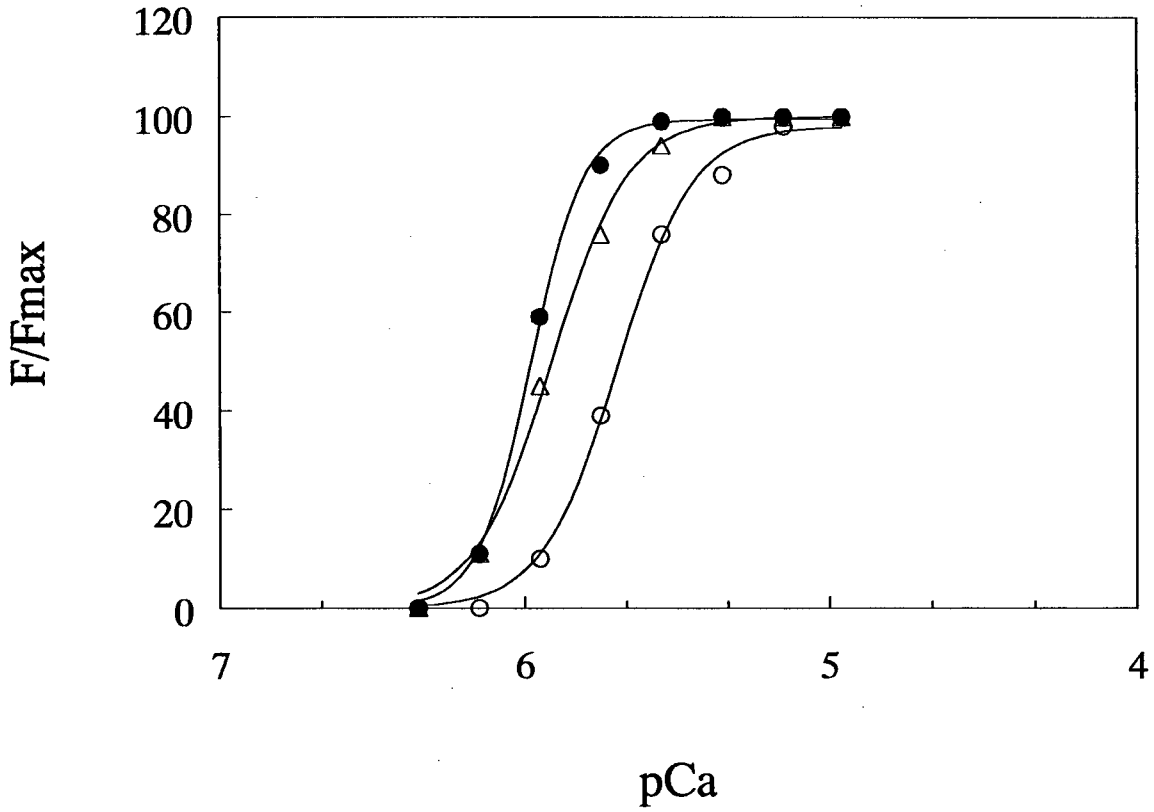


Figure 11b: Effects of TnC Extraction and Reconstitution on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves of the fibre in Figure 9a before extraction of endogenous TnC (●), after partial extraction of endogenous TnC (O) and after reconstitution with I130S mutant of TnC (Δ). Parameters of the Hill equation for each curve are given in Table 4.

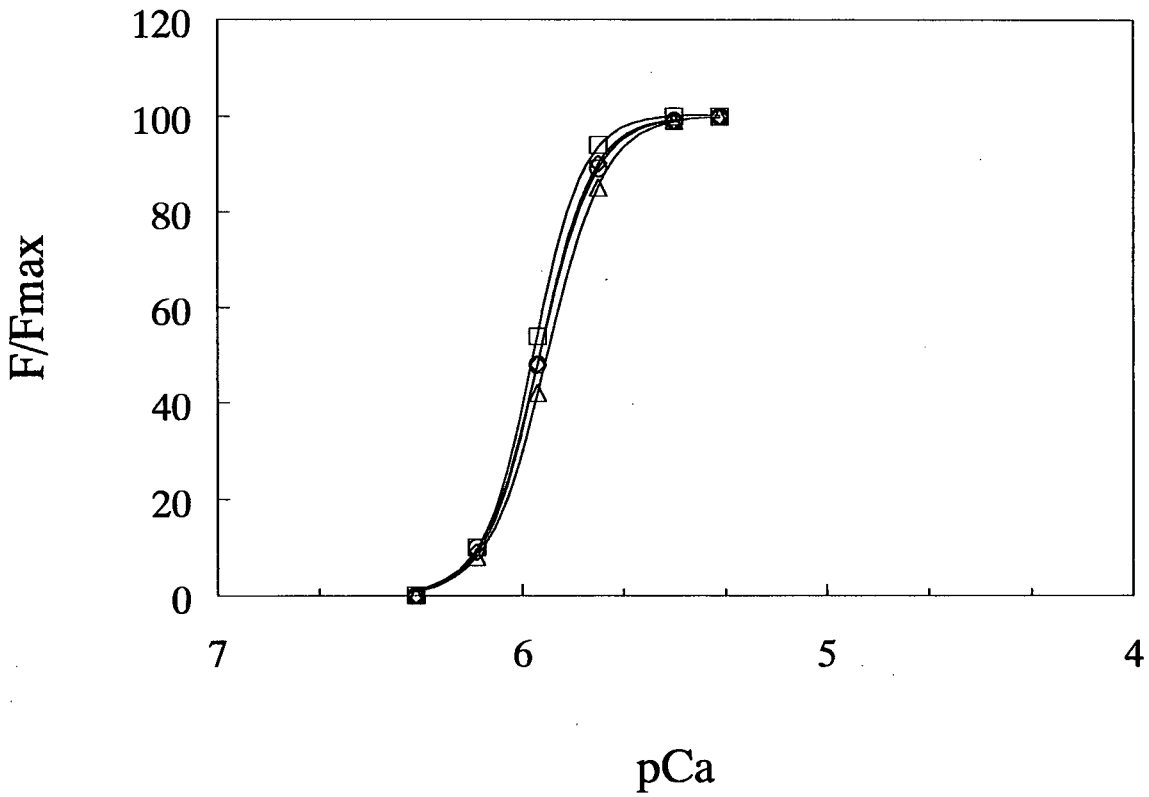


Figure 12: Effects of TnC Pre-soaks on the Calcium Sensitivity of Skinned Single Muscle Fibre Segments

Force-pCa curves acquired from a single fibre at a sarcomere length of 2.4 μm, before fibre treatment (●), after 3x10 second pre-soaks in I130S (□), after 4x10 seconds pre-soaks in I130S (Δ) and after 8x10 second pre-soaks in I130S (○). Parameters of the Hill equation for each trace are given in Table 4.

Effects of Extraction Medium on Contractile Performance

Figure 13 compares the force-pCa curves of untreated fibres with those of fibres having been partially extracted of endogenous TnC in a TFP-free medium. These results indicate a moderate but insignificant ($P \geq 0.05$) shift both in the values of pK and in n for these fibres after reconstitution with native rabbit skeletal muscle TnC (Table 7). Thus, one may conclude that this extraction technique has minimal effects on fibre performance, restoring contractile parameters of treated fibres to near control values. Although this extraction technique does not appear to be harmful to the fibre, its usefulness is limited by its inability to fully extract TnC from fibre segments. Figure 14 compares the mechanical and electrophoretic profiles of a single muscle fibre before and after partial extraction of endogenous TnC. As indicated by the tension transient, this fibre exhibited a significant reduction in force upon partial extraction of TnC. SDS-PAGE analysis of the same segment, however, revealed only a modest reduction in TnC content. Furthermore, intensity profiles of the fibre, before and after partial TnC extraction, quantitatively confirmed that force reduction is not directly proportional to decreases in the fibre's TnC content.

Therefore, in an attempt to more completely remove endogenous TnC from individual fibre segments, a TFP-containing extraction medium was utilized. Figure 15 compares the force-pCa curves of untreated fibres with those having been fully extracted of endogenous TnC in a TFP-containing solution. The resultant traces indicate a statistically insignificant shift both in pK and in n for these fibres after reconstitution in native rabbit skeletal muscle TnC. In fact, the magnitude of this shift is identical to that

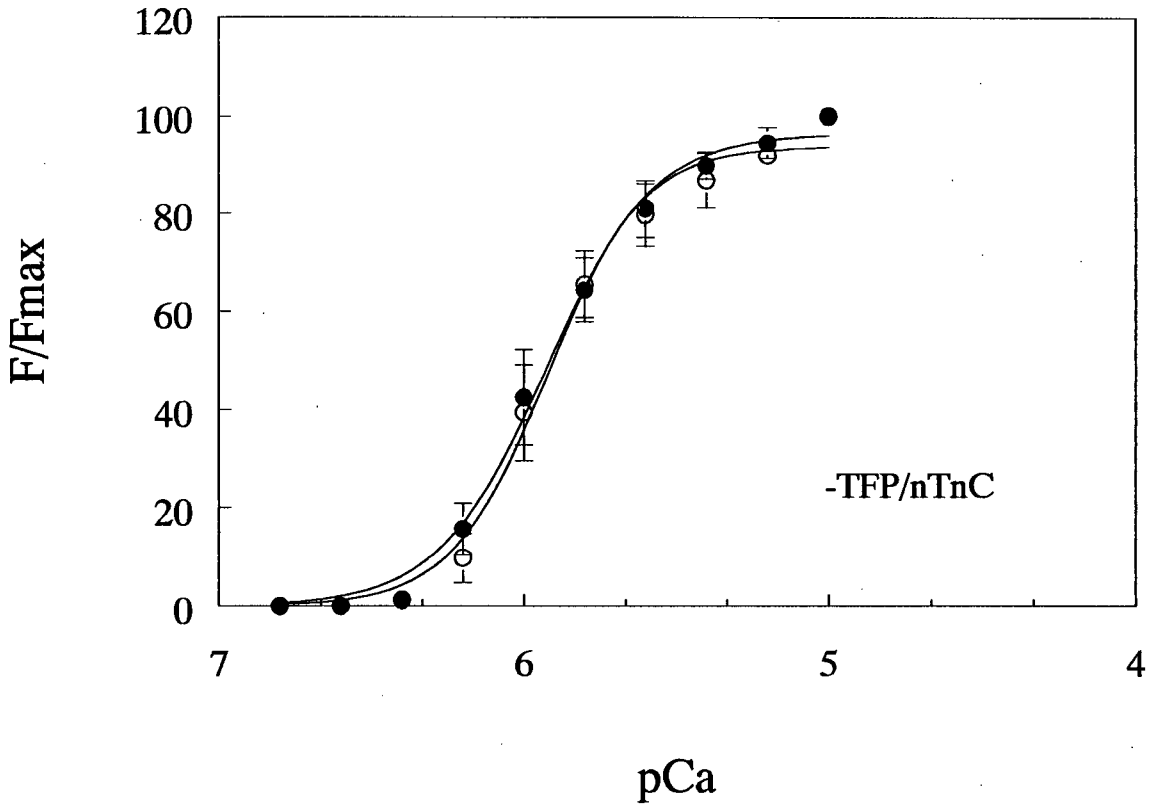
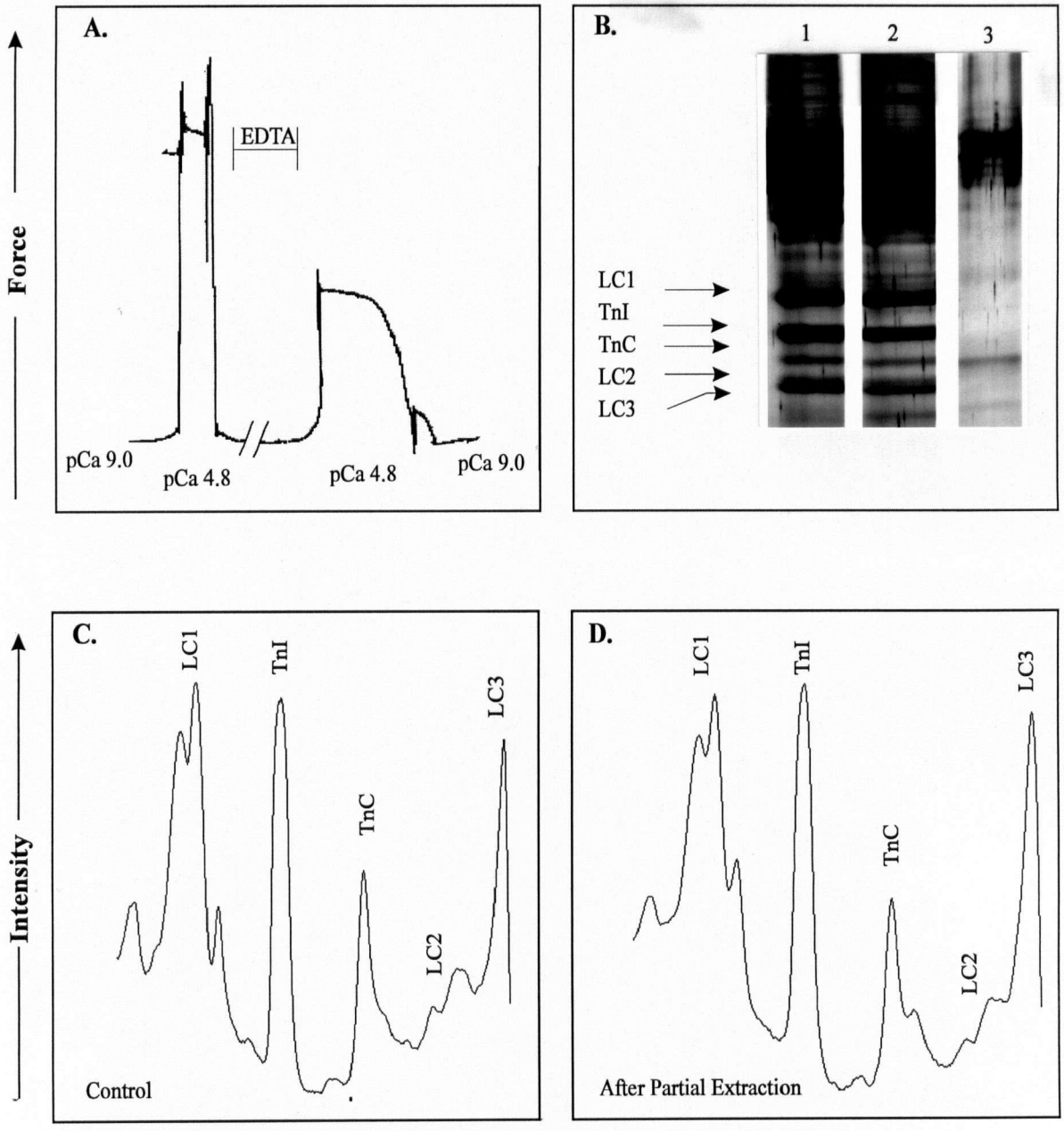


Figure 13: Effect of Non-TFP Containing Extraction Medium on the Calcium Sensitivity of Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves from untreated fibres (●) and fibres having been partially extracted of endogenous TnC in a TFP-free medium and reconstituted with native rabbit skeletal muscle TnC (○). Average TnC removed from these fibres as measured by force was 56.2 ± 3.3%, while reconstitution was 97.33 ± 6.8% of Fmax. Parameters of the Hill equation for each trace are given in Table 5. ● = 2.4 μm, 6 fibres and ○ = 2.4 μm, 6 fibres.

Figure 14: Qualitative and Quantitative Analysis of TnC Content and Force Production in Partially Extracted Single Fibre Segments

A) Original tension records obtained from a single skinned fibre segment showing maximal fibre activation before and after the partial extraction of endogenous TnC. Fmax has decreased by 50% after a 10-minute exposure to an EDTA-containing extraction solution. B) Silver stained polyacrylamide gels of the same fibre segment, before (Lane 1) and after (Lane 2) TnC extraction. Lane 3 contains native rabbit skeletal TnC only. C) Intensity profile of Lane 1 in Panel B; the ratio of TnC:TnI peak area is 0.29. D) Intensity profile of Lane 2 in Panel B; the ratio of TnC:TnI peak area is 0.27.


A.
Force
EDTA
pCa 9.0
pCa 4.8
pCa 4.8
pCa 9.0
B.
1
2
3
LC1
TnI
TnC
LC2
LC3
C.
Intensity
LC1
TnI
TnC
LC2
LC3
Control
D.
LC1
TnI
TnC
LC2
LC3
After Partial Extraction

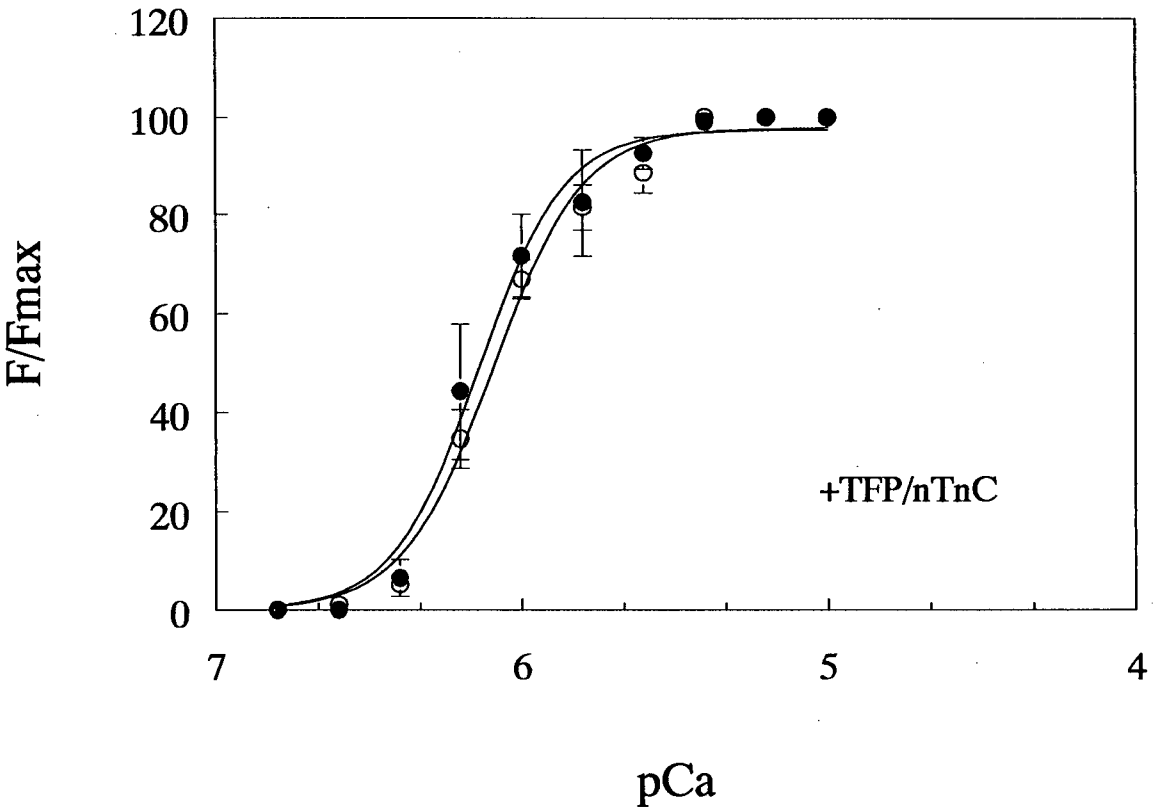


Figure 15: Effect of TFP Extraction Medium on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves in untreated fibres (●) and in fibres having been fully extracted of endogenous TnC in a TFP-containing medium and reconstituted with native rabbit skeletal muscle TnC (O). Average TnC removed from these fibres as measured by force was 87.0 ± 1.8%, while reconstitution was 100.3 ± 2.3% of Fmax. Parameters of the Hill equation for each trace are given in Table 5. ● = 2.4 μm, 6 fibres and O = 2.4 μm, 6 fibres.

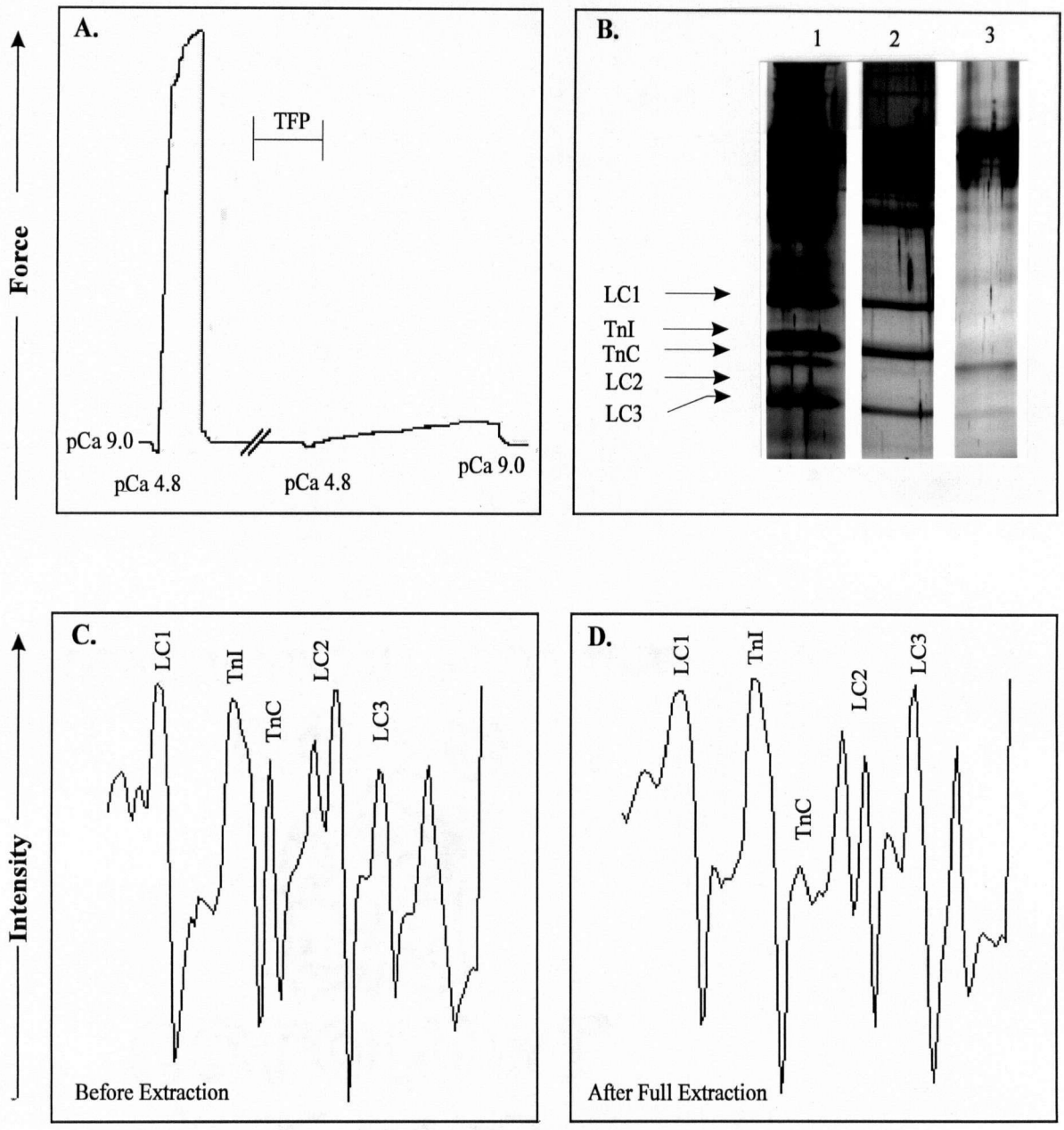
seen with fibres treated in a TFP-free medium (Table 7). The aforementioned mechanical studies suggest that complete extraction of TnC by solutions containing high concentrations of TFP causes neither significant damage nor alteration to the calcium-sensitive components of the contractile machinery. Electrophoretic examination of fibre segments before and after the extraction protocol confirmed a significant decrease in the TnC content of fibres after treatment with TFP (Figure 16). Similarly, intensity profiles of these gels provided quantitative evidence that this extraction technique results in a more complete removal of TnC from fibre segments.

Effects of Troponin C Mutants on Length-Dependent Calcium Sensitivity

Figures 17a, 17b and 17c show data obtained from single fibre segments after partial extraction of endogenous TnC and a subsequent reconstitution with I130S, I130G and I130 mutants of TnC respectively. A comparison of the change in pK values for each set of curves (Table 8) indicates that increasing the sarcomere length of reconstituted fibres altered their observed calcium sensitivity, though the magnitude of this shift was not as great as that seen in untreated fibres (Table 3). The force-pCa relations of these mutant-containing fibres suggests an intact 'length sensor', facilitating an increase in calcium sensitivity at long lengths of the sarcomere. To confirm the behavior of these high-affinity mutants, the same experiment was performed on fibres reconstituted with native rabbit skeletal muscle TnC. This experiment (Figure 17d) showed that native rabbit skeletal muscle TnC behaves identically to the high affinity TnC mutants upon reconstitution, exhibiting a shift both in pK and in n at long sarcomere lengths (Table

Figure 16: Qualitative and Quantitative Analysis of TnC Content and Force Production in Fully Extracted Single Fibre Segments

A) Original tension records obtained from a single skinned fibre segment showing maximal fibre activation, before and after the complete extraction of endogenous TnC. Fmax has decreased by 95% after a 5-minute exposure to a TFP-containing extraction medium. B) Silver-stained polyacrylamide gel of the same fibre segment, before (Lane 1) and after (Lane 2) TnC Extraction. Lane 3 contains native rabbit skeletal muscle TnC only. C) Intensity profile of Lane 1 in Panel B; the ratio of TnC:TnI peak area is 0.28. D) Intensity profile of Lane 2 in Panel B; the ratio of TnC:TnI peak area is 0.04.


A.
Force
TFP
pCa 9.0
pCa 4.8
pCa 4.8
pCa 9.0
B.
1
2
3
LC1
TnI
TnC
LC2
LC3
C.
Intensity
LC1
TnI
TnC
LC2
LC3
Before Extraction
D.
LC1
TnI
TnC
LC2
LC3
After Full Extraction

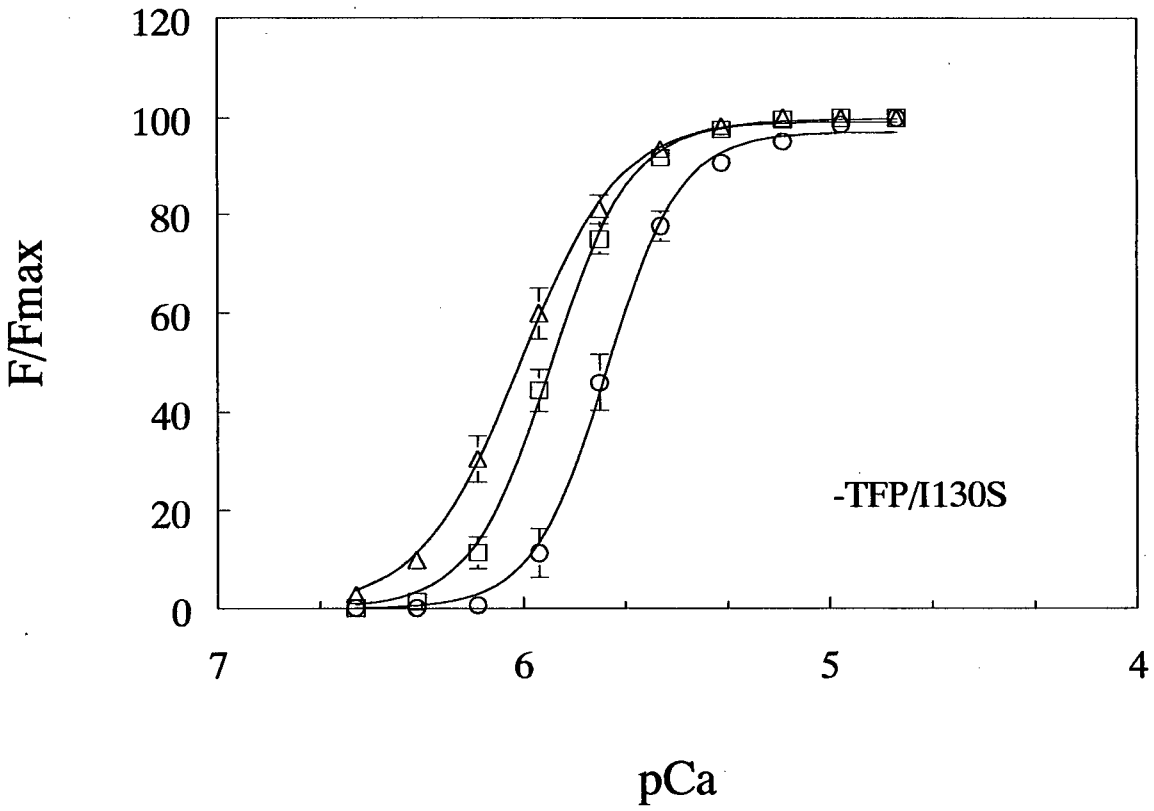


Figure 17a: Combined Effects of Partial Extraction of Endogenous TnC and Reconstitution with I130S on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibres

A comparison of the force-pCa curves for fibres after partial removal of endogenous TnC and reconstitution with a serine analog of TnC at short (O), intermediate (□), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 61.5 ± 1. 6%, while reconstitution was 87. 8 ± 2.5% of Fmax. Parameters of the Hill equation for each trace are given in Table 5. O = 2.0 μm, 8 fibres; □ = 2.5 μm, 16 fibres; Δ = 3.0 μm, 12 fibres.

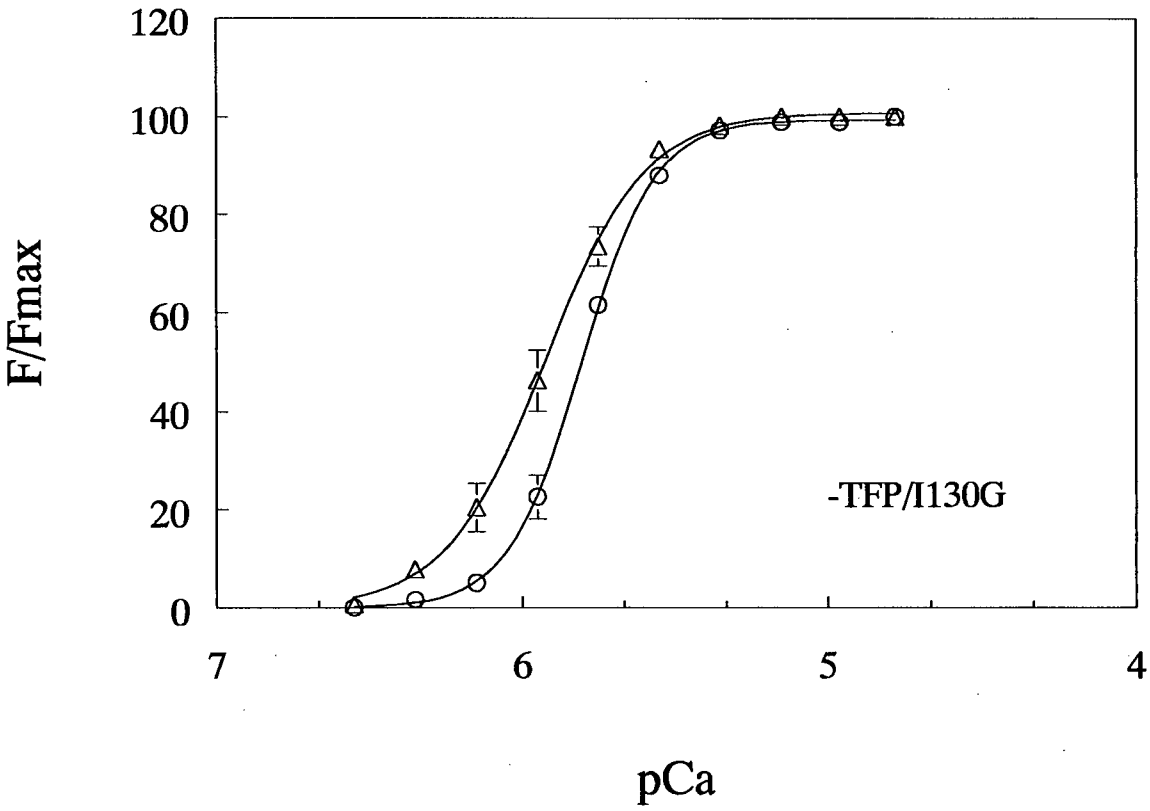


Figure 17b: Combined Effects of Partial Extraction of Endogenous TnC and Reconstitution with I130G on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibres

A comparison of the force-pCa curves for fibres after partial removal of endogenous TnC and reconstitution with a glycine analog of TnC at short (O), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 61.7 ± 4.1%, while reconstitution was 82.7 ± 1.3% of Fmax. Parameters of the Hill equation for each trace are given in Table 6. O = 2.4 μm, 7 fibres; Δ = 3.0 μm, 7 fibres.

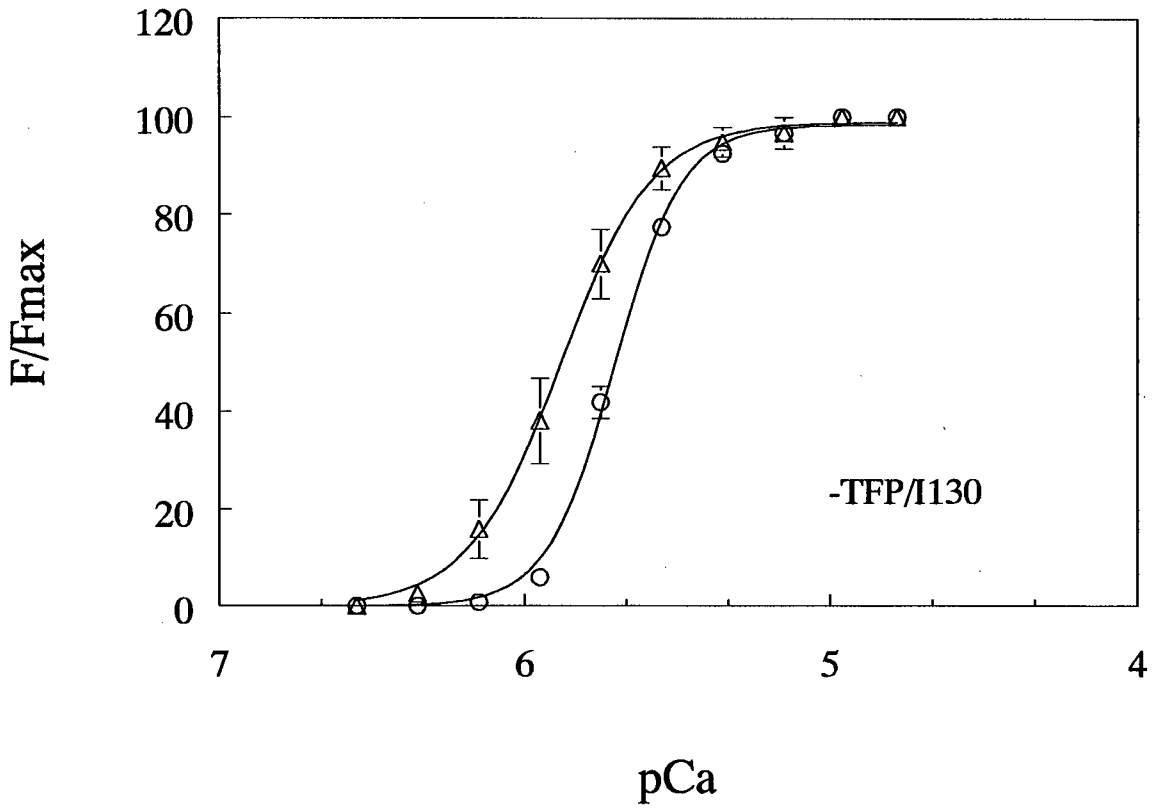


Figure 17c: Combined Effects of Partial Extraction of Endogenous TnC and Reconstitution with I130 on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibres

A comparison of the force-pCa curves for fibres after partial removal of endogenous TnC and reconstitution with a recombinant isoleucine analog of TnC at short (O), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 64.5 ± 4.5%, while reconstitution was 91. 0 ± 2.3% of Fmax. Parameters of the Hill equation for each trace are given in Table 5. O = 2.2 μm, 4 fibres; Δ = 3.0 μm, 4 fibres.

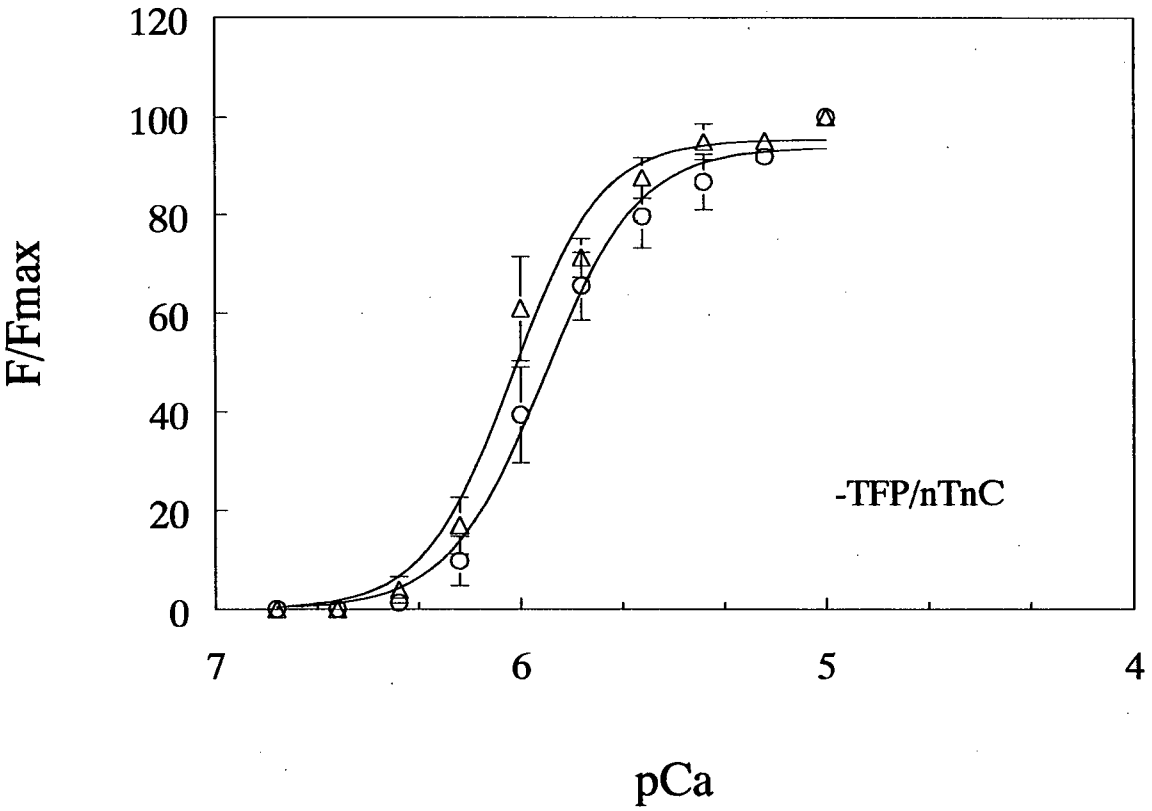


Figure 17d: Combined Effect of Partial Extraction of Endogenous TnC and Reconstitution with nTnC on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibres

A comparison of the force-pCa curves for fibres after partial removal of endogenous TnC and reconstitution with native rabbit skeletal muscle TnC at short (O), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 56.2 ± 3.3%, while reconstitution was 97.3 ± 6.8% of Fmax. Parameters of the Hill equation for each trace are given in Table 6. O = 2.4 μm, 6 fibres; Δ = 3.0 μm, 6 fibres.

8). Figure 18 shows the average reduction and recovery of force for fibres treated in this manner. The partial extraction technique resulted in an average force diminution of approximately 61.0 ± 1.4% (n=35), while recovery was 89.2 ± 1.9% of Fmax (n=35).

To assess the contribution of residual endogenous TnC within these partially extracted fibres, TFP was used to more completely remove TnC from fibre segments. Subsequent gel analysis confirmed that the magnitude of force reduction in partially extracted fibres does not correlate with TnC content. Therefore, to accurately assess the physiological implications of the current high-affinity TnC mutants, it was decided that full extraction of endogenous TnC was necessary. This protocol ensured that measurements of calcium sensitivity undertaken on fibres reconstituted with TnC mutants were not diluted by the presence of residual endogenous TnC.

Figure 19 graphically represents the average reduction in force for fibres treated with a TFP-containing extraction medium. Force decreased by 84.6 ± 1.5% after TFP treatment and recovered to 92.2 ± 2.9% of the control value for Fmax (n=21). The high force return of reconstituted fibres would suggest that TnC is able to successfully reassociate itself into the thin filament structure with an orientation that preserves subunit interactions. Figures 20a and 20b compare the force-pCa relationships for fibres having been fully extracted of endogenous TnC and reconstituted with I130S and I130G mutants of TnC at short and long sarcomere lengths, respectively. The parameters of the Hill equation for these curves (Table 9) indicate a statistically significant shift in the values of both pK and n at longer lengths of the sarcomere. These results confirm a length-dependent increase in calcium sensitivity of the myofilaments that is consistent with fibre

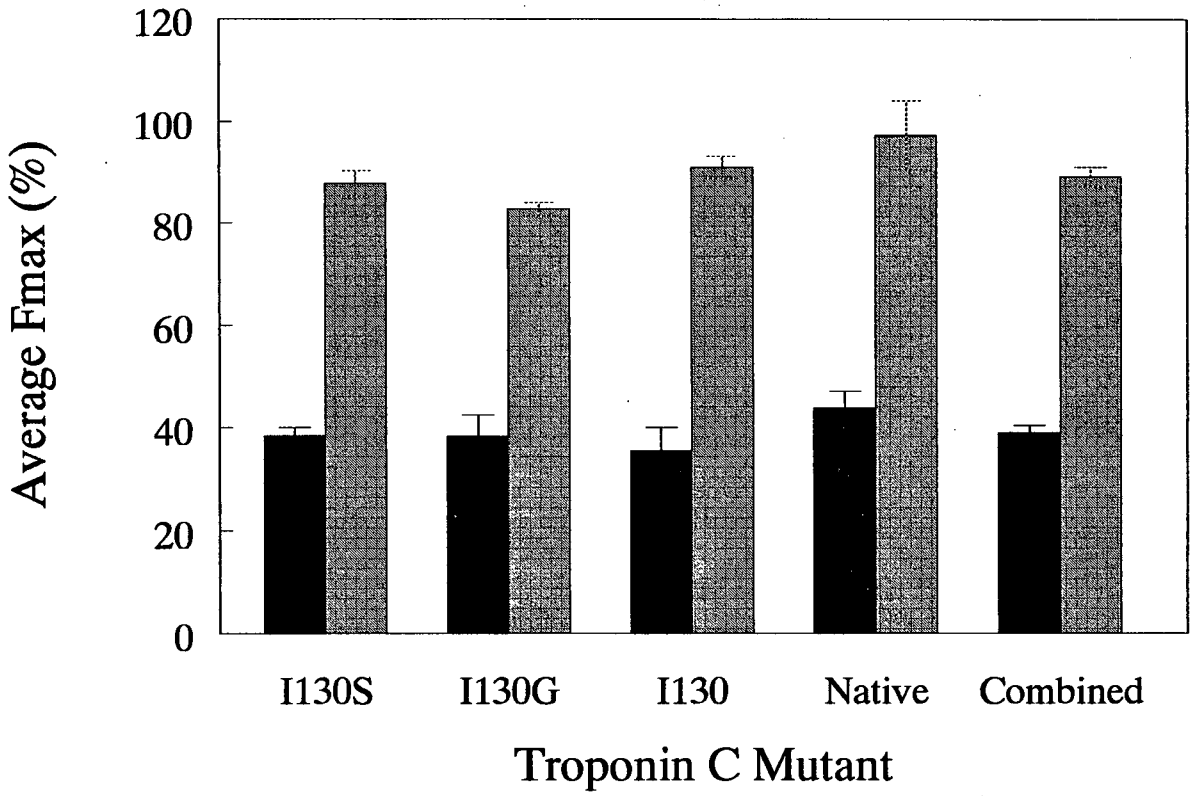


Figure 18: Effects of Partial TnC Extraction and Reconstitution on Maximal Force Development in Single Skinned Skeletal Muscle Fibre Segments

Comparison of the average reduction in maximal force of fibre segments after partial extraction of endogenous TnC (solid bars), and recovery of force upon reconstitution with either TnC mutants or native TnC (hatched bars). I130S = 18 fibres; I130G = 7 fibres; I130 = 4 fibres; Native = 6 fibres; Combined = 35 fibres.

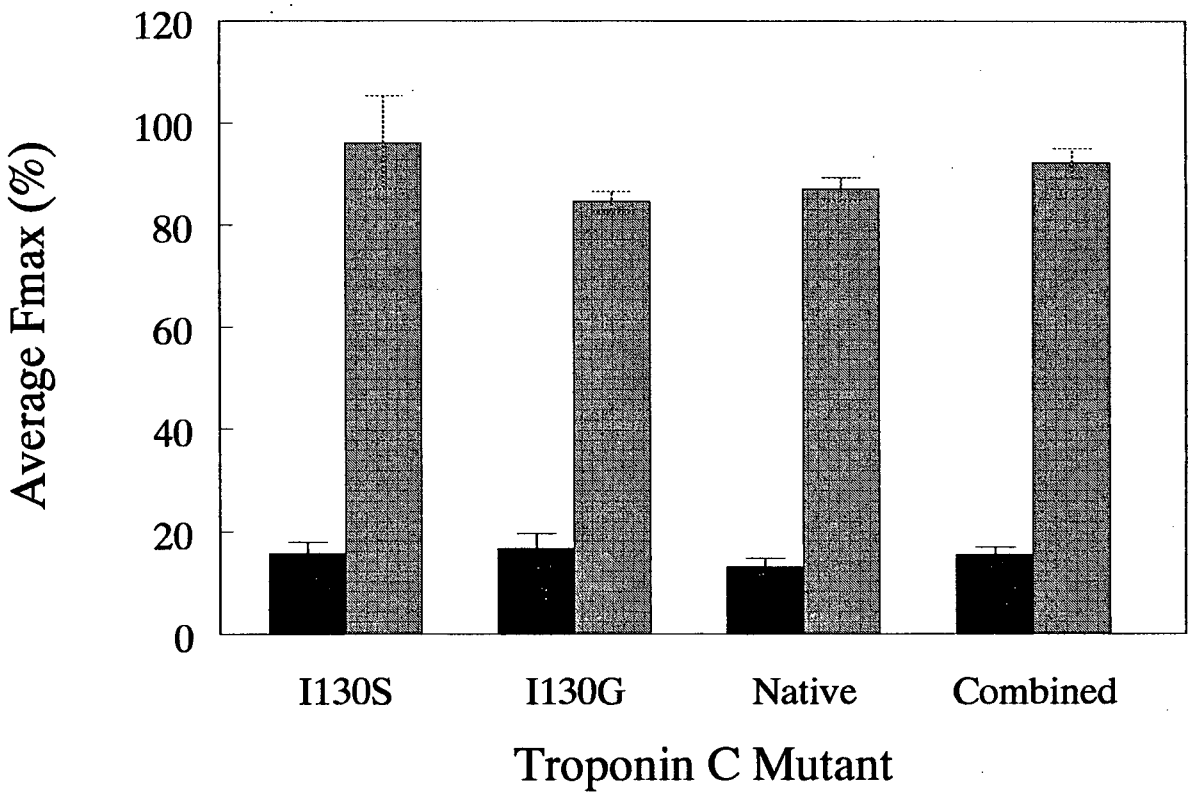


Figure 19: Effects of Full TnC Extraction and Reconstitution on Maximal Force Development in Single Skinned Skeletal Muscle Fibre Segments

Comparison of the average reduction in maximal force of fibre segments after extraction of endogenous TnC in a TFP containing medium (solid bars), and recovery of force upon reconstitution with either TnC mutants or native TnC (hatched bars). I130S = 6 fibres; I130G = 9 fibres; Native = 6 fibres; Combined = 21 fibres.

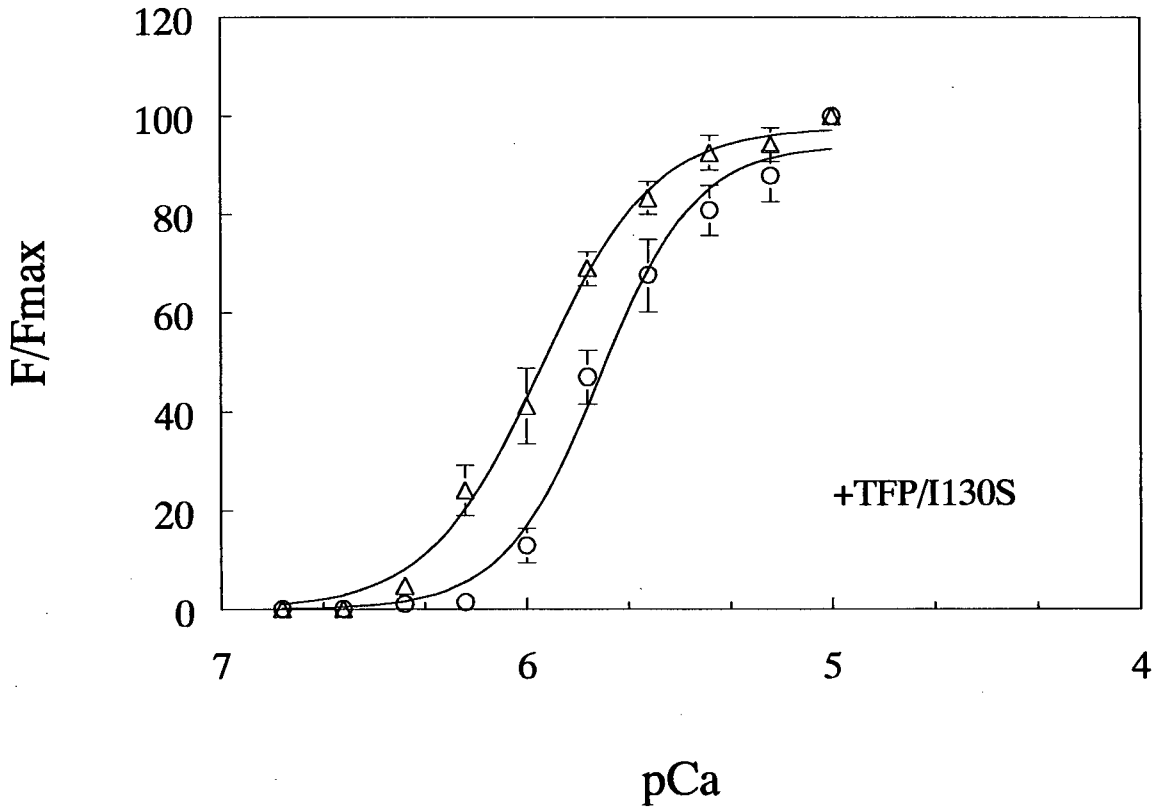


Figure 20a: Combined Effects of TFP and the I130S Mutant on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves for fibres after full extraction of endogenous TnC in a TFP-containing medium and reconstitution with a mutated serine-containing analog of TnC at short (O), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 84.2 ± 2.1%, while reconstitution was 96.2 ± 9.2% of Fmax. Parameters of the Hill equation for each trace are given in Table 7. O = 2.4 μm, 6 fibres; Δ = 3.0 μm, 6 fibres.

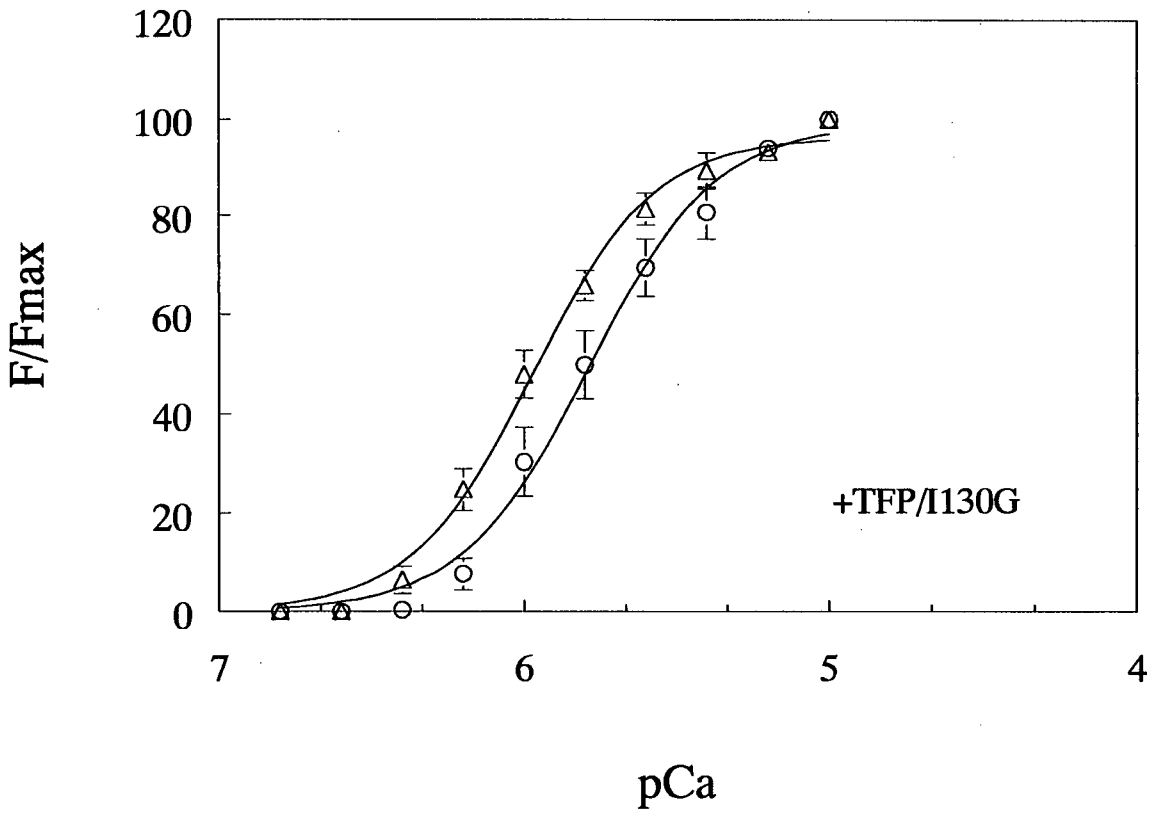


Figure 20b: Combined Effects of TFP and the I130G Mutant on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibres

A comparison of the force-pCa curves for fibres after full extraction of endogenous TnC in a TFP-containing medium and reconstitution with a mutated glycine-containing analog of TnC at short (O), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 83.2 ± 2.9%, while reconstitution was 84.6 ± 2.1% of Fmax. Parameters of the Hill equation for each trace are given in Table 7. O = 2.4 μm, 9 fibres; Δ = 3.0 μm, 9 fibres.

stretch. Performing this experimental series with native rabbit skeletal muscle TnC produced similar results (Figure 20c), indicating a shift in calcium sensitivity of the contractile machinery (Table 9).

SDS-PAGE has shown that TFP treatment of psoas muscle fibres results in a more complete extraction of TnC. It is shown here that mutant reconstitution of these TnC-depleted fibres occurs just as readily, with tension and fibre TnC content returning to near control values. Figure 21 shows the tension trace and gel profiles of a TFP-extracted fibre that has subsequently been reconstituted with a mutant TnC. Plotting the intensity profiles of the TnC bands, clearly shows that the TnC:TnI ratios in both the control and reconstituted stages retain a high degree of similarity. The recovery of both force and TnC:TnI ratios in reconstituted fibres suggests that the mutant TnC proteins are stoichiometrically reassociating themselves with other thin filament structures in a manner that preserves protein interactions.

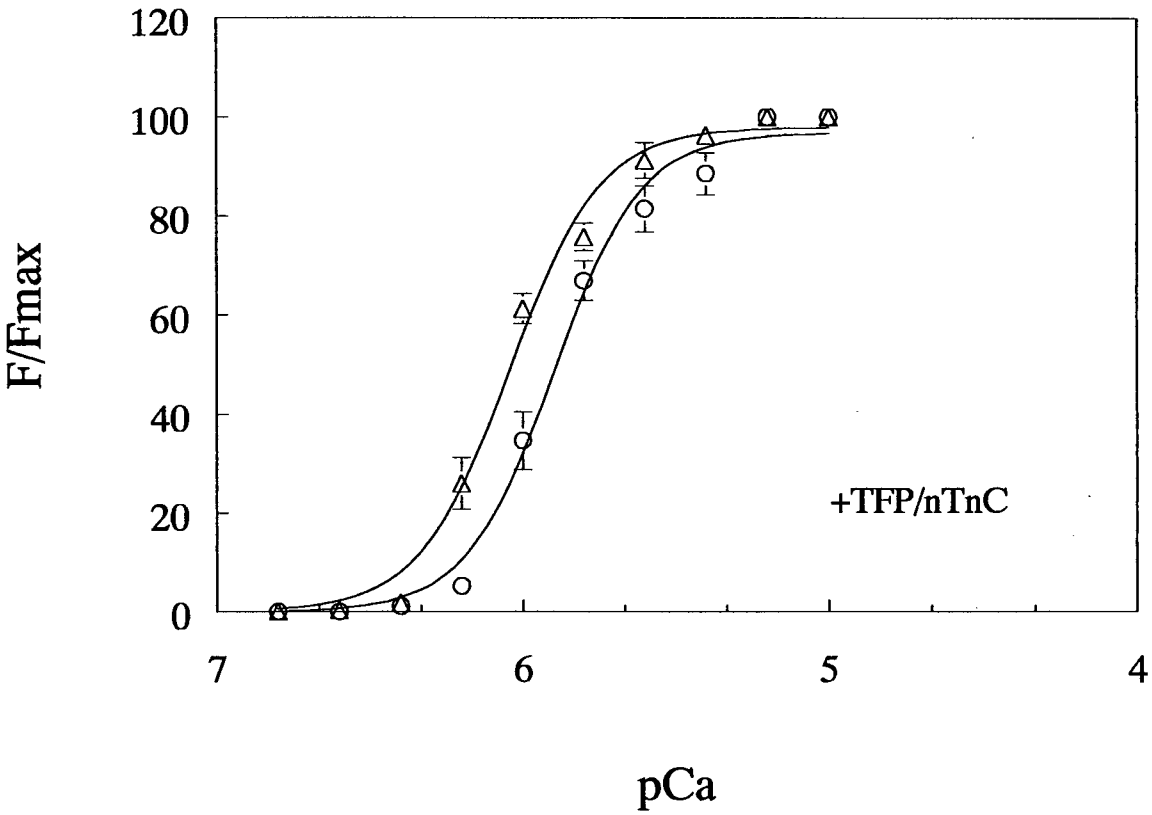


Figure 20c: Combined Effects of TFP and nTnC on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibres

A comparison of the force-pCa curves for fibres after full extraction of endogenous TnC in a TFP-containing medium and reconstitution with native rabbit skeletal muscle TnC at short (O), and long (Δ) sarcomere lengths. Average TnC removed from these fibres as measured by force was 87.0 ± 1.8%, while reconstitution was 100.3 ± 2.3% of Fmax. Parameters of the Hill equation for each trace are given in Table 7. O = 2.4 μm, 6 fibres; Δ = 3.0 μm, 6 fibres.

Figure 21: Qualitative and Quantitative Analysis of TnC Content and Force Production in Reconstituted Single Fibre Segments

A) Original tension records obtained from a single skinned fibre segment showing maximal fibre activation before extraction of endogenous TnC, and after reconstitution of the TnC-depleted fibre by I130G. Force recovers to 92% of Fmax after TFP extraction and I130G reconstitution.. B) Silver stained polyacrylamide gels of the same fibre segment, before extraction of TnC (Lane 1) and after reconstitution of fibre by an I130G mutant of TnC (Lane 2). Lane 3 contains native rabbit skeletal muscle TnC only. C) Intensity profile of Lane 1 in Panel B; the ratio of TnC:TnI peak area is 0.28. D) Intensity profile of Lane 2 in Panel B; the ratio of TnC:TnI peak area is 0.29.

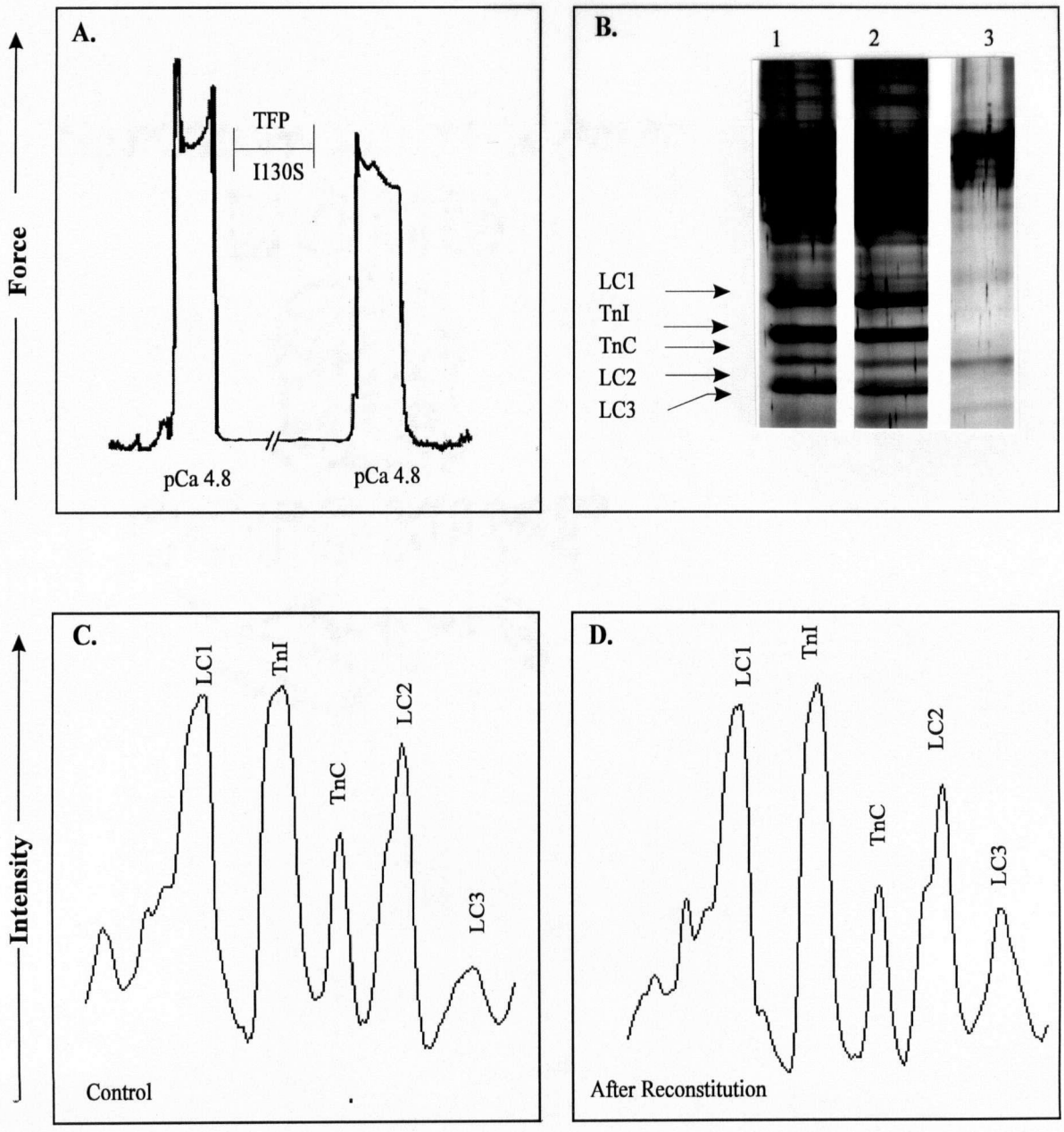

A.
Force
TFP
I130S
pCa 4.8
pCa 4.8
B.
1
2
3
LC1
TnI
TnC
LC2
LC3
C.
Intensity
LC1
TnI
TnC
LC2
LC3
Control
D.
LC1
TnI
TnC
LC2
LC3
After Reconstitution

Table 6: Parameters of the Hill Equation from Force-pCa Curves in Rabbit Psoas Muscle Fibres

Sarcomere Length (μm)	Fibre Treatment	Number of Fibres	pK		ΔpK	n	
2.0	none	8	5.80±0.26	(5.86)ǂ		4.30±0.43	(3.77)ǂ
2.5		11	5.96±0.14*	(5.97)	0.16	4.45±0.23	(4.21)
2.5	none	11	5.96±0.14	(5.97)		4.45±0.23	(4.21)
3.0		6	6.14±0.25*	(6.14)	0.18	3.37±0.31	(3.14)
2.4		1	6.00			5.00	
2.4 ex	-TFP/I130S	1	5.70*		0.30	3.51	
2.4 rr		1	5.96*		0.26	3.45	
2.4		3	5.96			5.29	
2.4 rr	3x10 sec I130S	3	5.94		0.02	4.94	
2.4 rr	4x10 sec I130S	3	5.91		0.03	4.53	
2.4 rr	8x10 sec I130S	3	5.94		0.03	4.69	

* Significant differance of pK upon fibre treatment using Student's t-test ($p \leq 0.05$)
ǂ Values in brackets are from a fit of average curves to the Hill Equation
rr Indicates reconstitutued fibres
ex Indicates TnC extracted fibres

Table 7: The Effects of Extraction Medium on the Parameters of the Hill Equation From Force-pCa Curves in Rabbit Psoas Fibres Reconstituted with Native Rabbit Skeletal Muscle TnC

Sarcomere Length (μm)	Fibre Treatment	Number of Fibres	pK		ΔpK	n	
2.4	-TFP	6	5.93±0.06	(5.93)ǂ		3.58±0.92	(2.46)ǂ
2.4 rr		6	5.89±0.08	(5.92)	0.04	3.77±0.84	(2.77)
2.4	+TFP	6	5.93±0.05	(5.95)		3.59±0.51	(3.20)
2.4 rr		6	5.89±0.04	(5.90)	0.04	3.10±0.24	(3.01)

* Significant differance of pK upon fibre treatment using Student's t-test ($p \leq 0.05$)

ǂ Values in brackets are from a fit of average curves to the Hill equation

rr Indicates reconstitutued fibres

Table 8: The Effects of Sarcomere Length on the Parameters of the Hill Equation From Force-pCa Curves in Partially-Extracted and TnC-Reconstituted Rabbit Psoas Muscle Fibres

Sarcomere Length (μm)	Fibre Treatment	Number of Fibres	pK		ΔpK	n	
2.0 rr	-TFP/I130S	8	5.73±0.03	(5.72)ǂ		3.94±0.30	(3.54)ǂ
2.5 rr		16	5.88±0.02*	(5.91)	0.15	3.88±0.24	(3.29)
2.5 rr	-TFP/I130S	16	5.88±0.02	(5.91)		3.88±0.24	(3.29)
3.0 rr		12	6.00±0.02*	(6.00)	0.12	3.13±0.34	(2.62)
2.2 rr	-TFP/I130	4	5.71±0.01	(5.71)		4.11±0.27	(3.38)
3.0 rr		4	5.88±0.06*	(5.88)	0.17	3.05±0.16	(3.29)
2.4 rr	-TFP/I130G	7	5.81±0.02	(5.85)		3.79±0.26	(4.28)
3.0 rr		7	5.95±0.05*	(5.92)	0.14	2.95±0.16	(2.65)
2.4 rr	-TFP/nTnC	6	5.89±0.08	(5.92)		3.58±0.92	(2.77)
3.0 rr		6	6.06+0.08*	(6.02)	0.17	4.85±1.83	(3.00)

* Significant differance of pK at different sarcomere lengths using Student's t-test ($p \leq 0.05$)
ǂ Values in brackets are from a fit of average curves to the Hill equation
rr Indicates reconstitutued fibres

Table 9: The Effects of Sarcomere Length on the Parameters of the Hill Equation From Force-pCa Curves in Fully-Extracted and TnC-Reconstituted Rabbit Psoas Muscle Fibres

Sarcomere Length (μm)	Fibre Treatment	Number of Fibres	pK		ΔpK	n	
2.4 rr	+TFP/I130S	6	5.76±0.04	(5.76)ǂ		3.08±0.62	(2.74)ǂ
3.0 rr		6	5.98±0.03*	(5.95)	0.22	2.34±0.16	(2.33)
2.4 rr	+TFP/I130G	9	5.77±0.06	(5.78)		2.45±0.20	(2.07)
3.0 rr		9	5.95±0.04*	(5.97)	0.18	2.16±0.13	(2.16)
2.4 rr	+TFP/nTnC	6	5.89±0.04	(5.90)		3.10±0.24	(3.01)
3.0 rr		6	6.04+0.03*	(6.04)	0.15	2.98±0.21	(2.92)

* Significant differance of pK at different sarcomere lengths using Student's t-test ($p \leq 0.05$)

ǂ Values in brackets are from a fit of average curves to the Hill equation

rr Indicates reconstitutued fibres

DISCUSSION

The data presented in this thesis confirms that altering sarcomere length has an effect on the calcium sensitivity of force production in skeletal muscle fibres. These observations show that the development of maximal force in single skinned rabbit psoas fibre-segments follows closely from the predictions of the tension-length curve (Gordon *et al.*, 1966). Tension generation appeared maximal between sarcomere lengths of 2.4 μm and 2.5 μm, but decreased significantly at both shorter (2.0 μm) and longer (3.0 μm) sarcomere lengths. This is likely due to repositioning of sarcomeres from the plateau of the tension-length curve to positions on the ascending and descending limbs, respectively. Thus, the degree of myofilament overlap in striated muscle fibres seems to play an important role in regulating force development.

As well, this study has confirmed that TnC plays an important role in regulating the absolute calcium sensitivity of skeletal muscle fibres. In conjunction with similar findings for cardiac muscle (Moss *et al.*, 1985; Babu *et al.*, 1986), it has become increasingly evident that the calcium-sensitive response of the myofilaments is mediated primarily by TnC's actions. Removal of endogenous TnC caused a dramatic shift in both the force development and the calcium sensitivity of treated fibres. This behavior is likely to reflect the inability of the thin filament to become 'turned-on' in the presence of vacant TnC sites resulting in limited tension development.

This study has also substantiated an increase in the calcium sensitivity of force in fibres at long sarcomere lengths and a decrease in sensitivity of these fibres at shorter

sarcomere lengths. The mechanisms that facilitate and regulate this observed length-dependent increase in calcium sensitivity of the myofilaments are addressed below.

Regulation of Calcium Sensitivity

It is well known that the calcium sensitivity of a muscle fibre is determined both by the affinity with which calcium binds to TnC and by the magnitude of force a fibre generates at a given calcium concentration. Investigators have examined each of these mechanisms and have determined that calcium sensitivity may be altered by modulating either of these parameters (Fuchs, 1995).

Increasing TnC's affinity for calcium allows muscle fibres to elicit a contractile response at much lower sarcoplasmic calcium concentrations. Regulation of the protein's calcium-affinity has been shown to occur either by direct substitution of the amino acid residues within the protein's ligand-binding sites (Putkey *et al.*, 1989) or by destabilization of the helices associated with each of the binding-loops (Shaw *et al.*, 1991; Trigo-Gonzalez *et al.*, 1993). The results presented in this study suggest that destabilization of the high-affinity binding sites of TnC by site-directed mutagenesis does not result in a concomitant decrease in the calcium sensitivity of the myofilaments. Whereas the current mutant proteins are known to display significantly depressed binding-affinities for calcium in solution (Trigo-Gonzales *et al.*, 1993), the effects of such behavior were not detectable in the present study within reconstituted fibres. The results of the current study would indicate that changing the calcium-affinity of sites III and IV in chicken skeletal muscle TnC does not have a measurable effect on the calcium sensitivity of reconstituted fibres.

One may speculate that the calcium-binding affinity of the mutant proteins was not depressed to the extent that it would cause thin filament destabilization in reconstituted fibres. Alternatively, a compensatory mechanism may exist within striated muscle tissue to overcome the deleterious effects associated with introduction of such mutants into the muscle fibre. Interestingly, an analogous mutation in the N-terminal domain of the TnC protein has been putatively shown in solution to cause a large decrease in the cooperative-binding of calcium at sites I and II (LeBlanc and Borgford, 1995). Further studies similar to the present one are suggested for this novel class of mutant proteins to facilitate further correlation of TnC behavior in solution with the effects of TnC in reconstituted fibres.

Whereas, changing the binding affinity of the current mutant TnC proteins did not appear to cause a corresponding change in muscle fibre performance, it has been shown by other investigators that TnC's affinity for calcium primarily determines the calcium-sensitive response of the myofilaments (Fuchs, 1995). Moreover, cardiac muscle TnC displays a higher binding-affinity for calcium than does skeletal muscle TnC (Putkey *et al.*, 1989). This difference between the two protein isoforms may explain the increased calcium sensitivity of the contractile apparatus in cardiac muscle as compared to that in skeletal muscle. Mutating cTnC proteins to possess an active calcium-binding site I, has been shown to result in a marked difference in the calcium sensitivity of reconstituted cardiac myocytes (Gulati *et al.*, 1990). As such, the force-pCa relationships of single cardiac muscle fibres reconstituted with this class of TnC mutant is depressed at all sarcomere lengths (Gulati *et al.*, 1990). This suggests that the differential ion-affinities of

the two TnC isoforms may account for the previously observed differences in contractile parameters between skeletal and cardiac muscle.

Most important for this study, TnC affinity has also been shown to possess a strong dependence on muscle fibre length (Kentish *et al.*, 1988). Rapidly changing fibre length during activation has been shown to generate local calcium fluxes in the region of the myofilaments (Allen and Kentish, 1988; Gordon and Ridgeway, 1993). This observation has been interpreted to reflect transient changes in the calcium-affinity of TnC at different lengths of the sarcomere. These changes in the protein's cation-binding capacity may, then, represent a component of the mechanism by which length-dependent calcium sensitivity of striated muscle fibres is regulated. However, a number of other mechanisms may also be directly involved.

In cardiac muscle, for example, bound myosin cross-bridges play a fundamental role in the modulation of calcium sensitivity by altering the observed calcium-binding affinity of TnC (Hibberd and Jewel, 1982; Hofman and Fuchs, 1987; Fuchs and Wang, 1991). Investigators have had a particular interest in determining whether this effect on calcium sensitivity is either a direct result of muscle fibre length or, rather, a function of the total force produced by the muscle fibre (Bremel and Weber, 1972; Allen and Kentish, 1988). Bremel and Weber (1972) showed that the development of rigor myosin cross-bridges in the presence of low MgATP caused a calcium-independent activation of thin filaments which resulted in ATP hydrolysis and an increased calcium affinity of troponin complexes. Initial myosin cross-bridge binding promoted the development of more tension-generating interactions and cooperatively enhanced the level of thin-filament

activation. In another study, Allen and Kentish (1988) measured calcium flux from the region of the myofilaments under conditions were length and force were regulated independently. Their findings also support a model for calcium sensitivity which is highly dependent on force production rather than on muscle fibre length. Taken together, these results suggest that both the binding-affinity of TnC and the sensitivity of the muscle fibre to calcium are dynamic properties of the muscle fibre which change constantly to reflect its force-producing state.

This has been confirmed by analysis of cTnC's calcium-affinity at different orientations of the myosin head (Guth and Potter, 1987; Saeki *et al.*, 1993). Different conformations of S1 are thought to reflect different force-producing states of the muscle fibre. As such, TnC affinity changes markedly as myosin cross-bridges move from conditions of relaxation, to those of actively cycling and to those of rigor. Furthermore, these changes in TnC affinity that accompany tension development appear to be caused by altered conformational states of the protein (Hannon *et al.*, 1992). Fluorescence studies have clearly shown a more compact conformation of TnC during rigor activation with a measured decrease in circular dichroism both during the myosin cross-bridge cycle and during periods of relaxation (Hannon *et al.*, 1992).

From these results, further experiments are warranted on the high-affinity TnC mutants which are designed to measure both instantaneous stiffness and maximum velocity of unloaded shortening (Vmax) in reconstituted muscle fibres. Clearly, bound myosin cross-bridges have an important regulatory role in determining TnC conformation and affinity. Bremel and Weber (1972) have shown that the cooperativity of thin filament

activation is highly dependent on the extent and magnitude of tension-generating interactions between the myofilaments. Thus, examination of cross-bridge dynamics in reconstituted muscle fibres may elucidate the role of the high-affinity binding sites of TnC in calcium-sensitive contractile processes. Both pH and low ionic-strength conditions have been shown to cause conformational changes in the TnC protein which correspond to an increase in helicity (Strang and Potter, 1992). This change in the protein's secondary structure has been reported to be similar to the conformational changes that TnC undergoes during muscle fibre activation. Therefore, utilization of the current TnC mutants is suggested under low ionic-strength conditions to examine the changes in TnC conformation that accompany force production. Conditions of low ionic strength and pH have been shown to increase the calcium-sensitive response of the myofilaments (Martyn and Gordon, 1988). Therefore, utilization of TnC mutants under these experimental conditions should provide direct evidence for a relationship between TnC affinity and the calcium sensitivity of the fibre.

Another important mechanism by which a fibre can regulate its calcium sensitivity is to increase the magnitude of force it develops at a given sarcoplasmic calcium concentration (Fuchs, 1995). The present study has shown that the partial extraction of endogenous TnC is accompanied by a significant reduction in the calcium sensitivity of the contractile myofilaments. This suggests that the thin filament is unable to become activated in the presence of vacant TnC sites, even under conditions of high intracellular calcium. The net effect is a significant decrease in the fibre's force-generating capacity and, subsequently, in the measured calcium sensitivity. Other investigators, however, have

examined mechanisms to increase force development and to promote a leftward shift in the force-pCa relationship for treated muscle fibres to reflect an increased calcium sensitivity of the myofilaments (Allen and Moss, 1987; Martyn and Gordon, 1988; Metzger and Moss, 1988). This has been achieved either through compression of the myofilament lattice or by lowering the ionic strength of the activation medium. In both cases, myosin cross-bridge formation was enhanced, resulting in a higher maximal force production per calcium cation and in a resultant increase in the muscle fibre's calcium sensitivity.

Interestingly, the changes in cTnC affinity that accompany force production in cardiac muscle cells have not been shown to occur in skeletal muscle fibres (Wang and Fuchs, 1994). Therefore, cardiac myocytes may display a higher degree of thin filament cooperativity by virtue of the modified structure of their troponin complex. Although both TnC isoforms exhibit a large degree of homology (Van Eyck *et al.*, 1991), the region of their binding interface with the other members of the troponin complex has been shown to possess considerable divergence between the two kinds of striated muscle fibres (Van Eyck *et al.*, 1991). It would appear that coordination of troponin subunits plays an important role in regulating force development, calcium sensitivity and thin filament cooperativity. For example, the efficiency by which TnI disinhibits the F-actin polymer, under conditions of muscle fibre activation, directly impinges upon maximal force generation and calcium sensitivity (Zot and Potter, 1987). Thus, the inappropriate coordination of TnC and TnI appears to result both in altered protein interactions and in a less effective activation of the thin filament. This has been demonstrated in experiments where endogenous cTnC was exchanged for sTnC resulting in a severe depression of both

force and calcium sensitivity in reconstituted muscle fibres (Gulati *et al.*, 1990). Alternatively, mutation of the naturally occurring TnC isoform to mimic sTnC did not appear to have as great an effect on these parameters as replacement of this protein for its skeletal muscle isoform (Gulati *et al.*, 1990; Putkey *et al.*, 1991). This was most likely due to undisturbed interactions with the remainder of the native troponin complex.

The high-affinity TnC mutants utilized in this study did not appear to significantly affect TnC-TnI interactions, despite their attenuated conformations. Investigators have hypothesized that the integrity of TnC-TnI interactions in this region of the protein are paramount to successful transduction of the calcium signal from TnC to the contractile myofilaments (Van Eyck *et al.*, 1991; Ngai *et al.*, 1994). However, it is becoming increasingly apparent that the conformational change which occurs in the TnC protein upon ligand binding to the low-affinity sites may be associated with the formation of a joint binding site between the two domains of TnC for the inhibitory peptide of TnI (Swenson and Fredricksen, 1992). Thus, release of TnI's inhibition of actin may reflect a cooperative interaction between the high-affinity and low-affinity domains of TnC which is only possible when TnC becomes activated. In light of this hypothesis, one may interpret the results of the current study to suggest that attenuation of the high-affinity domain of TnC does not significantly modify the nature of the TnIp binding site generated by the interaction of the protein's carboxy- and amino-terminal domains. As such, the effectiveness of thin filament activation is not diminished, resulting both in sustained force production and in calcium sensitivity of reconstituted fibres.

The tension- and length-dependent increase both in calcium-affinity and in sensitivity of striated muscle fibres has been examined by others in determining the effects of lattice spacing on contractile performance. Decreasing the lattice spacing to mimic long sarcomere lengths has been shown by some investigators to result both in increased force development and increased calcium sensitivity (Allen and Moss, 1987; Martyn and Gordon, 1988; Metzger and Moss, 1988). The mechanism of this response is thought to reflect a heightened probability of force-generating attachments between thick and thin myofilaments which accompany reduced interfilament distances (Martyn and Gordon, 1988). As a result, the same degree of thin filament activation in compressed muscle fibre segments results in more bound cross-bridges and more force development.

On the other hand, this mechanism does not explain the increased calcium sensitivity of the myofilaments which has been reported to occur in response to stretch (Bressler *et al.*, 1994). At long sarcomere lengths, myofilament overlap was minimized causing a net decrease in force production. However, at these long sarcomere lengths an increase in the calcium sensitive response of the myofilaments was still observed. Thus, it is hypothesized here, that while tension generating cross-bridges are reduced during periods of fibre stretch, the proportion of these bridges that are available for force production (i.e. bound to the thin myofilament) are greater than at resting sarcomere lengths due to a decrease in the interfilament lattice spacing. Indeed, the proportion of myosin cross-bridges bound to the thin filament regulates the calcium affinity of TnC and the calcium sensitivity of the muscle fibre (Bremel and Weber, 1972). Thus, sarcomere length governs the total number of myosin cross-bridges that may form, while the Ca^{2+}-

affinity of troponin is determined by the formation of tension-generating interactions (Kentish *et al.*, 1988).

To confirm this assumption that myosin cross-bridge binding is enhanced under conditions of decreased lattice spacing, the following experimental protocol is suggested to elucidate myosin cross-bridge attachment at different sarcomere lengths. The rigor stiffness of single muscle fibres is an accurate measure of the total number of cross-bridges available for force production, whereas active stiffness measures the proportion of those cross-bridges that are actually involved in force-generating events (Huxley and Simmons, 1971; Bressler and Clinch, 1974). Thus, determining the ratio of active stiffness to passive stiffness at different lengths of the sarcomere should reflect the number of cross-bridges bound to the thin filament at any given time. It is predicted that the results of the suggested study would confirm a ratio of active to rigor stiffness at long lengths of the sarcomere that approximates 1, while this ratio would decrease proportionally with reduced sarcomere lengths.

Accordingly, a hypothesis is presented here for calcium sensitivity in striated muscle which is firmly dependent on force-producing interactions between thick and thin myofilaments. It is suggested that tension development is transduced through the myosin cross-bridge to thin filament structures, resulting in an increased calcium-affinity of TnC and, subsequently, in an increased calcium sensitivity of the muscle fibre. As muscle fibres are stretched to long sarcomere lengths, the myofilament-lattice spacing may decrease in a manner that promotes the development of force-generating interactions between thick and thin myofilaments. The formation of these tension-generating cross-bridges would then

potentiate the already heightened affinity of TnC for calcium at long lengths of the sarcomere. Moreover, with increased force development there is a proportional increase in TnC affinity for calcium, resulting both in heightened thin filament activation and in elevated force-generation per calcium cation. Thus, this system may be envisioned to possess a high degree of positive feedback and cooperativity between myofilaments.

It would appear that the length-dependent calcium sensitivity of striated muscle fibres is highly regulated by a number of different mechanisms. The calcium binding-affinity of TnC (Allen and Kentish, 1988), the myofilament lattice spacing (Hofmann and Fuchs, 1988) and the formation of force producing cross-bridges (Fuchs and Wang, 1991; Wang and Fuchs, 1994) are all parameters of the muscle fibre that change significantly at different lengths of the sarcomere. Indeed, the regulation of calcium sensitivity in striated muscle fibres is not exclusively dependent upon any one of these parameters, but rather, on an interaction between all of them (Fuchs, 1995)

Analysis of TnC Extraction Techniques

The data presented in this study provides evidence that there is no significant change in the calcium sensitivity of myofilaments in response to TnC extraction and subsequent reconstitution with native TnC. Furthermore, the data also shows that the method of endogenous TnC extraction is not a significant determinant of the muscle fibre's subsequent performance.

Partial extraction techniques have been reported by some workers to minimize experimentally-induced trauma to the muscle fibre (Moss, 1992). Ensuring the absence of

potentially toxic reagents, such as TFP, from the extraction medium has been thought to preserve the calcium sensitivity and cooperativity of treated muscle fibres after TnC reconstitution (Moss *et al.*, 1991). However, a problem with this protocol is the presence of endogenous TnC within extracted muscle fibres (Moss *et al.*, 1986). As a result, these investigators were unable to determine whether TnC was extracted from 'trigger-binding sites' or whether it was removed preferentially from regions of the thin myofilament which were not involved in myofilament overlap. The significance of this scenario is that TnC may not have been extracted from regions of the thin myofilament which had been involved in calcium-regulatory processes under isometric conditions. Furthermore, both TnC content and force production do not appear to follow a linear relationship in skeletal muscle fibres (Moss *et al.*, 1986). As such, while partial extraction techniques result in force reduction which is approximately half maximal, the TnC content of muscle fibres under these conditions remains high. This is supported by the results of the present study which shows that a 50% reduction in force may be correlated with an 8-10% decrease in a muscle fibre's TnC content as measured by TnC:TnI ratios on silver-stained polyacrylamide gels. Therefore, the observed effect of any mutant TnC protein which is subsequently reconstituted into a muscle fibre is likely to be diluted by the presence of large amounts of endogenous TnC. Furthermore, there is no indication that this TnC reassociates with regions of the thin myofilament which are participating in muscle contraction under isometric conditions. From these observations, one suggests caution in analyzing results obtained from experiments using this partial extraction technique.

The effectiveness of complete extraction techniques has been questioned because TFP may present harmful side effects to the muscle fibre, diminishing its calcium-sensitive response upon reconstitution (Moss *et al.*, 1991). The drug TFP is a phenothiazine tranquilizer which functions physiologically as a calmodulin antagonist (Massom *et al.*, 1990). Due to the high sequence homology between TnC and calmodulin, TFP may also bind to TnC by the displacement of divalent cations. The net effect is the dislodging of TnC from the thin filaments by a non-specific detergent mechanism (Massom *et al.*, 1990). This study has clearly shown that calcium sensitivity is restored both after complete extraction of TnC and subsequent fibre reconstitution. It has further been shown that the fibre retains its length-dependent calcium sensitivity upon reconstitution with native rabbit skeletal muscle TnC. The shift in calcium sensitivity of the fibre at long sarcomere lengths appears to resemble that observed in an unextracted control muscle fibre. Moreover, the results from electrophoresis show that the reduction in force ensuing after full extraction of TnC is nearly proportional to the decrease in the muscle fibre's TnC content. They also demonstrate that this effect is completely reversible upon reconstitution of the muscle fibre. Therefore, TFP does not inactivate thin myofilament binding sites for TnC after protein extraction. Furthermore, fibre deterioration was not shown to accelerate after treatment with TFP. Thus, the integrity of the experimental protocol was not minimized by this extraction technique. Interestingly, at very low concentrations, TFP has been shown to increase TnC's affinity for Ca^{2+} and to increase the sensitivity of the myofilaments during contraction (Kurebayashi and Ogawa, 1988).

The results presented in this thesis support the utilization of TFP as an effective means to extract endogenous TnC, without compromising the contractile properties of experimentally manipulated muscle fibres. This method of TnC extraction will be a necessary component of further studies designed to address the effects of mutant TnC proteins on contractile parameters. Full extraction of endogenous TnC from striated muscle fibres is the only mechanism by which the contribution of reconstituted proteins can be truly assessed with a high degree of confidence.

REFERENCES

Allen D. and Kentish J. (1988) The Cellular Basis of the Length-Tension Relation in Cardiac Muscle. *Journal of Molecular and Cellular Cardiology.* **17**:821-840.

Allen D. and Kentish J. (1988) Calcium Concentration in the Myoplasm of Skinned Ferret Ventricular Muscle Following Changes in Muscle Length. *The Journal of Physiology.* **407**:489-503.

Allen J. and Moss R. (1987) Factors Influencing the Ascending Limb of the Sarcomere Length-Tension Relation in Rabbit Skinned Muscle Fibres. *The Journal of Physiology.* **390**:119-136.

Babu A., Lehman W. and Gulati, J. (1989) Characterization of the Ca^{2+} Switch in Skeletal and Cardiac Muscles. *Federation of European Biochemical Societies.* **251**:177-182.

Babu A., Pemrick S. and Gulati J. (1986) Ca^{2+} Activation of Troponin C Extracted Vertebrate Striated Fast Twitch Muscle Fibers. *Federation of European Biochemical Societies.* **203**:20-24.

Babu A., Scordilis S., Sonnenblick E. and Gulati J. (1987) The Control of Myocardial Contraction With Skeletal Fast Muscle Troponin C. *The Journal of Biological Chemistry.* **262**:5815-5822.

Babu A., Sonnenblick E. and Gulati J. (1988) Molecular Basis for the Influence of Muscle Length on Myocardial Performance. *Science.* **240**:74-76.

Babu A., Su H., Ryu Y. and Gulati J. (1992) Determination of Residue Specificity in the EF-Hand of Troponin C for Ca^{2+} Coordination, by Genetic Engineering. *The Journal of Biological Chemistry.* **267**:15469-15474.

Brandt P., Cox R. and Kawai M. (1980) Can the Binding of Ca^{2+} to Two Regulatory Sites on Troponin C Determine the Steep pCa/Tension Relationship of Skeletal Muscle? *Proceedings of the National Academy of Sciences.* **8**:4717-4720.

Bremel R. and Weber A. (1972) Cooperation Within Actin Filaments in Vertebrate Skeletal Muscle. *Nature.* **238**:97-101.

Brenner B., Yu L. and Podolsky R. (1984) X-Ray Diffraction Evidence for Cross-Bridge Formation Relaxed Muscle at Various Ionic Strengths. *Biophysical Journal.* **46**:299-306.

Bressler B. and Clinch N. (1974) The Compliance of Contracting Skeletal Muscle. *The Journal of Physiology.* **237**:477-493.

Bressler B. and Morishita L. (1991) Variation of Calcium Sensitivity of Skinned Rabbit Psoas Fibres at Long Sarcomere Lengths is Not Related to the Change in Lattice Spacing. *Biophysical Journal.* **59**: 47a.

Bressler B. and Morishita L. (1992) Tension Responses and Calcium Sensitivity of Skinned Skeletal Muscle Fibres in Solutions of Decreased Ionic Strength. *Biophysical Journal.* **61**:293a.

Bressler B., Morishita L., Gauthier L., Borgford T. and Virani S. (1994) Mutation of the High Affinity Calcium Binding Sites of Skeletal Muscle Troponin C. *Biophysical Journal.* **66**:310a.

Chandra M., Fidalgo da Silva E., Sorrenson M., Ferro J., Pearlstone J., Nash B., Borgford T., Kay C. and Smillie L. (1994) The Effects of N-Helix Deletion and Mutant F29W on the Ca^{2+} Binding and Functional Properties of Chicken Skeletal Muscle Troponin C. *The Journal of Biological Chemistry.* **269**:14988-14994.

Chandra M., McCubbin W., Kay C. and Smillie L. (1994) Ca^{2+}, Mg^{2+}, and Troponin I Inhibitory Peptide Binding to a Phe-154 to Trp Mutant of Chicken Skeletal Muscle Troponin C. *Biochemistry.* **33**:2961-2969.

Collins J., Greaser M., Potter J. and Horn M. (1977) Determination of the Amino Acid Sequence of Troponin C from Rabbit Skeletal Muscle. *The Journal of Biological Chemistry.* **252**:6356-6362.

Cooley J. and Tukey J. (1965) An Algorithm for the Machine Computation of Simplex Fourier Series. *Math Comput.* **19**:297-301.

Cox J., Comte M. and Stein E. (1981) Calmodulin-Free Skeletal Muscle Troponin C Prepared in the Absence of Urea. *Biochemical Journal.* **195**:205-211.

Edman K. and Reggiani C. (1987) The Sarcomere Length-Tension Relation Determined in Short Segments of Intact Muscle Fibres of the Frog. *The Journal of Physiology.* **385**:709-732.

Endo M. (1972) Stretch Induced Increases in Activation of Skinned Muscle Fibre by Calcium. *Nature.* **237**:211-213.

Fabiato A. and Fabiato F. (1978) Myofilament Generated Tension Oscillations During Partial Activation and Activation Dependence of the Sarcomere Length Tension Relation in Skinned Cardiac Cells. *Journal of General Physiology.* **72**:667-699.

Fidalgo da Silva E., Sorrenson M., Smillie B., Barrabin H. and Scofano H. (1993) Comparison of Calmodulin and Troponin C With and Without its Amino-Terminal

Helix (Residues 1-11) in the Activation of Erythrocyte Ca^{2+}-ATPase. *The Journal of Biological Chemistry.* **268**:26220-26225.

Francois J., Gerday C., Prendergast F. and Potter J. (1993) Determination of the Ca^{2+} and Mg^{2+} Affinity Constants of Troponin C From Eel Skeletal Muscle and Positioning of the Single Tryptophan in the Primary Structure. *The Journal of Muscle Research and Cell Motility.* **14**:585-593.

Fuchs F. (1995) Mechanical Modulation of the Ca^{2+} Regulatory Protein Complex in Cardiac Muscle. *News in Physiological Sciences.* **10**:6-11.

Fuchs F. and Wang Y. (1991) Force, Length and Ca^{2+} Troponin C Affinity in Skeletal Muscle. *The American Journal of Physiology.* **261**:C787-C792.

Fujimori K., Sorenson M., Herzberg O., Moult J. and Reinach F. (1990) Probing the Calcium-Induced Conformational Transition of Troponin C With Site-Directed Mutants. *Nature.* **345**:182-184.

Fujisawa T., Ueki T. and Iida S. (1989) Structural Change of the Troponin C Molecule Upon Ca^{2+} Binding Measured in Solution by the X-Ray Scattering Technique. *The Journal of Biochemistry.* **105**:377-383.

George S., Su Z., Fan D. and Means A. (1993) Calmodulin-Cardiac Troponin C Chimeras. *The Journal of Biological Chemistry.* **268**:25213-25220.

Godt R. and Maughan D. (1981) Influence of Osmotic Compression on Calcium Activation and Tension in Skinned Muscle Fibres of the Rabbit. *The European Journal of Physiology.* **391**:334-337.

Gordon A., Huxley A., and Julian F. (1966) The Variation in Isometric Tension With Sarcomere Length in Vertebrate Muscle Fibres. *The Journal of Physiology.* **184**:170-192.

Gordon A. and Ridgeway E.: Cross-Bridges Affect Both TnC Structure and Calcium Affinity in Muscle Fibres, in Sugi and Pollack (eds.): *Mechanism of Sliding in Muscle Contraction.* New York, Plenum Press, 1993, pp 183-194.

Grabarek Z., Leavis P. and Gergely J. (1986) Calcium Binding to the Low Affinity Sites in Troponin C Induces a Conformational Change in the High Affinity Domain. A Possible Route of Information Transfer in Activation of Muscle Contraction. *The Journal of Biological Chemistry.* **261**:608-613.

Grabarek Z., Tan R., Wang J., Tao T. and Gergely J. (1990) Inhibition of Mutant Troponin C Activity by an Intra-Domain Disulphide Bond. *Nature.* **345**:132-135.

Greaser M. and Gergely J. (1973) Purification and Properties of the Components of Troponin. *The Journal of Biological Chemistry.* **248**:2125-2133.

Guilian G., Moss R. and Greaser L. (1983) Improved Methodology for Analysis and Quantitation of Proteins on One-Dimensional Silver-Stained Slab Gels. *Analytical Biochemistry.* **129**:277-287.

Gulati J., Babu A. and Putkey J. (1989) Down-Regulation of Fast-Twitch Skeletal Muscle Fibres With Cardiac Troponin C and Recombinant Mutants. *Federation of European Biochemical Societies.* **248**:5-8.

Gulati J. and Babu A. (1985) Critical Dependence of Calcium-Activated Force on Width in Highly Compressed Skinned Fibres of the Frog. *Biophysical Journal.* **48**:781-787.

Gulati, J. Sonnenblick E. and Babu A. (1990) The Role of Troponin C in the Length Dependence of Ca^{2+}-Sensitive Force of Mammalian Skeletal and Cardiac Muscles. *The Journal of Physiology.* **441**:305-324.

Guo X., Wattanapermpool J., Palmiter K., Murphy A. and Solaro R. (1994) Mutagenesis of Cardiac Troponin I: Role of the Unique NH_2-Terminal Peptide in Myofilament Activation. *The Journal of Biological Chemistry.* **269**:15210-15216.

Guth K. and Potter J. (1987) Effect of Cycling Cross-Bridges on the Structure of Troponin C and on the Ca^{2+} Affinity of the Ca^{2+}-Specific Regulatory Sites in Skinned Rabbit Psoas Fibres. *The Journal of Biological Chemistry.* **262**:13627-13635.

Hannon J., Martyn D. and Gordon A. (1992) Effects of Cycling and Rigor Cross-Bridges on the Conformation of Cardiac Troponin C. *Circulation Research.* **71**:984-991.

Harrison S., Lamont C. and Miller D. (1988) Hysteresis and Length Dependence of Ca^{2+} Sensitivity in Chemically Skinned Rat Cardiac Muscle. *The Journal of Physiology.* **401**:115-143.

Hatakenaka M. and Ohtsuki I. (1992) Effect of Removal and Reconstitution of Troponins C and I on the Ca^{2+}-Activated Tension Development of Single Glycerinated Rabbit Skeletal Muscle Fibres. *European Journal of Biochemistry.* **205**:985-993.

Hatakenaka M. and Ohtsuki I. (1991) Replacement of Three Troponin Components With Cardiac Troponin Components Within Single Glycerinated Skeletal Muscle Fibres. *Biochemical and Biophysical Research Communications.* **181**:1022-1027.

Heidorn D. and Trewhella J. (1988) Comparison of the Crystal and Solution Structures of Calmodulin and Troponin C. *Biochemistry.* **27**:909-915.

Herzberg O. and James M. (1985) Structure of the Calcium Regulatory Muscle Protein Troponin C at 2.8 Å Resolution. *Nature.* **313**:653-659.

Herzberg O., Moult J. and James M. (1986) A Model for the Ca^{2+}-Induced Conformational Transition of Troponin C. *The Journal of Biological Chemistry.* **261**:2638-2644.

Hibberd M. and Jewell B. (1982) Calcium and Length-Dependent Force Production in Rat Ventricular Muscle. *The Journal of Physiology.* **329**:527-540.

Hofmann P. and Fuchs F. (1988) Bound Calcium and Force Development in Skinned Cardiac Muscle Bundles: Effect of Sarcomere Length. *Journal of Molecular and Cellular Cardiology.* **20**:667-677.

Huxley A. (1957) Muscle Structure and Theories of Contraction. *Annual Review of Biophysics and Biophysical Chemistry.* **7**:255-318.

Huxley A. and Neidergerke R. (1954) Structural Changes in Muscle During Contraction: Interference Microscopy of Living Muscle Fibres. *Nature.* **173**:971-973.

Huxley A. and Simmons R. (1971) Proposed Mechanism of Force Generation in Striated Muscle. *Nature.* **233**:533-538.

Huxley H. (1973) Muscular Contraction and Cell Motility. *Nature.* **243**:445-449

Huxley H. and Hanson J. (1954) Changes in the Cross-Striations of Muscle During Contraction and Stretch and Their Structural Interpretation. *Nature.* **173**:973-977.

Johnson J., Nakkula R., Vasulka C. and Smillie L. (1994) Modulation of Ca^{2+}-Exchange With the Ca^{2+}-Specific Regulatory Sites of Troponin C. *The Journal of Biological Chemistry.* **269**:8919-8923.

Julian F. and Moss R. (1981) Effects of Calcium and Ionic Strength on Shortening Velocity and Tension Development in Frog Skinned Fibres. *The Journal of Physiology.* **311**:179-199.

Kentish J., ter Keurs H. and Allen D. :The Contribution of Myofibrillar Properties to the Sarcomere Length-Force Relationship of Cardiac Muscle, in ter Keurs and Noble (eds.): *Starling's Law of the Heart Revisited.* Boston, Kluwer Academic Publishers, 1988, pp 1-14.

Koboyashi T., Tao T., Gergely J. and Collins J. (1994) Structure of the Troponin Complex. *The Journal of Biological Chemistry.* **269**:5725-5729.

Kurebayashi N. and Ogawa Y. (1988) Increase by Trifluoperazine in Calcium Sensitivity of Myofibrils in a Skinned Fibre From Frog Skeletal Muscle. *The Journal of Physiology.* **403**:407-424.

Laemmli R. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature.* **227**:680-685.

LeBlanc L. and Borgford T. (1995) N-Cap Mutants in the Regulatory Domain of Chicken Troponin C. *Biophysical Journal.* **68**:58a.

Li M., Chandra M., Pearlstone J., Racher K., Trigo-Gonzalez G., Borgford T., Kay C. and Smillie L. (1994) Properties of Isolated Recombinant N and C Domains of Chicken Troponin C. *Biochemistry.* **33**:917-925.

Li H. and Fajer P. (1994) Orientational Changes of Troponin C Associated With Thin Filament Activation. *Biochemistry.* **33**:14324-14332.

Lymn R. and Taylor E. (1971) Mechanism of Adenosine Triphosphate Hydrolysis by Actomyosin. *Biochemistry.* **10**:4617-4624.

Lynch G., and Williams D. (1994) The Effect of Lowered pH on the Ca^{2+}-Activated Contractile Characteristics of Skeletal Muscle Fibres From Endurance-Trained Rats. *Experimental Physiology.* **79**:47-57.

Martyn D. and Gordon A. (1988) Length and Myofilament Spacing-Dependent Changes in Calcium Sensitivity of Skeletal Fibres: Effects of pH and Ionic Strength. *The Journal of Muscle Research and Cell Motility.* **9**:428-445.

Martyn D. and Gordon A. (1992) Force and Stiffness in Glycerinated Rabbit Psoas Fibres. *Journal of General Physiology.* **99**:795-816.

Massom L., Lee H. and Jarrett H. (1990) Trifluoperazine Binding to Porcine Calmodulin and Skeletal Muscle Troponin C. *Biochemistry.* **29**:671-681.

Maughan D. and Godt R. (1979) Stretch and Radial Compression Studies on Relaxed Skinned Muscle Fibres of the Frog. *Biophysical Journal.* **28**: 391-402.

Mehler E., Pascual-Ahuir J. and Weinstein H. (1991) Structural Dynamics of Calmodulin and Troponin C. *Protein Engineering.* **4**:625-637.

Metzger J. and Moss R. (1988) Thin Filament Regulation of Shortening Velocity in Rat Skinned Skeletal Muscle: Effects of Osmotic Compression. *The Journal of Physiology.* **398**:1165-175

Metzger J. and Moss R. (1991) Kinetics of a Ca^{2+}-Sensitive Cross-bridge State Transition in Skeletal Muscle Fibres: Effects Due to Variations in Thin Filament Activation by Extraction of Troponin C. *Journal of General Physiology.* **98**:233-248.

Metzger J., Parmacek M., Barr E., Pasyk K., Lin W., Cochrane K., Field L. and Leiden J. (1993) Skeletal Troponin C Reduces Contractile Sensitivity to Acidosis in Cardiac Myocytes From Transgenic Mice. *Proceedings of the National Academy of Sciences.* **90**:9036-9040.

Morano I. and Ruegg J. (1991) What Does TnC_{DANZ} Fluorescence Reveal About the Thin Filament State? *The European Journal of Physiology.* **418**:333-337.

Morimoto S. and Ohtsuki I. (1994) Role of Troponin C in Determining the Ca^{2+}-Sensitivity and Cooperativity of the Tension Development in Rabbit Skinned and Cardiac Muscles. *Journal of Biochemistry.* **115**:144-146.

Morimoto S., Fujiwari T. and Ohtsuki I. (1988) Restoration of Ca^{2+}-Activated Tension of CDTA-Treated Single Skeletal Muscle Fibres by Troponin C. *Journal of Biochemistry.* **104**:874-874.

Morimoto S. and Ohtsuki I. (1987) Ca^{2+} and Sr^{2+}-Sensitivity of ATPase Activity of Rabbit Skeletal Myofibrils: Effect of the Complete Substitution of Troponin C With Cardiac Troponin C, Calmodulin and Parvalbumins. *Journal of Biochemistry.* **101**:291-301.

Moss R. (1979) Sarcomere Length-Tension Relations of Frog Skinned Muscle Fibres During Calcium Activation at Short Lengths. *The Journal of Physiology.* **292**:117-202.

Moss R. (1992) Calcium Regulation of Mechanical Properties of Striated Muscle. *Circulation Research.* **70**:865-883.

Moss R., Guilian G. and Greaser M. (1982) Physiological Effects Accompanying Removal of Myosin LC_2 From Skinned Skeletal Muscle Fibres. *The Journal of Biological Chemistry.* **257**:8588-8591.

Moss R., Guilian G. and Greaser M. (1985) The Effects of Partial Extraction of TnC Upon the Tension-pCa Relationship in Rabbit Skinned Skeletal Muscle Fibres. *The Journal of General Physiology.* **86**:585-600.

Moss R., Lauer M., Guilian G. and Greaser M. (1986) Altered Ca^{2+} Dependence of Tension Development in Skinned Skeletal Muscle Fibres Following Modification of Troponin by Partial Substitution with Cardiac Troponin C. *The Journal of Biological Chemistry.* **261**:6096-6099.

Moss R., Nwoye L. and Greaser M. (1991) Substitution of Cardiac Troponin C Into Rabbit Muscle Does Not Alter the Length Dependence of Ca^{2+} Sensitivity of Tension. *The Journal of Physiology.* **440**:273-289.

Murray J. and Weber A. (1974) The Cooperative Action of Muscle Proteins. *Scientific American.* **230**: 59-72.

Negele J., Dotson., Liu W., Sweeney H. and Putkey J. (1992) Mutation of the High Affinity Calcium Binding Sites in Cardiac Troponin C. *The Journal of Biological Chemistry.* **267**:825-831.

Ngai S. and Hodges R. (1992) Biologically Important Interactions Between Synthetic Peptides of the N-Terminal Region of Troponin I and Troponin C. *The Journal of Biological Chemistry.* **267**:15715-15720.

Ngai S., Sonnichsen F. and Hodges R. (1994) Photochemical Cross-linking Between Native Rabbit Skeletal Troponin C and Benzoylbenzoyl-Troponin I Inhibitory Peptide, Residues 104-115. *The Journal of Biological Chemistry.* **269**:2165-2172.

Nishita K., Tanaka H. and Ojima T. (1994) Amino Acid Sequence of Troponin C From Scallop Striated Adductor Muscle. *The Journal of Biological Chemistry.* **269**:3464-3468.

Nowak E., Borovikov Y., Khoroshev M. and Dabrowska R. (1991) Troponin I and Caldesmon Restrict Alterations in Actin Structure Occurring on Binding of Myosin Subfragment 1. *Federation of European Biochemical Societies.* **281**:51-54.

Oakley B., Kirsch D. and Morris N. (1980) A Simplified Ultrasensitive Silver Stain for Detecting Proteins in Polyacrylamide Gels. *Analytical Biochemistry.* **105**:361-363.

Ovasaka M. and Taskinen J. (1991) A Model for Human Cardiac Troponin C and for Modulation of Its Ca^{2+} Affinity by Drugs. *Proteins: Structure, Function, and Genetics.* **11**:79-94.

Park H., Gong B. and Tao T. (1994) A Disulfide Crosslink Between Cys^{98} of Troponin-C and Cys^{133} of Troponin-I Abolishes the Activity of Rabbit Skeletal Troponin. *Biophysical Journal.* **66**:2062-2065.

Parmacek M. and Leiden J. (1991) Structure, Function and Regulation of Troponin C. *Circulation.* **84**:991-1003.

Pearlstone J., Borgford T., Chandra M., Oikawa K., Kay C., Herzberg O., Moult J., Herklotz A., Reinach F. and Smillie L. (1992) Construction and Characterization of a Spectral Probe Mutant of Troponin C: Application to Analyses of Mutants with Increased Ca^{2+} Affinity. *Biochemistry.* **31**:6545-6553.

Phan B. and Reisler E. (1993) Aluminum Flouride Interactions with Troponin C. *Biophysical Journal.* **65**:2511-2516.

Potter J. and Gergely J. (1975) The Calcium and Magnesium Binding Sites of Troponin and Their Role in the Regulation of Myofibrillar Adenosinetriphosphate. *The Journal of Biological Chemistry.* **250**:4628-4633.

Potter J. and Johnson J. (1982) Troponin, in Cheung (ed.) *Calcium and Cell Function, vol. II.* New York, Academic Press, 1982, pp 145-173.

Putkey J., Sweeney H. and Campbell S. (1989) Site-Directed Mutation of the Trigger Calcium-Binding Sites in Cardiac Troponin C. *The Journal of Biological Chemistry.* **264**:12370-12378.

Putkey J., Dotson D. and Mouwad P. (1993) Formation of Inter- and Intramolecular Disulfide Bonds Can Activate Cardiac Troponin C. *The Journal of Biological Chemistry.* **268**:6827-6830.

Putkey J., Liu W. and Sweeney H. (1991) Function of the N-Terminal Calcium-Binding Sites in Cardiac/Slow Troponin C Assessed in Fast Skeletal Muscle Fibers. *The Journal of Biological Chemistry.* **266**:14881-14884.

Ramsey R. and Street S. (1940) The Isometric Length-Tension Diagram of Isolated Muscle Fibres of the Frog. *Journal of Cell Comp and Physiology.* **15**:11-34.

Rudel R. and Zite-Ferenczy F. (1980) Efficiency of Light Diffraction by Cross-Bridge Striated Muscle Fibers Under Stretch and During Isometric Contraction. *Biophysical Journal.* **30**:507-516.

Ruegg J. (1986) Troponin, the On-Off Switch of Muscle Contraction in Striated Muscle, in Burggren, Ishii, Johansen, Langer, Neuweiler and Randal (eds.) *Calcium in Muscle Activation.* New York, Springer-Verlag, 1986, pp 83-113.

Ruegg J., Zeugner C., Van Eyck J., Kay C. and Hodges R. (1989) Inhibition of TnI-TnC Interaction and Contraction of Skinned Muscle Fibres by the Synthetic Peptide TnI [104-115]. *The European Journal of Physiology.* **414**:430-436.

Saeki Y., Kurihara K., Hongo K. and Tanaka E. (1993) Alterations in Intracellular Calcium and Tension of Activated Ferret Papillary Muscle in Response to Step Length Changes. *The Journal of Physiology.* **463**:291-306.

Shaw G., Hodges R. and Sykes B. (1991) Probing the Relationship Between α-Helix Formation and Calcium Affinity in Troponin C: H NMR Studies of Calcium Binding to Synthetic and Variant Site III Helix-Loop-Helix Peptides. *Biochemistry.* **30**:8339-8347.

Shiraishi F., Kambara M. and Ohtsuki I. (1992) Replacement of Troponin Components in Myofibrils. *Journal of Biochemistry.* **111**:61-65.

Shiraishi F. and Yamamoto K. (1994) The Effect of Partial Removal of Troponin I and C on the Ca^{2+}-Sensitive ATPase Activity of Rabbit Skeletal Myofibrils. *Journal of Biochemistry.* **115**:171-173.

Slawnych M., Morishita L. and Bressler B. (1995) Sarcomere Homogeneity in Short Muscle Fibre Segments. *Biophysical Journal.* **In press.**

Smith L., Greenfield N. and Hitchcock-deGregori S. (1994) The Effects of Deletion of the Amino-terminal Helix on Troponin C Function and Stability. *The Journal of Biological Chemistry.* **269**:9857-9863.

Stephenson D. and Wendt I. (1984) Length Dependence of Changes in Sarcoplasmic Calcium Concentration and Myofibrillar Calcium Sensitivity in Striated Muscle Fibres. *The Journal of Muscle Research and Cell Motility.* **5**:243:272

Stephenson D. and Williams D. (1982) Effects of Sarcomere Length on the Force-pCa Relation in Fast- and Slow-Twitch Skinned Muscle Fibres From the Rat. *The Journal of Physiology.* **333**:637-653.

Stiene G., Papp Z. and Elzinga G. (1993) Calcium Modulates the Influence of Length Changes on the Myofibrillar Adenosine Triphosphate Activity in Rat Skinned Cardiac Trabeculae. *The European Journal of Physiology.* **425**:199-207.

Strang P. and Potter J. (1992) A Monoclonal Antibody That Recognizes Different Conformational States of Skeletal Muscle Troponin C and Other Calcium Binding Proteins. *The Journal of Muscle Research and Cell Motility.* **13**:308-314.

Sweeney H., Brito R., Rosevear P. and Putkey J. (1990) The Low-Affinity Ca^{2+} Binding Sites in Cardiac/Slow Skeletal Muscle Troponin C Perform Distinct Functions: Site I Alone Cannot Trigger Contraction. *Proceedings of the National Academy of Sciences,* **87**:9538-9542.

Swenson C. and Fredricksen R. (1991) Interaction of Troponin C and Troponin C Fragments with Troponin I and the Troponin I Inhibitory Peptide. *Biochemistry.* **31**:3420-3429.

Talbot J. and Hodges R. (1981) Synthetic Studies of the Inhibitory Region of Rabbit Skeletal Troponin I. Relationship of Amino Acid Sequence to Biological Activity. *The Journal of Biological Chemistry.* **256**:2798-2802.

Trigo-Gonzalez G., Racher K., Burtnick L. and Borgford T. (1992) A Comparative Spectroscopic Study of Tryptophan Probes Engineered Into High- and Low-Affinity Domains of Recombinant Chicken Troponin C. *Biochemistry.* **31**:7009-7015.

Trigo-Gonzalez G., Awang G., Racher K., Neden K. and Borgford T. (1993) Helix Variants of Troponin C With Tailored Calcium Affinities. *Biochemistry.* **37**:9826-9831.

Tsai C. and Frasch C. (1982) A Sensitive Silver Stain for Detecting Lipopolysaccharides in Polyacrylamide Gels. *Analytical Biochemistry.* **119**:115-119.

Van Eyck J., Kay C. and Hodges R. (1991) A Comparative Study of the Interactions of Synthetic Peptides of the Skeletal and Cardiac Troponin I Inhibitory Region With Skeletal and Cardiac Troponin C. *Biochemistry.* **30**:9974-9981.

Van Eyck J. and Hodges R. (1991) The Biological Importance of Each Amino Acid Residue of the Troponin I Inhibitory Sequence 104-115 in the Interaction With Troponin C and Tropomyosin-Actin. *The Journal of Biological Chemistry.* **263**:1726-1732.

Wang Y. and Fuchs F. (1994) Length, Force and Ca^{2+}-Troponin C Affinity in Cardiac and Slow Skeletal Muscle. *American Journal of Physiology.* **266**:C1077-C1082.

Wang C., Zhan Q., Tao T. and Gergely J. (1987) pH-Dependent Structural Transition in Rabbit Skeletal Troponin C. *The Journal of Biological Chemistry.* **262**:9636-9640.

Xu G. and Hitchcock-deGregori S. (1988) Synthesis of a Troponin C cDNA and Expression of Wild Type and Mutant Proteins in *E.Coli. The Journal of Biological Chemistry.* **263**:13962-13970.

Yates L. and Greaser M. (1983b) Troponin Subunit Stoichiometry and Content in Rabbit Skeletal Muscle and Myofibrils. *The Journal of Biological Chemistry.* **258**:5770-5774.

Zot A. and Potter J. (1987) Structural Aspects of Troponin-Tropomyosin Regulation of Skeletal Muscle Contraction. *Annual Review of Biophysics and Biophysical Chemistry.* **16**:535-559.

Zot H. and Potter J. (1982) A Structural Role for the Ca^{2+}-Mg^{2+} Sites on Troponin C in the Regulation of Muscle Contraction. *The Journal of Biological Chemistry.* **257**:7678-7683.

APPENDIX I

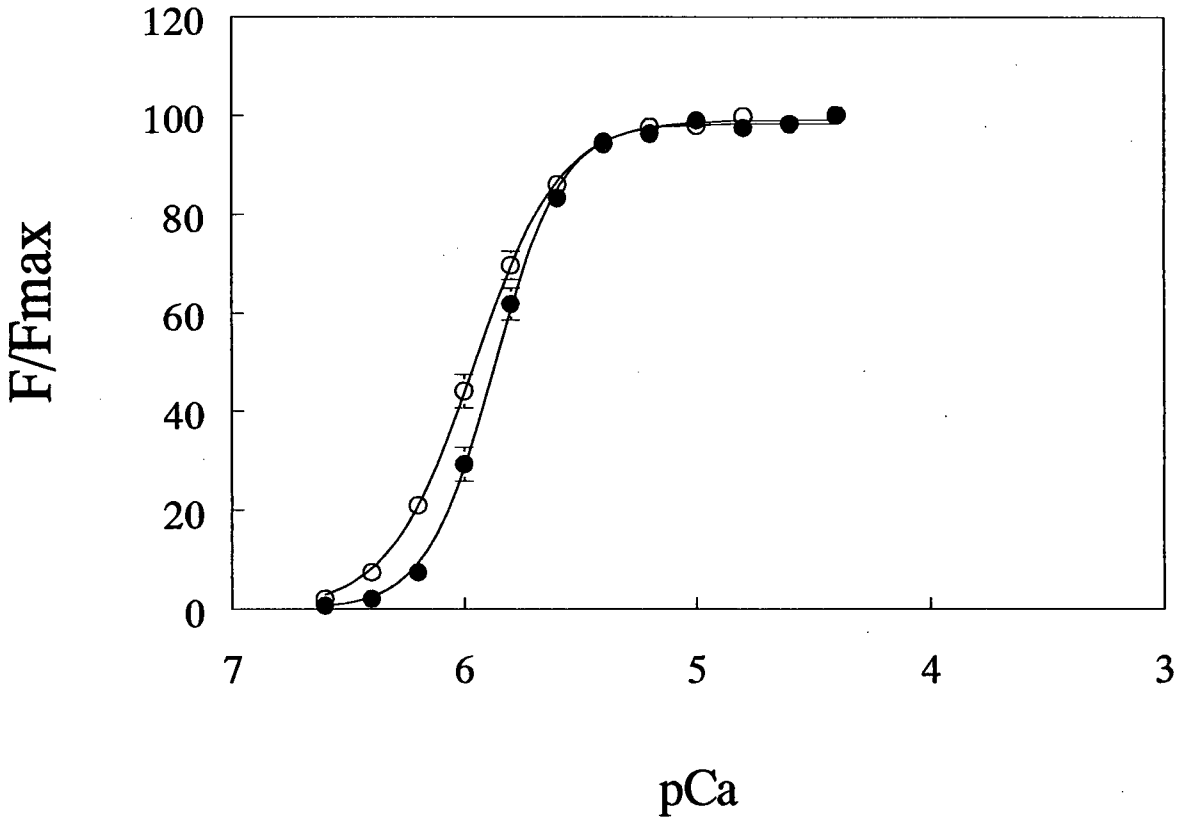


Figure 3a: Effects of Osmotic Compression on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves for skinned single fibre segments before (●) and after (○) treatment with 5% Dextran T-500. Parameters of the Hill equation for each trace are given in Table 8. ● = 2.2 μm, 29 fibres; ○ = 2.2 μm, 29 fibres.

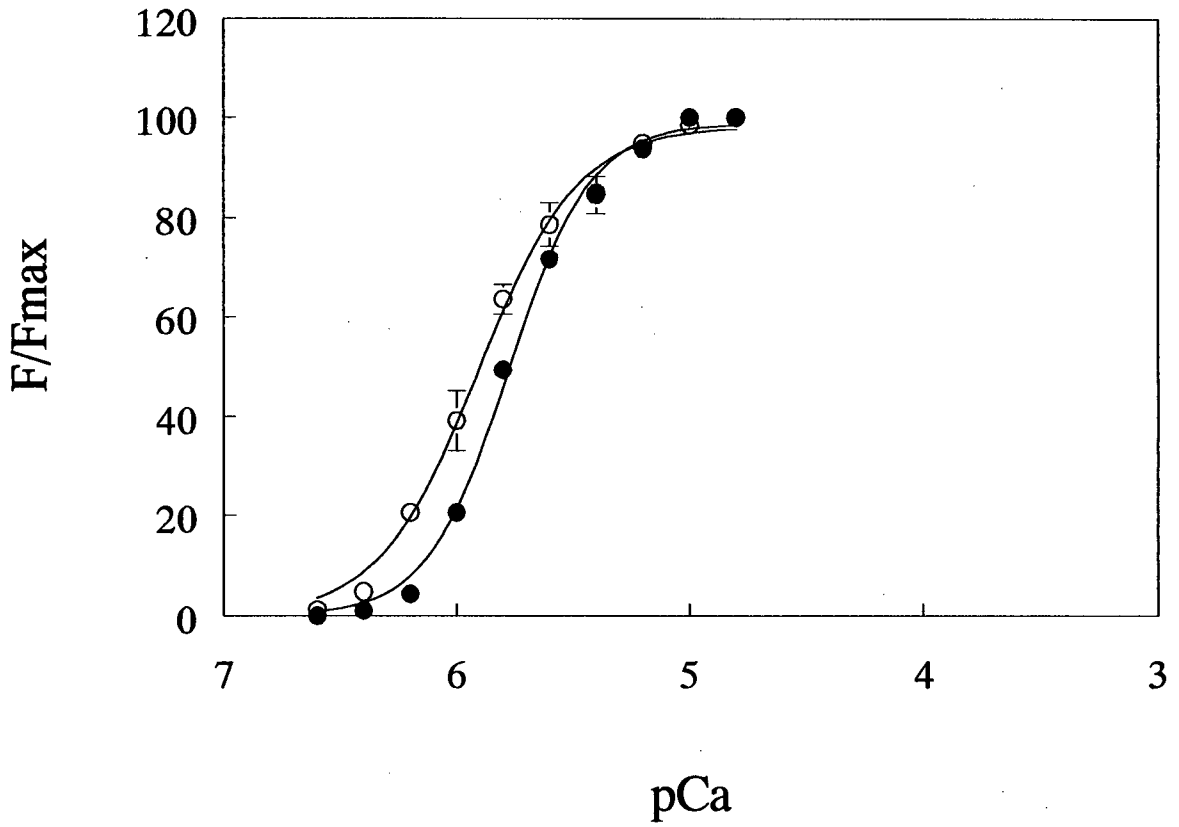


Figure 3b: Combined Effects of Osmotic Compression and Sarcomere Length on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves for skinned single fibre segments treated with 11.8% Dextran T-70at short (●) and long (○) sarcomere lengths. Parameters of the Hill equation for each trace are given in Table 8. ● = 2.4 μm, 8 fibres; ○ = 3.2 μm, 6 fibres.

Table 1: The Effects of Osmotic Compression and Sarcomere Length on the Parameters of the Hill Equation in Force-pCa Curves From Rabbit Psoas Muscle Fibres

Sarcomere Length (μm)	Fibre Treatment	Number of Fibres	pK		ΔpK	n	
2.2		29	5.86±0.02	(5.87)ǂ		3.44±0.13	(2.97)ǂ
2.2 dex[1]	5% Dextran	29	5.94±0.02*	(5.96)	0.09	2.63±0.81*	(2.36)
2.2 dex[2]		6	5.77±0.02	(5.77)		2.51±0.14	(2.49)
3.2 dex[2]	11.8% Dextran	8	5.84±0.03*	(5.91)	0.14	1.92±0.27*	(2.05)

* Significant differance of pK and n at different sarcomere lengths using Student's t-test ($p \leq 0.05$)

ǂ Values in brackets are from a fit of average curves to the Hill equation

dex[1] Indicates fibres treated with Dextran T-500

dex[2] Indicates fibres treated with Dextran T-70

APPENDIX II

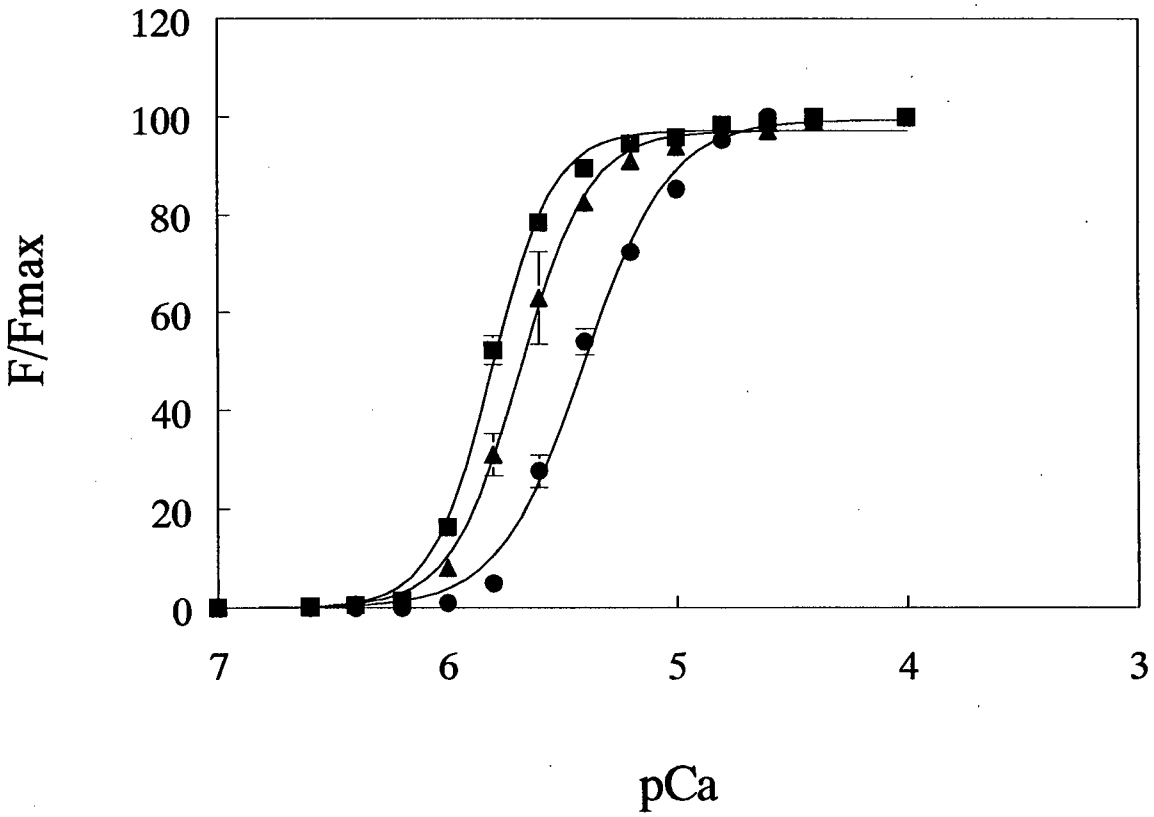


Figure 4a: Effects of Low Ionic Strength Activating Solution on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the Force-pCa curves for skinned single fibre segments at 200 mM ionic strength (●) at 150 mM ionic strength (Δ) and at 125 mM ioinc strength (■). Parameters of the Hill equation for each trace are given in Table 9. ● = 2.2 μm, 19 fibres; Δ = 2.2 μm, 19 fibres; ■ = 2.2 μm, 19 fibres.

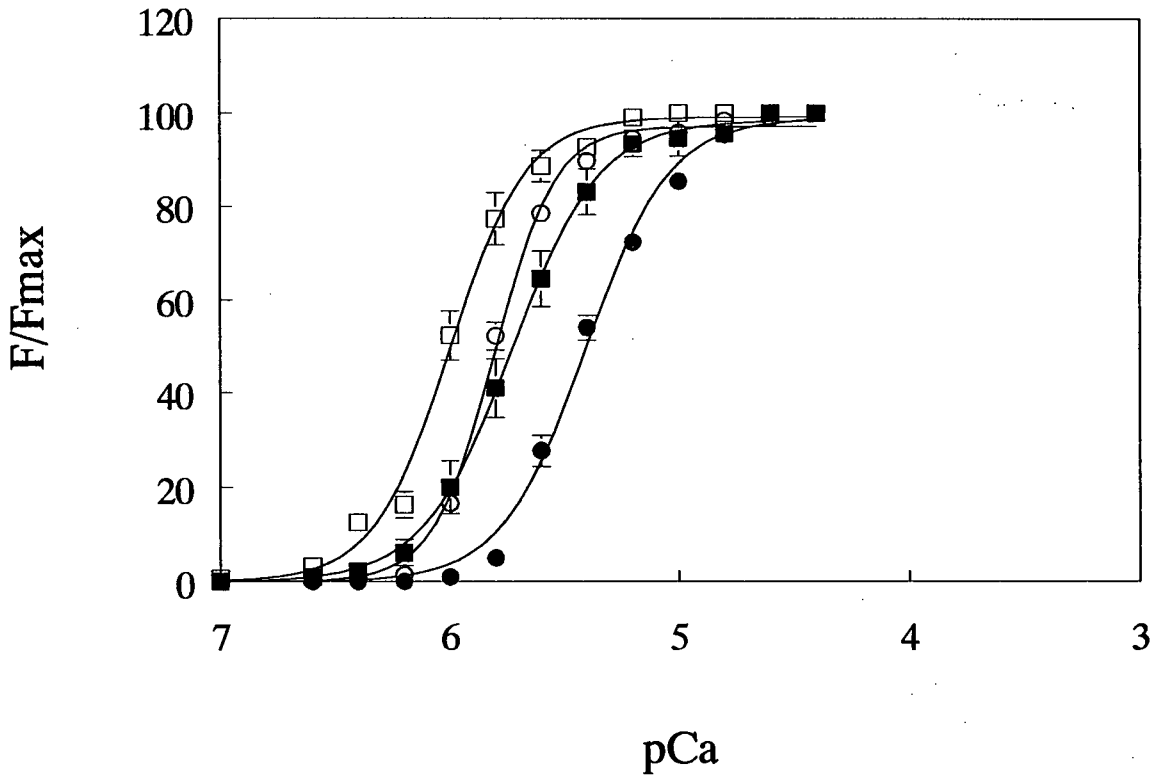


Figure 4b: Combined Effects of Low Ionic Strength Activating Solution and Sarcomere Length on the Calcium Sensitivity of Single Skinned Skeletal Muscle Fibre Segments

A comparison of the force-pCa curves for skinned single fibre segments at 200 mM ionic strength and short sarcomere length (●), at 125 mM ionic strength and short sarcomere length (○), at 200 mM ioinc strength and long sarcomere length(■) and at 125 mM ionic strength and long sarcomere length (□). Parameters of the Hill equation for each trace are given in Table 9. ● = 2.2 μm, 10 fibres; ○ = 3.2 μm, 6 fibres; ■ = 2.2 μm, 10 fibres; □ = 3.2 μm, 6 fibres.

Table 2: The Effects of Low Ionic Strength Activating Solution and Sarcomere Length on the Parameters of the Hill Equation in Force-pCa Curves From Rabbit Psoas Muscle Fibres

Sarcomere Length (μm)	Fibre Treatment	Number of Fibres	pK		ΔpK	n	
2.2	200 mM IS	19	5.44±0.02	(5.40)ǂ		2.78±0.12	(2.34)ǂ
2.2	150 mM IS	19	5.65±0.02*	(5.68)	0.20	3.07±0.21*	(2.87)
2.2	200 mM IS	19	5.43±0.02	(5.40)		2.78±0.10	(2.34)
2.2	125 mM IS	19	5.80±0.02*	(5.80)	0.38	3.12±0.13*	(3.22)
2.2	125 mM IS	6	5.80±0.02	(5.80)		3.38±0.27	(3.26)
3.2	125 mM IS	6	5.97±0.03*	(6.00)	0.18	2.77±0.20*	(2.61)
2.2	200 mM IS	10	5.54±0.08	(5.40)		3.19±0.15	(2.35)
3.2	200 mM IS	10	5.87±0.09*	(5.72)	0.36	2.65±0.18*	(2.24)

* Significant differance of pK and n at different sarcomere lengths using Student's t-test ($p \leq 0.05$)

ǂ Values in brackets are from a fit of average curves to the Hill equation

IS Indicates ionic strength of activating solutions