REGULATION OF MUSCLE CONTRACTION BY TROPONIN C

By

LOUISE ELLEN LEBLANC

B.Sc. (Honours), Simon Fraser University, 1993
M.Sc., Simon Fraser University, 1996

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Department of _Anatomy_

The University of British Columbia
Vancouver, Canada

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ABSTRACT

It is generally believed that calcium binding to Troponin C (TnC) in striated muscle acts as a simple switch, enabling tropomyosin to unblock myosin-binding sites on actin filaments. TnC has two domains, each containing a pair of structurally homologous sites. The high-affinity Ca^{2+}/Mg^{2+} binding sites are believed to have a primarily structural role, anchoring the protein into the troponin complex. The number of available myosin binding sites on the thin filament is determined by the intrinsic properties of the second pair of relatively low-affinity, calcium-specific sites. While TnC is found in all striated muscle, only one functional low-affinity site is common to all types, leading us to believe that it plays a crucial regulatory role in muscle contraction.

A series of genetically engineered proteins that modify the calcium-binding properties at site II of the regulatory domain have been introduced into single cell segments of chemically skinned skeletal rabbit psoas muscle fibres. The role of TnC in regulating tension, force development and the maximal rate at which muscle fibers can shorten under zero load, has been investigated.

The graded decrease in force with the TnC variants was mutant dependent. Measurements of the maximum shortening velocity using the slack test method produced biphasic plots at pCa 4.0. A graded decrease in force redevelopment (k_{tr}), measured using a quick release with restretch protocol, correlated with the decrease in force. The addition of phosphate to the activating solution decreased force to a greater extent than stiffness and increased k_{tr}. However the early fast phase of the biphasic shortening velocity was unexpectedly slowed in a TnC dependent manner with no increase in the later slow phase of shortening as would be expected if the mutant TnC had simply

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decreased the level of thin filament activation. Tension transients confirmed that the decrease in tension with the mutant TnC was due to a decrease in the number of bound cross-bridges and not a shift in the force per bridge. This study provides the first evidence that TnC regulates a step in the cross bridge cycle beyond thin filament activation showing that TnC is not just a simple switch.

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To Mom and Dad, for your unwavering confidence and support through all the years. I think this means as much to you as it does to me. On those days when I wondered if it was all worth it I just reminded myself that you were there cheering me on. I am very happy that I could share this with you.

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REGULATION OF MUSCLE ACTIVATION BY TROPONIN C

1.0 INTRODUCTION

It is generally believed that the binding of calcium to troponin C (TnC) in skeletal muscle acts as a simple "switch" that results in a shift in the position of tropomyosin over the surface of the actin strand to unblock myosin binding sites on the thin filament (H. E. Huxley, 1973). The number of unblocked myosin binding sites determines the level of activation (Ford, 1991, Metzger, 1995, Wahr et al, 1997). The binding of calcium to the low-affinity calcium-binding domain of TnC is critical in activating the regulatory function of the protein (Holroyde et al, 1980; Sweeney et al, 1990; Fujimori et al, 1990). Past studies (Guth & Potter, 1987; Walsh et al, 1985; Brandt et al,1990; Da Silva et al,1993) have implicated the importance of the dynamic equilibrium among calcium, TnC, and calcium-bound TnC in determining the degree of activation in muscle suggesting a possible role for the calcium-TnC interaction in modulating cross-bridge kinetics. Because of the rapid rate of calcium dissociation from TnC under physiological conditions, the concentration of calcium-TnC complex and the level of activation, may be determined by the dynamic equilibrium of this reversible binding of calcium to TnC. Active binding sites may therefore become transiently inactive. If the number of sites on the thin filaments is not at its maximum, even at maximally activating calcium concentrations, there is the potential for up- or down-regulation of the level of activation. While TnC is found in all striated muscle, only one functional low-affinity site, site II, is common to all types, leading us to believe that it plays a crucial regulatory role in muscle contraction.

1.1 Organization of Muscle Fibre Proteins

Whole muscle is arranged in groups of fibre bundles that contain striated muscle cells known as muscle fibres. Muscle cells contain myofibrils that consist of a repeating unit known as the sarcomere. Every sarcomere is mainly composed of two types of filaments, namely the thick and thin filaments. The large myosin molecules are the major protein component of the thick filaments, while actin is the major constituent of the thin filaments. Myosin molecules are arranged such that the "tail" forms the core of the filament and the "head" projects outward. Cross-bridges are formed when the myosin head from the thick filament binds to the actin filament at regular intervals, to bridge a $130A^{\circ}$ gap between them, as occurs in the mechanical process of muscle contraction. Before this interaction can occur, other proteins bound to the thin filament must undergo a conformational change. It is well established that the thin filament carries a repeating unit of proteins, troponin (Tn) and tropomyosin (Tm), that regulate the attachment of the myosin heads to the myosin binding sites on actin (Ebashi and Endo, 1968). Tm is a double stranded helical protein that sits outside the groove of the double stranded actin filament, blocking the attachment of myosin. This steric inhibition is regulated by the Tn complex of proteins. These three troponins, TnC, troponin-I (TnI) and troponin-T (TnT) decorate the thin filament at 38-nm intervals. At relatively low intracellular concentrations of calcium, the Tn complex inhibits muscle contraction by preventing a productive interaction between actin and myosin. Contraction is initiated by the release of calcium from the sarcoplasmic reticulum, into the myofilament space. This transient increase in the intracellular calcium is first communicated to the proteins of the muscle fibre through a change in the structure of TnC from a relatively closed conformation to a

more open conformation (Gagne et al, 1994). As TnC binds calcium the nature of its interaction with TnI changes, causing Tm to shift its position into the groove between the actin filaments to expose the active sites on actin, which the myosin head can now bind.

1.1.1 The Structure of Troponin C

TnC consists of two domains arranged as four helix-loop-helix motifs (figure 1); sites I and II are calcium specific while sites III and IV in the C-terminal domain bind both calcium and magnesium. *In vivo*, TnC sites III and IV bind either calcium or magnesium immediately upon synthesis to maintain the structural integrity of the troponin complex (Zot & Potter, 1982). The binding affinity for these sites is $2x10^7M^{-1}$ for calcium and $2x10^3M^{-1}$ for magnesium (Gagne et al,1994). However, the calcium dissociation rates for sites III and IV are too slow for the time scale on which muscle contraction occurs.

The ability to bind and release calcium ions rapidly in the physiological concentration range of 0.1 to 10 μM is observed in the N-terminal domain of the protein at sites I and II (Sorenson et al, 1995). These sites are therefore more likely to be involved in the regulation of muscle contraction and relaxation.

Cardiac TnC (cTnC) unlike skeletal TnC (sTnC), has a single functional calcium specific site (i.e. site II). It has been shown that restoration of the calcium binding ability of inactive site I in cTnC by site directed mutagenesis imparts fast skeletal TnC-like properties in terms of cooperativity (i.e. a marked increase in cooperativity of calcium regulation in slow skeletal and cardiac muscles), but cannot fully activate fast skeletal muscle myofibrils (Putkey et al, 1989). Therefore, the lower degree of coupling between
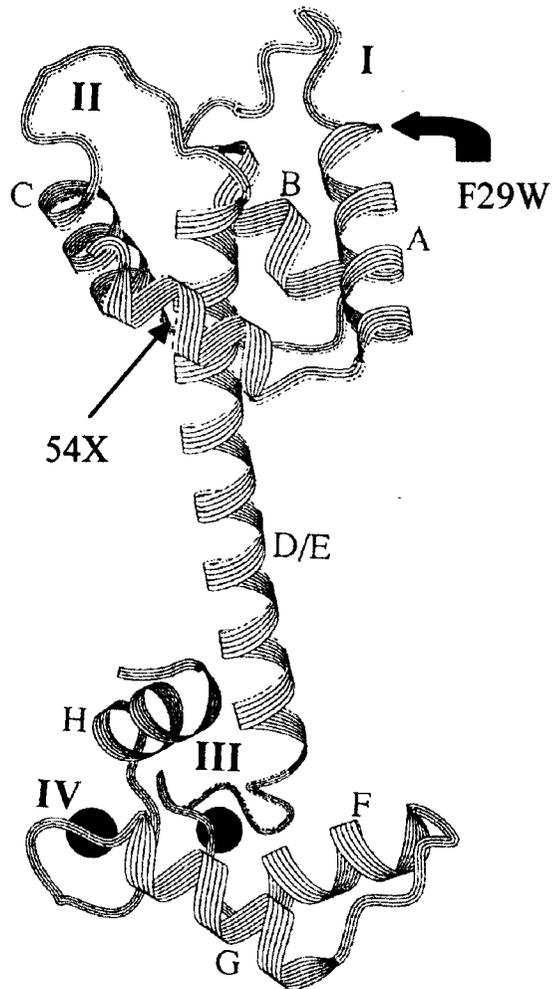
Figure 1. **Ribbon structure of troponin C**

A single molecule of troponin C is represented in this ribbon diagram. The

position of the tryptophan reporter group used in steady state fluorescence

studies (Leblanc et al, 2000) is indicated with the red arrow. The native Thr

residue at position 54, indicated by the black arrow, was substituted with

either a Ser, Val, Ala or Gly residue to generate a range of calcium affinities.

myosin and cTnC is not due to the deficient calcium coordination of site I of cTnC (Morimoto & Ohtsuki, 1996). It has been shown that the reactivation of calcium binding site I of cTnC with the simultaneous disruption of site II results in the inability to regulate muscle contraction (Hannon et al, 1993). The preparation of a chimera in which the first 96 N-terminal cardiac amino acids were combined with the remaining skeletal residues produced a construct that yielded a tension response indistinguishable from that of wild-type cTnC (Akella et al, 1997). This means that calcium binding to site II is an integral part of the function of TnC in regulating muscle contraction.

Using site-directed mutagenesis, a series of mutant skeletal TnC proteins were recently prepared by serial substitution of the N-cap position (figure 1) of the C-helix of chicken skeletal TnC (Leblanc et al, 2000). An alpha helix is, by definition (Aurora & Rose, 1998), a series of consecutive residues in a protein or peptide with backbone dihedral angle approximating $\phi = -60 \pm 15°$ and $\psi = -40 \pm 15°$. The residues that define the boundaries of an alpha helix are known as the N-cap, for the amino terminal cap (Richardson & Richardson, 1988; Harper & Rose, 1993) and C-cap for the carboxyl terminal cap. The N-cap and C-cap are the first and last residues of the series with an N – H(i) C = O(i-4) backbone hydrogen bond. It is now well recognized that the ends of α-helices are stabilized by capping motifs, or capping boxes, that involve the N-cap and/or residues on either side of the N-cap. For example, the N-cap residue frequently contributes, through its side chain, an H-bond to the backbone amide at position N+3 (the third position of the C-terminal side of the N-cap). The amide at this position precedes the first hydrogen bond made through the backbone. In instances where the side chain of the N-cap residue is incapable of hydrogen bonding, the backbone amide at N + 3 must

be satisfied by a hydrogen bond with another residue, with water, or simply left un-bonded. Surveys of protein structure reveal a preference for certain amino acids at the ends of helices. For example, threonine, serine and asparagine are most often the N-cap, because of the side chain hydrogen bonding that these residues offer. Notably, glutamic acid is the most preferred amino acid at positions N + 1, N + 2, and N + 3 where this residue is able to participate in capping motifs and stabilize the macro-dipole of the helix. The apparent preference for certain N-cap residues, observed in surveys of protein structure, is well correlated with the ability of amino acid residues to stabilize helices in proteins, determined by mutagenic substitutions (Serrano et al,1992).

For the purpose of this study five amino acid residues were selected at the N-cap position: the native threonine residue, serine, valine, alanine or glycine, providing a range of calcium affinities of the regulatory domain (Leblanc et al, 2000, Leblanc, 1996) in decreasing order as follows: Ser $\geq$ Thr > Ala > Val > Gly. Extensive biochemical and spectroscopic analyses were made of the five mutants in an earlier study (Leblanc et al, 1996) which made use of a fluorescent reporter group at position 29 (F29W) (Pearlstone et al, 1992). These studies confirmed that the calcium ion affinity was attenuated with a concomitant change in the measured calcium off-rates and calculated on-rates (Leblanc et al, 2000). Far UV Circular Dichroism spectra obtained for each of the mutants in an earlier study (Leblanc, 1996) showed that the 2° structure of all but one of the mutant proteins was essentially the same. The exception, serine, showed a large decrease in the $[\Theta]_{222nm}$ indicating a less frayed helical conformation even in the absence of calcium (figure 2). It was found that a serine residue at the N-cap of the C-helix stabilizes both the calcium-free and the calcium saturated folded states of the protein (Leblanc, 1996).
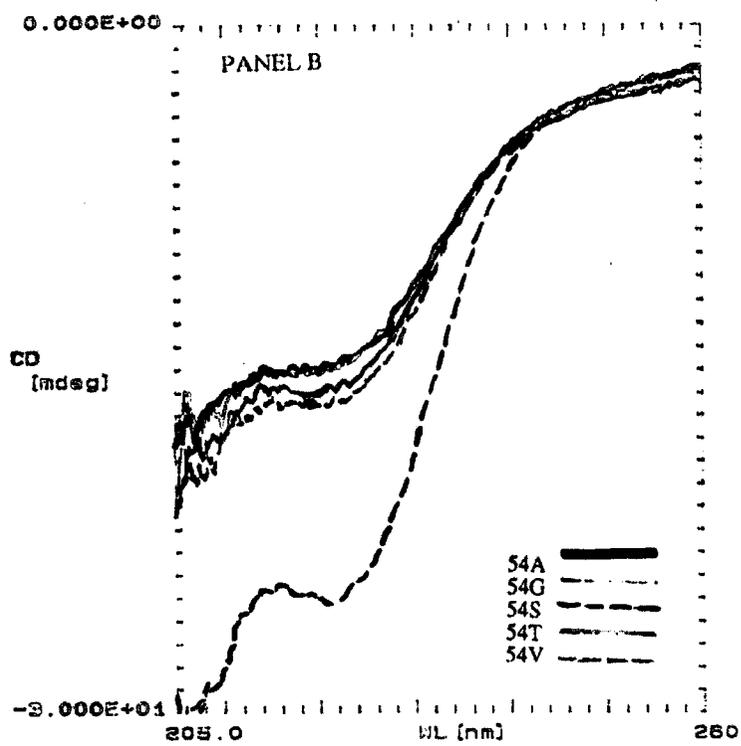
Figure 2. Circular dichroism measurements of the calcium-free mutant TnC proteins in solution.

The secondary structure of the mutant TnC proteins in solution assessed by far UV CD spectroscopy provides evidence for a more tightly folded protein when serine is the n-cap residue of the C-helix, even in the absence of calcium. Image taken from Leblanc, 1996.

In earlier work (Hannon et al, 1993) the inactivation of calcium binding to loop II was found to disrupt thin filament activation, showing that the principle of an all-or-none mechanism of activation of the thin filament was dependent on calcium first binding to TnC. The guiding hypothesis of this study was that the TnC mutants of skeletal TnC, with the attenuation of calcium binding to loop II, would show a change in the maximum tension that in turn related to the level of activation (i.e. the recruitment of thin filament sites available for cross-bridge attachment). A four-state cross-bridge reaction mechanism for skeletal muscle (Regnier et al, 1998) has been proposed, based on the four-state model for cardiac muscle introduced by Landesberg and Sideman (1994). In this model the number of force generating cross-bridges is theorized to depend on the kinetics of calcium binding to TnC. An equilibrium is modeled to exist between two non-force producing cross-bridge states, one without and one with calcium bound to TnC as well as two force-producing states, again one with and one without calcium bound to TnC. The forward rate through the cross-bridge cycle, from a bound low-force state to a force-producing state through to detachment again, would be influenced by the binding affinity of calcium to TnC.

1.1.2 Length Dependent Calcium Sensitivity of Skeletal Muscle Fibres

It is well established that elevating the concentration of calcium within a muscle cell, whether naturally or by bathing chemically skinned fibres in a bath of increasing calcium concentration, increases the level of thin filament activation resulting in the generation of force. The use of skinned fibres allows the experimenter to control the immediate environment of the fibre and vary any one of a number of parameters, singly

or in combination, which might directly alter the relationship between tension and the effective level of activation of the fibre. These include ionic strength (Gordon et al, 1973), pH (Fabiato and Fabiato, 1979; Chase & Kushmerick, 1988), MgATP (Godt, 1974), and ADP (Cooke & Pate, 1985) just to name a few. Mechanical parameters such as sarcomere length (Gordon et al, 1966) and lattice spacing (Moss, 1987) have also been investigated singly or in conjunction with other parameters listed above.

Fluorescence studies of calcium bound skeletal muscle fibres have demonstrated that decreasing the sarcomere length does not decrease the amount of calcium bound to TnC, even though the apparent calcium sensitivity of the fibre decreases (Guth and Potter, 1987). More recently, with the use of rhodamine-labelled TnC it was determined that a change in the sarcomere length and lattice spacing had no effect on the calcium-bound conformation of TnC (Martyn and Gordon, 2001). Contrary to earlier findings (Martyn and Gordon, 1988) these later studies confirmed that the lattice spacing does not alter either the myoplasmic calcium concentration nor the amount of calcium bound to the thin filament of skeletal muscle fibres unlike cardiac muscle where the amount of calcium bound is dependent on the sarcomere length (Fuchs and Wang, 1991; Martyn and Gordon, 2001). This means that cardiac muscle can likely use the change in sarcomere length to fine tune the force exerted by calcium dissociation from the thin filament. This mechanism however is not available to skeletal muscle leaving unanswered the question of how the length dependent calcium sensitivity of these fibres is controlled.

The Hill equation (section 3.2) is used to fit the increase in force with increasing calcium concentration. It describes a cooperative interaction rather than a simple linear

relationship. The Hill coefficient, "n_H", is a measure of the cooperativity of activation along the length of the thin filament. There are several cooperative interactions that contribute to the overall measure of cooperativity. These include the cooperativity of calcium binding to the two calcium-specific regulatory binding sites of TnC. The binding of calcium to TnC at one site enhances the binding of calcium to adjacent sites as well as the interactions between the troponin-tropomyosin units extending along the length of the thin filament in skeletal muscle (Brandt et al, 1987). This was demonstrated with the use of partial extraction of TnC (Moss et al, 1985; Brandt et al, 1987). It was found that n_H decreased by 26% with the extraction of one TnC molecule from each regulatory strand while tension only decreased by 6.6% (Brandt et al, 1987).

Strongly bound cross-bridges may also increase cooperativity by increasing calcium binding to TnC in cardiac muscle (Hofmann & Fuchs, 1987) but there is no evidence that this mechanism exists in skeletal muscle. Strongly attached bridges do however enhance the binding of neighbouring cross-bridges either within a regulatory unit or in an adjacent regulatory unit (7 actin, 1 Tn, 1 Tm). Rigor cross-bridges were even found to activate skinned skeletal fibres in the absence of calcium (Reuben et al, 1971) suggesting that there is an allosteric mechanism enhancing the movement of Tm in adjacent regulatory units without the need for enhanced binding of calcium to TnC.

In more recent studies, the use of N-ethylmaleimide modified S1 (NEM-S1) reduced the cooperative calcium-induced thin filament activation (Swartz & Moss, 2001). NEM-S1 is thought to function by binding with high affinity to actin sites, switching the thin filament into a more fully activated state, so that at low calcium levels (pCa > 6) it increased steady-state tension and reduced the dependence of calcium activation by TnC.

1.1.3 Troponin C as a Switch

TnC is the effecter of calcium activation of the thin filament and its removal decreases both tension and cooperativity between regulatory units, with the reduction in the maximum calcium-induced tension being related to the amount of TnC extracted (Brandt et al, 1990). The cooperative binding of calcium to TnC should affect the rate at which myosin-binding sites become available. These cross-bridges then act in a cooperative manner to propagate the formation of cross-bridges along an activated monomer (i.e. a troponin complex, 7 actin units and its associated tropomyosin) as well as to adjacent monomers (Iwamoto, 1998, Brandt et al, 1997). The factors contributing to cooperative interactions along the length of the fibre, as indicated above, are numerous. The relationship between calcium bound TnC and force is not a linear one (Moss, 1992) and no one factor is expected to be the only control point. Some redundancy would be expected for muscle to continue to function with minor variations in protein structure. More recent studies have suggested that fine-tuning of the TnC calcium off-rate is required for some specialized muscle functions as seen in the toadfish mating call, where the swim bladder vibrates at a rate of 200Hz (Rome et al, 1996).

Bremel and Weber (1972) reported that binding of calcium to the thin filament was increased at low concentrations of ATP. The "rigor" bridge formed at low ATP concentration facilitated calcium binding to TnC. This result implied that under normal physiological conditions there are "reserved" binding sites on the thin filaments; thus it challenges the notion that maximum binding sites are made available when the muscle is fully activated. Seow and Ford (1997) recently showed that the force increase associated with lowering ATP concentration is largely due to increased activation of the muscle and

only a small fraction of the force increase can be attributed to redistribution of the cross-bridges among the attached states. The result is consistent with the hypothesis that the level of activation (i.e. the number of sites on the filaments available for myosin binding) is not at its maximum even when the muscle is maximally activated with calcium. There is therefore room for up- or down-regulation of the level of activation in muscle. Calcium binding to TnC provides a possible mechanism for this regulation. If the presence of strongly bound cross-bridges increases the near neighbour binding of additional cross-bridges to cooperatively increase the level of activation, it is also plausible that a decrease in the number of bound cross-bridges would have a negative effect on the level of thin filament activation.

Early studies using various mutants of skeletal TnC demonstrated that increasing (da Silva et al, 1993) or decreasing (Fujimori et al, 1990) the calcium affinity in solution was translated into an up- or down-regulation in the sensitivity of the skinned fibres. The conclusion drawn is that the calcium induced conformational change in the N-terminal domain of TnC is an integral part of the pathway used to transmit the activating signal from TnC to the acto-myosin interface. It is therefore not unreasonable to speculate that if the calcium affinity of the regulatory binding sites is decreased, an associated loss of near neighbour cooperativity may down-regulate thin filament activation. Is TnC therefore just a simple switch in the process of thin filament activation?

1.2 The Cross-bridge Model of Contraction

Throughout the animal kingdom the basic mechanism of contraction (i.e. movement) involves an interaction of two proteins, actin and myosin, which is more or less precisely

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controlled by the intracellular calcium ion concentration (Ruegg, 1986). While mechanical studies have contributed significantly to our formulation of a model for contraction, the essential basis of the contemporary approach to muscle mechanics, the sliding filament theory, was first proposed on the basis of morphological studies (H.E. Huxley & Hanson, 1954, A.F. Huxley & Niedergerke, 1954). With light microscopy it was shown that when the length of the preparation was altered either passively or by active contraction, the width of the I-band changed, but the width of the A-band remained constant except under conditions of extreme shortening. The conclusion drawn from this, and later confirmed by electron microscopy, was that there are two sets of interdigitating filaments, myosin and actin, that were responsible for tension generation.

In 1970, Huxley and Simmons showed that when an active muscle was suddenly released the tension dropped instantaneously (T_1) and then recovered to an intermediate value in about 2 msec (T_2), followed by a more gradual redevelopment of tension. This suggested an undamped elastic element in series with a damped one. Studies of this quick phase of the series elastic component in single muscle fibres, at different lengths beyond the rest length, revealed that as the length of the muscle was increased and the isometric tetanic tension decreased, the measured instantaneous stiffness also decreased due to a reduction in the total number of cross-bridges formed on activation (Huxley & Simmons, 1971). This suggested that the cross-bridges were regions of relatively high compliance in contracting muscle (Huxley& Simmons, 1971, Clinch and Bressler, 1971, Bressler and Clinch, 1974). A new model was therefore proposed to account for the location and function of the elasticity of the cross-bridges (Huxley & Simmons, 1971). This revised model of the cross-bridge mechanism was based on two major findings:

1) the appearance of the T_1 and T_2 phases of the tension transients and 2) the measured stiffness was tension and length dependent. They had observed that the redevelopment of tension, after quick release (1 msec) in a single muscle fibre, occurred in several stages. The instantaneous drop in tension corresponding to the release was followed by a quick tension recovery and then a more gradual redevelopment. They proposed that a cross-bridge is attached to the myosin backbone by an undamped elastic element and that tension generation is the result of the myosin head attaching to actin and rotating ("the rocking cross-bridge"). This cyclic interaction of myosin cross-bridges with the actin filaments was also later supported with biochemical evidence of several intermediate states in the acto-myosin interaction (Lymm & Taylor, 1971, Eisenberg & Greene, 1980). A diagram of the cross-bridge cycle based on the strain dependent model of Eisenberg and Greene (1980) is shown in figure 3.

1.2.1 Tension Transients

The work by Huxley & Simmons (1971) and Bressler & Clinch, (1971, 1974, 1975) provided direct experimental evidence that the instantaneous stiffness obtained with rapid length changes, in an isometric tetanus, is a measure of the number of active force-producing acto-myosin cross-bridges in contracting skeletal muscle.

Since the number of available cross-bridges is limited by the number of open (i.e. unblocked) acto-myosin binding sites, it was predicted that the mechanical manifestation of activation should correlate to the extent of calcium binding to TnC, which in turn is determined by the molecular structure of the mutant proteins. Titration of the calcium specific sites of the TnC mutant proteins revealed a shift in the pCa_{50} values when

A.M* ⟷ A + M

+ATP ↕ 1   +ATP ↕ 1'

7 ↱↓

A.M*.ADP   A-M.ATP ⟷ M.ATP   2

↕ 6   3' ↕   3 ↕

A.M*.ADP.P$_i$ A-M.ADP.P$_i$ ⟷ M.ADP.P$_i$ 4

5 ↱   4' ↕

                                 M + ADP + P$_i$
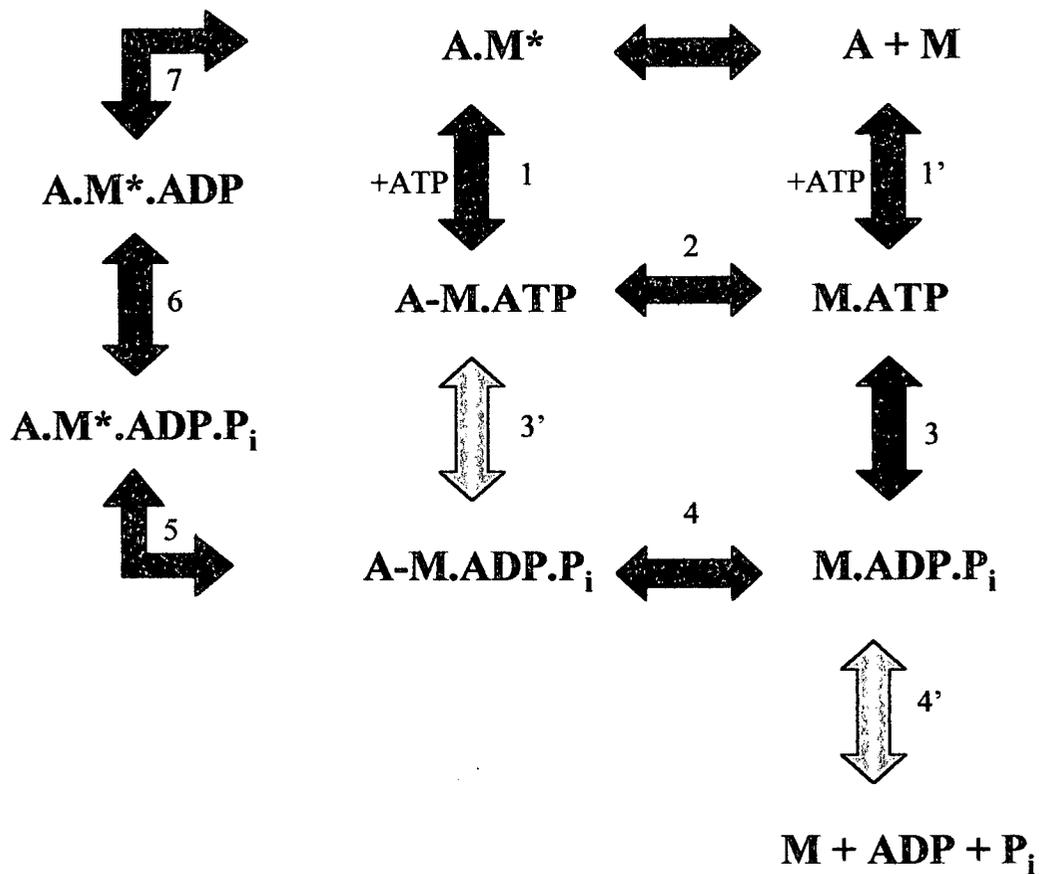
Figure 3. The cross-bridge cycle

This diagrammatic representation of the cross-bridge cycle illustrates the cyclic attachment and detachment of myosin (M) to the actin (A) filament. Steps which are shown with outlined arrows (steps 3'and 4') are included even though they occur at a much slower rate than the comparable steps shown with the filled arrows. The asterix denotes a strongly bound, force producing state. The A-M.ADP.Pi is a weakly bound conformation that is quickly formed after ATP hydrolysis. This cross-bridge state undergoes an isomerization to form a strongly bound state (step 5) from which Pi is released. This transition is a rate-limiting step that is reported to be calcium sensitive.

compared to the wild-type TnC (Leblanc et al, 2000). If an increase in the dissociation constant reflects a change in the equilibrium between the calcium-free and calcium-bound state, then the level of activation conferred on the thin filament might be determined by the association and dissociation rates of calcium to the mutant proteins.

1.2.2 Rate of Force Redevelopment (k_{tr})

Force redevelopment is characterized by following the increase in force that occurs after a period of isotonic shortening with immediate restretch to the original filament overlap where the muscle is then held isometrically (Brenner & Eisenberg, 1986, Brenner, 1988). In early studies of force redevelopment, the half-time to P_o (maximum isometric tension) was observed to be calcium sensitive (Brenner, 1988). This however could be explained by the decreased number of cross-bridges with decreasing [Ca^{2+}], imposing a steric constraint on the rate of force development due to the partial activation of the thin filament. However, when the force development was followed after a simple release step the analysis was further complicated by the fact that the shortening velocity decreased in the isotonic phase of the contraction as the sarcomere length decreased. This was resolved by keeping the sarcomere length constant so that the number of potential cross-bridges would remain unchanged.

The redevelopment of force from zero force maximum isometric tension measures the rate of formation of strongly bound intermediates such that the rate of ATP hydrolysis would limit the rate at which cross-bridges go from the low force state to the high force state. The rate constant, k_{tr}, reflects the isometric cross-bridge turnover kinetics. Relating this to the two-state model of muscle contraction proposed by A.F. Huxley

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(1957), $k_{tr} = f_{app} + g_{app}$. These two rate constants characterize the transitions from the non-force-generating states to the force-generating states and back again to the non-force-generating states (Brenner, 1988).

To measure the rate of force redevelopment (Brenner & Eisenberg, 1986) a maximally activated fibre that has reached steady state tension is allowed to shorten at its maximum velocity by introducing a release of 10-15% of the total fibre length for 10-20 ms (Huxley, 1957). While shortening at maximum velocity, the number of attached cross-bridges in rabbit psoas fibres is only about 20% of the total available (Brenner, 1988). This is followed by a rapid restretch to the fibre's initial length to mechanically dissociate the myosin cross-bridges from actin (Metzger et al, 1989), or at least shift them to the low-force state. If the step is fast enough, the attachment of the cross-bridges followed by the cycling of these same bridges is observed as a single exponential rise from zero force to a maximum force level for the selected experimental conditions. Returning the fibre to its original length avoids the complication of sarcomere length dependent changes (i.e. calcium sensitivity and force). This simplifies the interpretation of the results and allows k_{tr} to be derived by fitting the tension trace to an exponential equation of the form:

$$P = P_{init} + A\exp(-k_{tr}\, t), \qquad\qquad (1)$$

where P is the tension, P_{init} is the initial tension, t is time and A is the amplitude of the redeveloped tension (Brenner, 1988).

In 1991 Metzger and Moss reported that the level of activation of fibres could be altered either by 1) varying the $[Ca^{2+}]_{free}$ or 2) by extracting TnC to various levels while keeping the $[Ca^{2+}]$ constant. A change in the rate appeared to correlate with

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the $[Ca^{2+}]$ suggesting that calcium may directly attenuate a rate constant that limits the formation of a strongly bound cross-bridge state. The decrease in tension due to partial extraction of TnC was attributed to a decrease in the calcium sensitivity of isometric tension with no concomitant change in the rate of force redevelopment. This would indicate that the cross-bridge transition from the weakly bound state to the force-generating state was unaffected by the disruption of the thin filament cooperativity resulting from the partial extraction of TnC. In recent studies using NEM-S1 at low calcium levels, the addition of these strongly attached, non-cycling cross bridges eliminated the calcium dependence of k_{tr} (Swartz & Moss, 2001) providing further evidence that the rate of force redevelopment is calcium regulated by the thin filament regulatory system. The use of NEM-S1 also eliminated the cooperative activation by neighbouring regulatory units not unlike that observed with partial extraction of TnC. Together these studies suggest that the control of the rate of force redevelopment lies within the regulatory unit and not between regulatory units of the thin filament.

Experiments with a modified, calcium insensitive form of cardiac TnC (aTnC) demonstrated that while k_{tr} depended on the level of activation (Chase et al, 1994), it was independent of the calcium concentration. Graded activation in fibres reconstituted with variable ratios of sTnC and cTnC showed a non-linear or curvilinear dependence on calcium. Unlike the earlier observations of Metzger and Moss in 1991, these results indicated that k_{tr} reflects the dynamics of activation of individual thin filament regulatory units and that k_{tr} is modulated by calcium binding to TnC (Chase et al, 1994).

More recently, calmidazolium, a calcium-sensitizing compound that stabilizes the interaction between TnC and calcium, was used to increase the rate of cross-bridge

transition from non-force-bearing to force-bearing states (Regnier et al, 1996). Taken together, these results suggest that k_{tr} is related to calcium activation of the thin filament. This question was recently addressed with the use of an N-helix deletion mutant of TnC exhibiting a decrease in calcium affinity. This N-del mutant of TnC, with reduced calcium affinity, was used to relate the rate of calcium-dissociation from TnC to a decrease in the rate of tension redevelopment (Regnier et al, 1999). As with control fibres, adding calmidazolium did increase k_{tr}, however, increasing the rate of calcium dissociation from TnC did not cause a further decrease in k_{tr}, suggesting a basal level that can only be fine-tuned in one direction. The question that begs to be asked is whether the N-helix mutant is not downregulated due to a change in the interaction with TnI as a result of the missing N-terminal helix, rather than the differential calcium binding properties displayed by the mutant.

Additional studies to investigate steps in the cross-bridge cycle under the control of calcium that might affect k_{tr} have included the addition of an increasing concentrations of orthophosphate (P_i) at both maximal and submaximal calcium concentrations (Metzger and Moss, 1991). The finding that there is an increase in k_{tr} suggests that the calcium sensitivity of k_{tr} involves, at least in part, an effect of calcium on the phosphate release step or alternatively a state transition or isomerization that is in rapid equilibrium with the P_i release step.

1.2.3 Maximum Velocity of Unloaded Shortening (V_{max})

For many muscles, the steady velocity of contraction depends on the load. The velocity, which is at a maximum (V_{max}) when the muscle bears no load, declines toward

zero as the load approaches P_o. A muscle only performs work when it shortens. When exerting its isometric tension, or contracting at its maximum velocity, a muscle does no external work but it continues to utilize chemical energy and hence has zero efficiency.

The maximum shortening velocity is strongly dependent on the rate of cross-bridge dissociation from the thin filaments (Huxley, 1957). According to this model of muscle contraction, V_{max} is highly sensitive to the detachment rate constant (g_2) of strongly bound cross-bridges, and functions to limit the accumulation of negatively strained cross-bridges that oppose contraction. Biochemical studies of the dissociation of ADP from acto-myosin subfragment 1 show a strong correlation between this ADP dissociation constant and the maximum shortening velocity (Siemankowski et al, 1985). This is consistent with the finding that the addition of MgADP to the activating solution causes a marked slowdown of the shortening velocity of skinned fibres (Metzger, 1996). It is generally believed that the myosin binding sites on the thin filaments remain active throughout the period that the muscle is activated. The rate of cross-bridge detachment from the thin filaments therefore depends on factors such as mechanical strain on the cross-bridges or ADP release from the bridges.

In a series of experiments to look at the effect of calcium on V_{max} (Metzger & Moss, 1991), it was reported that the calcium sensitivity of V_{max} was related to the level of thin filament activation which also varied with $[Ca^{2+}]$ which, based on their finding that k_{tr} did not vary with thin filament activation suggested that the mechanisms underlying k_{tr} and V_{max} were different. This is consistent with the idea that the rate of tension development and maximum shortening velocity are rate limited at different points in the cross-bridge cycle (attachment versus detachment) (Potma & Stienen, 1996). V_{max}

has been correlated with the rate of release of ADP from the acto-myosin complex, which is subsequent to the transition(s) hypothesized to limit k_{tr} (i.e. transition to strongly bound states). Martyn et al (1994) however, found that unloaded shortening was not the result of calcium binding to sites other than TnC. They used a calcium independent form of TnC to turn the filament "on" and then varied the calcium concentration. They found that neither the force nor the V_{max} was altered demonstrating that the average level of thin filament activation and V_{max} was affected by calcium binding to TnC only.

Studies which have measured V_{max} using untreated skinned fibres activated at submaximal calcium concentrations or after partial extraction of TnC at maximal calcium concentrations have led to the conclusion that V_{max} is sensitive to the degree of thin filament activation in muscle fibres (Metzger,1996).

In light of our hypothesis that the active state is a dynamic equilibrium between the on- and off-rates of calcium binding to TnC, it is possible that an active binding site can transiently become inactive. This would effectively increase the rate of cross-bridge detachment from the thin filaments and as a result increase the maximum velocity of shortening. The reduced cooperativity of binding may however impede the ability of the thin filament from becoming maximally activated, reducing V_{max}. Since the cooperativity of calcium binding was shown to be dependent on the amino acid substitution at the N-cap of the C-helix (Leblanc et al, 2000) it may be possible to differentiate between the relative importances of these two parameters.

1.2.3.1 The Effect of Phosphate on Shortening Velocity During Maximal and Submaximal Activation

Unloaded shortening velocity (V_{us}) is a mechanical parameter that characterizes the kinetics of the acto-myosin (A-M) interaction during filament sliding. In living muscle fibres, this property, which can be measured using the slack test (Edman, 1979), is found to be proportional to the ATPase rate for the myosin from that muscle type (Barany, 1967). This suggests that the maximum rate at which fibres can shorten depends on the cycling rate of the cross-bridges; more specifically how quickly they can detach and become available for re-attachment.

Recent studies on shortening velocity using chemically skinned fibres have allowed the direct control of the level of activation of a muscle cell by varying the calcium concentration of the bathing solution. At maximally activating calcium concentrations, a plot of the length shortened versus time is fit with a single straight line. However, at submaximal calcium concentrations, the data are best described with a biphasic curve fit with two straight lines of different slopes: an initial high velocity phase (V_{o1}) which reflects the enzymatic ATPase rate followed by a slower phase (V_{o2}) which is dependent on the level of thin filament activation (Moss, 1986). At low calcium levels there are fewer attached cross-bridges with a greater proportion in low force states. The addition of calcium causes more cross-bridges to attach, favoring a relative shift to the high force state and an increase in shortening velocity (Martyn et al, 1994) Adding 10mM P_i to sub-maximally activated skinned fibres also increases the rate of the slower phase so that only one phase, V_{o1}, is identified (Metzger, 1996). It has been proposed that the addition of P_i acts to back up the bridges from a strongly attached force

generating state to a low force state, depressing the force (Eisenberg & Greene, 1980, Martyn & Gordon, 1992). The shifting of the population toward positively strained bridges while decreasing the number of negatively strained bridges, results in an increase of the measured unloaded shortening velocity.

1.3 Hypothesis

The spectroscopic studies of the structure of a series of five mutant proteins of skeletal TnC, undertaken in an earlier study, (Leblanc, 2000) suggested that their conformation in solution was essentially unchanged. Unlike other studies of fibres reconstituted with mutant TnC proteins, such as the N-del mutant of TnC with the N-terminal helix removed (Regnier et al, 1999), it was expected that these five proteins would interact normally with the troponin complex of proteins.

The first objective of this study was to test this prediction in skinned rabbit skeletal muscle fibres reconstituted with each of the chicken skeletal mutant TnC proteins in turn. Does a change in the calcium affinity necessarily predict a change in the calcium sensitivity of the muscle fibre.

Next, the role of TnC in the length dependent calcium sensitivity of skeletal muscle fibres was investigated. In recent studies (Martyn Gordon, 2001) it was shown that unlike cTnC, sTnC does not release calcium as muscle fibres shorten, precluding this as the mechanism for fine tuning the level of force as the sarcomere length of the fibre changes. However, the mechanism of the length dependent calcium sensitivity in skeletal muscle remains unanswered.

Using this series of mutant TnC proteins, with their range of calcium affinities, the question of whether TnC plays a dynamic role in the kinetics of the cross-bridge cycle as hypothesized by Regnier et al, (1998) and Landesberg and Sideman (1994) could be investigated. Since the level of thin filament activation is proposed to influence the rate of force redevelopment (Chase et al, 1994) as well as unloaded shortening velocity (Metzger et al, 1996), this study offered the possibility of determining whether a simple

calcium effect was the rate-limiting factor in the kinetics of each of these steps or whether the calcium binding properties of TnC were important in the regulation of one or more of these steps. In other words, is Troponin C just a simple switch?

2.0 MATERIALS AND METHODS

2.1 Buffers and Solutions

Relaxing Solution: 0.1M KCl
9mM $MgCl_2$
5mM EGTA   + 10 pellets KOH
10mM MOPS
4mM Na_2ATP
pH to 7.0 with KOH
5% dextran

Skinning Solution: 50% vol/vol relaxing solution:glycerol

Activating Solutions: 5mM ATP
3mM Mg^{2+}
15mM phosphocreatine
40mM MOPS
15mM EGTA
133mM Na^+ and K^+
$[Ca^{2+}]$ varied by amount of calcium proprionate added while
maintaining a total concentration of 0.200mM at pH 7.0 and $10^{\circ}C$.
5% dextran

Activating Solutions with phosphate:
5mM ATP
3mM Mg^{2+}
15mM phosphocreatine
40mM MOPS
15mM EGTA
131mM Na^+ and K^+
10mM Phosphate
$[Ca^{2+}]$ varied by amount of calcium proprionate added while
maintaining a total concentration of 0.200mM at pH 7.0 and $10^{\circ}C$.
5% dextran

Extraction Solution: 5mM EDTA
20mM immidazole
0.5mM TFP
pH 7.0

The activating solutions, with and without phosphate were prepared using a computer program described by Martyn and Gordon (1988).

2.1.1 Abbreviations:

ATP	adenosine triphosphate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethelene glycol bis (β-aminoethyl ether) N,N,N',N' -tetraacetic acid
MOPS	3-N-Morpholinopropanesulfonic acid
n_H	Hill coefficient
NEM-S1	N-ethylmaleimide modified S1
nm/hs	nanometers per half sarcomere
PMSF	phenylmethyl sulfonyl fluoride
RR	reconstituted fibre
S1	Myosin Subfragment 1
SL	sarcomere length
TFA	trifluoroacetic acid
Tm	tropomyosin
TnC	troponin C
Tn	troponin complex consisting of troponins C, I and T.
TFP	trifluoperazine hydrochloride

2.2 Preparation and Storage of Rabbit Psoas Fibres

Adult New Zealand white rabbits were euthanized with CO_2. The psoas muscles were removed and stored in relaxing solution for 2 hours at $4^{\circ}C$. Each muscle was then dissected into small bundles and tied onto wooden sticks at *in situ* length and placed in plastic tubes filled with skinning solution. The muscle bundles were stored overnight at $4^{\circ}C$. After approximately 20 hours the tubes were decanted and refilled with fresh skinning solution. The fibres were stored at $-20^{\circ}C$ for up to 6 weeks.

2. 3 Preparation of Fibres for Force-pCa Curves

Single fibres of approximately 6mm in length were isolated from a bundle and mounted by winding the fibre onto 2 hooks attached to an immovable arm and a force transducer so that the final length of the fibre was between 2 to 3mm in length. To prevent slippage, the hooks were prepared by etching them with nitric acid (Moss, 1992).

2.4 Preparation of Fibres for Mechanical Experiments

Single psoas muscle fibres were isolated from a muscle fibre bundle and cut into 2 to 3mm segments. To minimize end compliance, both ends of the segment were chemically fixed with the localized application of a 5% gluteraldehyde solution in glycerol (Chase & Kushmerick, 1988). The ends were then secured in two 1mm T-shaped clips cut from heavy-duty aluminum foil (Ford et al, 1977). The fibre was then mounted on two stainless steel hooks projecting from the servo-motor and force transducer respectively.

2.5 Preparation and Purification of Native Troponin C

The troponin complex was first isolated from rabbit psoas muscle (Potter, 1982) to the point of a pelleted ether extract. The ether extract was then resuspended in a minimal volume of a solution consisting of 6M urea, 1mM EDTA, 50mM Tris and 1mM DTT, at a pH of 8.0. After extracting overnight with constant stirring at 4°C, the solution was centrifuged at 3,500rpm for 20 minutes. The supernatant was then loaded onto a DE52 anion exchange column (Whatman) that was washed until baseline was achieved. The flow through is TnI. Using a salt gradient from 0M to 0.35M NaCl (in the same buffer used to re-suspend the ether pellet) and a gradient volume of 6 to 10X the total column volume, the TnT and TnC were eluted. The TnT was recovered first (between 0 and 0.20M NaCl) followed by the TnC between 0.25M and 0.30 M NaCl. The fractions were identified using SDS PAGE and purified Tn subunits graciously provided by Dr. Donald A. Martyn at the University of Washington in Seattle, Washington, U.S.A.. The TnC fractions were then desalted by gel filtration on a G25 column (Pharmacia) with 0.1% trifluoroacetic acid (TFA) in ddH$_2$O. If any impurities appeared on the gels, the TnC was purified further using FPLC and a Q-sepharose column. Alternatively, S-sepharose could be used to purify away any impurities at pH 4.0.

2.6 Preparation of Chicken Skeletal Troponin C Mutant Proteins

All of the mutant proteins used in this study were prepared in Dr. Thor Borgford's lab at Simon Fraser University. The preparation of the clones as the pLcII-Fx-TnC fusion vector is described elsewhere (Leblanc et al, 2000). The proteins were expressed by inoculation a 2 mL culture with a single colony from a 36 hour plate of QY13

transformed with the plasmid DNA of one of the clones. After a 12 hour incubation at 30°C, a 1 mL aliquot was drawn from the culture and heat shocked in a 42°C water bath for 10 minutes followed by a 2 hour incubation at 37°C in a shaker incubator. The cells were then harvested by centrifugation at 15,000 rpm for 10 minutes and re-suspended in 50μL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) with 1 μL of 0.5 PMSF. The cells were lysed and the supernatant analyzed by SDS PAGE. The remaining 1 mL of the culture was used to inoculate 200mL of 2xTY-AMP broth which was incubated at 30°C to an $O.D._{600}$ of 0.5. The culture was then heat shocked as above and incubated at 37°C for 4 hours after which time the culture was cooled on ice and centrifuged to collect the cell pellet. The pellet was stored at –60°C. Lysis of the pellet followed by the purification of the mutant TnC fusion proteins expressed from pLcII-Fx-TnC gene is described in detail elsewhere (Trigo-Gonzalez et al, 1992). The purification of the fusion protein was verified using SDS page. Digestion with Factor X_a removed the λcII protein connected to the recombinant TnC protein through the Factor X_a tetrapeptide recognition sequence. The TnC protein was purified using anion exchange chromatography on a Pharmacia FPLC system equipped with a Mono Q 10/10 column.

2.7 Equipment for Mechanical Experiments

A Cambridge Technology Model 308 servo motor modified with a lighter aluminum arm to achieve a step duration of 160μs for steps as large as 100μm, was used for all mechanical measurements. Two different models of force transducer were used in the course of the mechanical experiments. One was a modified Akers AE801 with a silicon beam that had been shortened by 1mm to increase the sensitivity to 40mV/mg and

a measured resonance frequency of 24kHz with a signal to noise ratio of 20. The second force transducer was a photoelectric type force transducer with a resonant frequency of 12kHz, a sensitivity of 10mV/mg and a signal to noise ratio of about 20 (Seow and Ford, 1997).

An automated data acquisition program written using Microsoft Visual Studio and Component Works (Measurement Studio, National Instruments) to control a NiDAQ multifunction input/output board (PC16035E, National Instruments) simultaneously controlled the servo motor while collecting data from the transducer in 10□s increments.

A milled aluminum plate mounted on the stage of an inverted microscope (Nikon) held the servo-motor and force transducer which were mounted on adjustable positioners to allow for movement in both the vertical and horizontal directions. The activation of the fibres was accomplished by raising and lowering the assembly, consisting of the motor, force transducer and fibre, into a series of plexiglass wells containing about 1.3mL of solution. A video camera mounted to the microscope was used to display the fibre on a computer screen. The measurement of the sarcomere spacing was performed in real time with the ImageProPlus software program as described in more detail below.

2.8 Extraction of Troponin C from Rabbit Fibres

The native and mutant TnC proteins were extracted from the fibres by soaking the fibres mounted on the experimental apparatus in an extraction solution of 0.5mM trifluoperazine (TFP) which removes the calcium from TnC thereby destabilizing its attachment to the thin filament (Metzger et al, 1989, Metzger & Moss, 1991). To optimize the removal of TnC while minimizing the possibility of fibre damage, fibres

were treated a maximum of 10 minutes, in two 5-minute increments. Since the procedure was carried out at 10°C, and TFP precipitates out of solution below room temperature, the TFP solution was exchanged after 5 minutes. TnC was extracted to greater than 95% by this method while reducing the exposure of the fibre to TFP. The effective extraction fraction was immediately confirmed by measuring the maximum tension achieved by the fibre in an activating solution of pCa 4.0 (see 2.1).

2.9 Basic Experimental Protocol

The untreated skinned fibre was mounted onto the hooks of the motor and transducer and was tested prior to extraction of the native TnC. A "real time" image of the fibre was viewed on a computer screen using an imaging software package (Image Pro Plus). A calibrated cursor drawn down the length of the acquired image of the fibre measured the mean value for the sarcomere length. Micromanipulators on the motor and transducer mounts allowed the fibre to be lengthened or shortened, as required, to set the desired sarcomere length. The fibre was then maximally activated in an activating solution of pCa 4.0. This provided a control value of the maximum force for each untreated fibre. Once the plateau of force was achieved, the fibre was placed in a 1nM $[Ca^{2+}]$ activating solution (pCa 9.0). The sarcomere length was re-measured. If the sarcomere length deviated by more than 5%, the entire procedure was repeated a second time. If the sarcomere length deviated again the fibre was discarded. Otherwise the sarcomere length was adjusted to 2.6µm before proceeding with the next step in the protocol detailed below.

A 0.5mM solution of TFP was used to completely extract the native TnC protein. Fibres with a level of activation below 5% of the untreated fibre were subsequently used. Reconstitution with a mutant TnC or with native rabbit TnC was achieved with four 30-second washes using a 1mg/mL solution of protein alternating with 1 minute washes in pCa 9.0 solution. The fibre was re-tested in an activating solution at pCa 4.0. Experiments that involved a single activation after reconstitution with the mutant TnC, were re-extracted and reconstituted with native rabbit TnC isolated from rabbit tissue. The maximum force was measured and compared to the maximum force measured for the untreated control fibre.

2.9.1 Force-pCa Curves

Force-pCa data were collected with fibres mounted as described above, by moving the fibre into consecutive baths with increasing calcium concentrations starting from pCa 9.0 and increasing to pCa 4.0. Traces were collected on a Tektronix TDS 420 four channel digitizing oscilloscope and a printed copy was obtained. Using the Image Pro Plus software the sarcomere length of the fibre was set at either 2.0, 2.6 or 3.0µm. Two protocols outlined below were used to acquire the force-pCa data of fibres reconstituted with the glycine mutant of TnC. A statistical analysis of the results demonstrated that the simpler protocol (b) could be used for the remaining mutant proteins.

a) Dissected fibres were cut into two pieces using one half as the control fibre while reconstituting the second half with the glycine mutant of TnC. Force-pCa curves at each sarcomere length were averaged.

b) A force-pCa trace was first collected with a control fibre after which the native TnC was extracted from the same fibre with TFP. Force was measured at pCa 4.0. If the force was less than 5% of the untreated fibre, it was reconstituted with one of the mutant TnC proteins and a second trace force-pCa trace was collected.

2.9.2 Mechanical Experiments

2.9.2.1 Tension Transients

A small, rapid length step completed in $160\mu s$ was applied at the plateau of a contraction. The corresponding tension response, the tension transient, after a small rapid length step applied at the plateau of contraction, was recorded (figure 4) and measurements of the T_1 (i.e. extreme tension reached at the end of the step) were made (Huxley & Simmons, 1972). The ratio of T_1 versus the step size was determined to obtain an estimate of the fibre stiffness. It has been shown (Bagni el al., 1990, Huxley et al, 1994, Wakabayashi et al, 1994) that the cross-bridges are not the only source of compliance (inverse of stiffness) in a fibre, with the non-overlapped portion of the thin filaments contributing significantly to fibre compliance. Fibre length was held constant under both control and test condition so as to eliminate the length dependent stiffness variation.
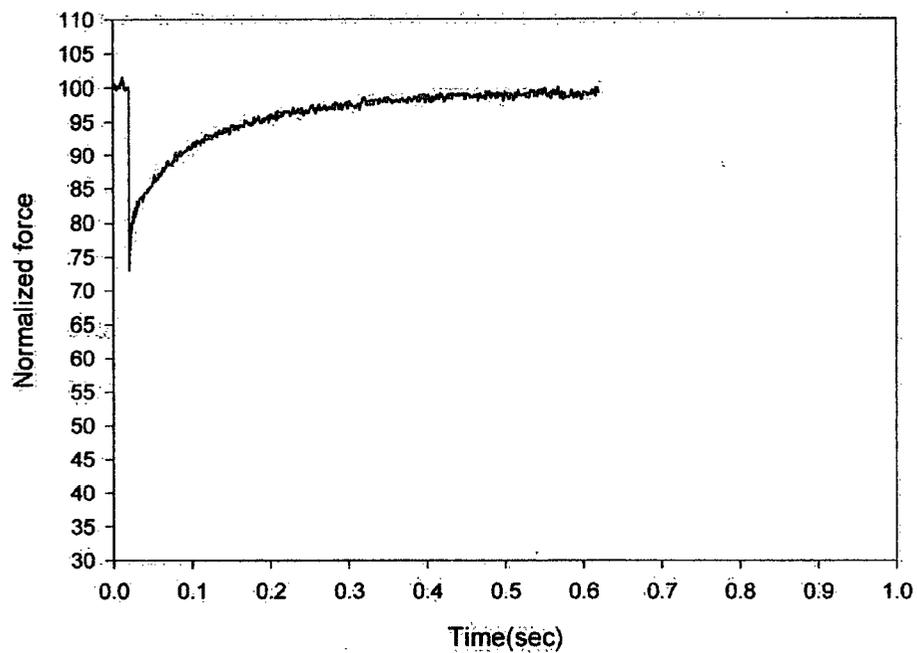
Figure 4. Average tension transient after a small release step.

An average tension transient (n=20) using skinned rabbit psoas fibres, after

a small, rapid release step of 8nm/hs, is shown. Force was normalized to

maximum tension prior to averaging. Measurements were made at 10°C.

2.9.2.2 Rate of Force Redevelopment (k_{tr})

Prior to activation of the fibres at pCa 4.0, the sarcomere length was set at 2.6μm. At the plateau of tension development, a large release step of 200nm/hs was introduced followed by a re-stretch to the original fibre length after 20msec. The large release with restretch has the effect of reducing the isometric tension to near zero by detaching most of the strongly bound cross-bridges and by forcing those that remain attached into the weakly bound state (Brenner, 1988). The rate at which force redeveloped towards the original isometric level was then recorded and normalized. In this study, sarcomere length control was not used, therefore the rate constant will be calculated from the time to redevelop one-half of the maximum force ($t_{1/2}$) (Chase et al, 1994). Based on the recent findings of Brenner (1998) and Martyn and Gordon (2001) it can be assumed that the release and restretch does not affect thin filament activation of skeletal muscle fibres.

2.9.2.3 Maximum Velocity of Unloaded Shortening (V_{max})

The slack test described by Edman (1979) was used to measure the unloaded shortening velocity of both the untreated fibre and after reconstitution of the TnC extracted fibre with a mutant TnC protein. The fibres were activated in pCa 4.0 solution, either with or without 10mM phosphate. A series of 9 different step releases ranging from 100nm/hs to 300nm/hs in 25nm/hs increments were carried out, followed by a re-stretch to the original length, 200 to 600msec after the release. The step sizes were imposed randomly. The slack time was taken as the time between the end of the length step and the time at which the force began to redevelop. The latter time point was chosen as the intersection point between the force baseline and the least-squares regression of the

initial linear rise of force (Martyn et al, 1994). The force baseline was determined by a large amplitude release that caused the fibre to become slack. Three to six recordings were carried out at each amplitude of release (ΔL). The data was pooled, averaged and plotted against the slack time (Δt). The data was fit with a regression line using the least-squares method, with the slope of the line providing a measure of the unloaded shortening velocity.

3.0 RESULTS

3.1 Maximum Tension at pCa 4.0

The maximum force of a single skinned fibre was measured. Following the extraction and reconstitution protocol outlined in the methods, the maximum force at pCa 4.0 was measured. Fibres were rejected if the force after extraction was greater than 5% of the initial force. Using Sigma plot the mean force value $\pm$ the standard error for each mutant protein was calculated (Table 1). The tension ranged from $0.59P_o$ to 0.85 P_o with P_o being the maximum force of the control fibres. In all experiments, the control refers to the untreated fibres with native rabbit TnC.

Re-extraction of the mutant TnC and reconstitution with the native rabbit TnC resulted in an increase in force to the original force level observed with the untreated fibre as seen in figure 5. This protocol verified that the extraction process had not damaged the fibre and that the force measured with the mutant TnC was not an artifact of the extraction protocol. The range in force values observed with the mutant TnC confirms that the drop in force cannot be attributed to the reporter group (F29W) inserted into the sequence to study the calcium-binding properties of the proteins.

Table 1. Relative force of fibres reconstituted with mutant troponinC at pCa 4.0

Mutant Protein of Troponin C	P/P_o
T54S	0.85 ± 0.017
T54	0.77 ± 0.041
T54V	0.71 ± 0.021
T54A	0.69 ± 0.020
T54G	0.59 ± 0.015

P/P_o represents the ratio of the average force of a fibre reconstituted with a mutant TnC relative to the maximum tension of the control fibres.
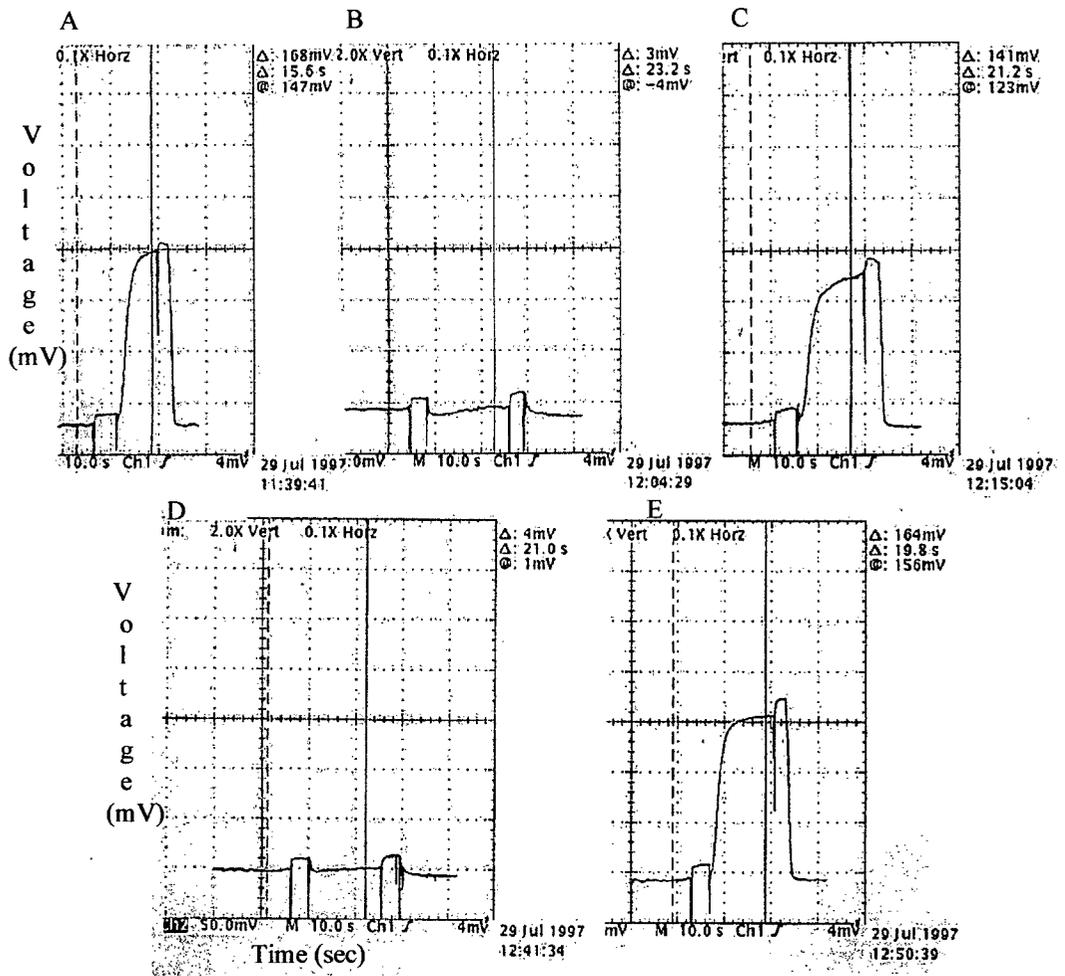
Figure 5. The extraction protocol of single skinned rabbit psoas fibres

The maximum force at pCa 4.4 (A) was measured prior to extraction of the native TnC (B).

Following reconstitution with the mutant TnC, force was measured (C). Re-extraction of the

mutant TnC (D) followed by reconstitution with the native rabbit skeletal TnC (E) confirms

that the change in force is the result of the mutant TnC. Traces were printed from the oscillo-

scope. The vertical scale records the force signal from the transducer as a voltage (mV). The

horizontal scale is time with each division representing 10 seconds.

3.2 Force-pCa Curve Analysis

Single skinned skeletal rabbit psoas fibres were mounted between two hooks, one of which was attached to a force transducer. The sarcomere length of the fibre was set to 2.2 μm while it was kept in a relaxing solution at pCa 9.0, with 5% dextran (T70). The fibre was then placed in a series of solutions of increasing calcium concentration while a trace of the increase in tension was recorded.

The sarcomere length was incrementally increased to provide a record of each fibre at 2.2, 2.6 and 3.0 μm. The force-pCa curve was then analyzed using the following form of the Hill equation which assumes that pCa = $-\log_{10}[Ca^{2+}]$,

$$F = \frac{F_o}{[1+ 10^{n(pCa_{1/2} - pCa)}]} . \qquad (2)$$

where F is the measured force at each calcium concentration used, F_o is the maximum measured force, pCa is defined above, and $pCa_{1/2}$ is the pCa_{50} value which is the midpoint of the titration where the fibre displays 50% of the maximum force (Gordon et al, 2000). The equation is used to fit a line to the data points showing the change in force with increasing calcium concentration.

The extraction and reconstitution protocol in the acquisition of force-pCa curves is demonstrated in figure 6. The graph summarizes the findings of an average of 4 fibres, from which native TnC had been extracted by 98%, followed by reconstitution with purified native rabbit TnC (figure 7). The successive force-pCa curves at a sarcomere length of 2.6μm followed by 3.0μm demonstrate that any changes seen with the mutant TnC proteins can be interpreted as originating with the mutant protein and not the extraction and reconstitution protocol.
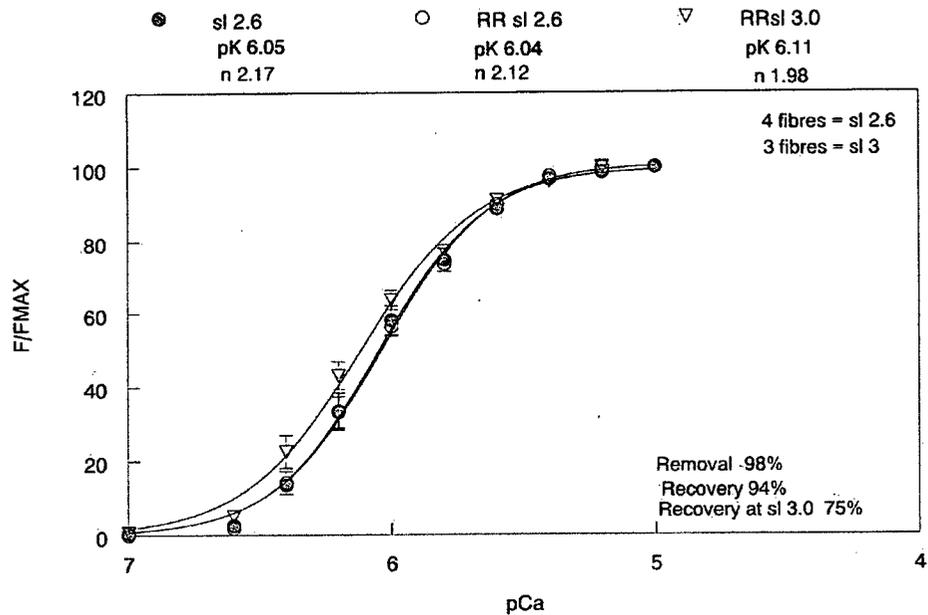
Figure 6. Reconstitution of TnC extracted fibres with native rabbit TnC

Fibres were extracted of native TnC and reconstituted (RR)with purified native TnC to

test the extraction and reconstitution protocols. Standard error bars are shown. Force-

pCa curves were obtained at a sarcomere length (SL) of 2.6μm both before extraction

and after reconstitution. The sarcomere length was then increased to 3.0μm to test the

integrity of the length dependent calcium sensitivity of the reconstituted fibres. The

"n" value represents the Hill coefficient of the curve fitted to the data using a modified

Hill plot (equation 2).
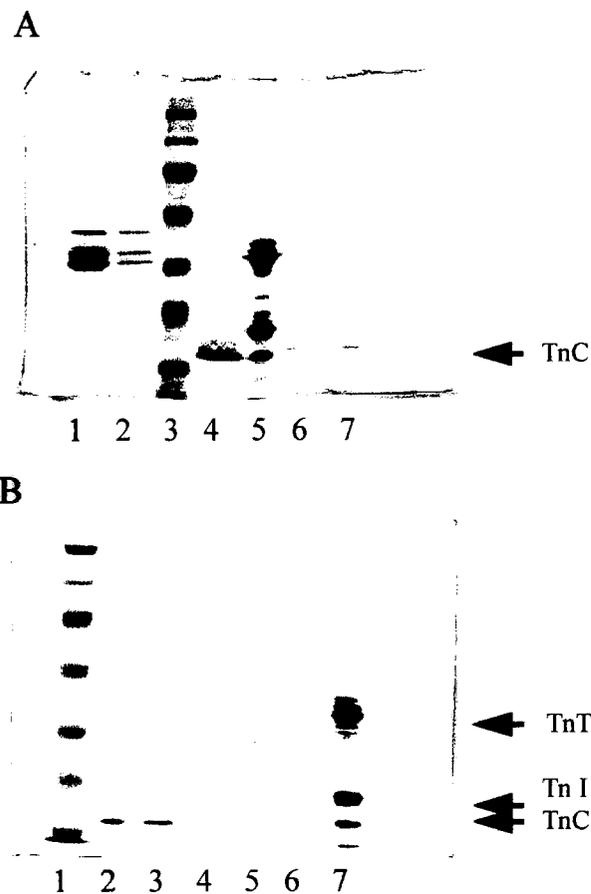
Figure 7. SDS PAGE analysis of the purification of native TnC.

Panel A: Initial purification of native TnC from the ether extract of the skeletal muscle tissue

(Potter, 1982). Fractions were eluted from a DE52 (Whatman) chromatography column with

6M urea, 50mM Tris, (pH 8.0), 1mM EDTA, 1mM DTT using an NaCl gradeint from 0 to

0.5M. TnC eluted between 0.25M and 0.30M NaCl. Columns 1 and 2, tropomyosin fraction;

column 3, molecular weight (MW) marker; columns 4, 6 and 7, eluted native TnC fractions;

column 5, troponin standard.

Panel B: A G25 column was used to desalt the TnC fractions shown above. Column 1,

MW marker; column 2 and 3, purified native TnC fractions; column 7, troponin standard.

The successful extraction of the native rabbit TnC protein and the reconstitution with a mutant TnC protein was also confirmed by SDS PAGE analysis (figure 8) of fibres run on a 0.5 mm thick gel and stained with Silver Stain Plus (Biorad).

A plot of the average force-pCa curve is shown in figures 9 and 10 with a tabulated summary of the mean pCa_{50} values and the average Hill coefficients (n_H) (Table 2 and 3). The glycine mutant is presented separately (figure 10), since it was analyzed using fibres from a second rabbit using 2 different experimental methods for the selection of fibres for reconstitution: paired versus unpaired fibres. The error bars in figures 9 and 10 represent the standard error at each pCa value.
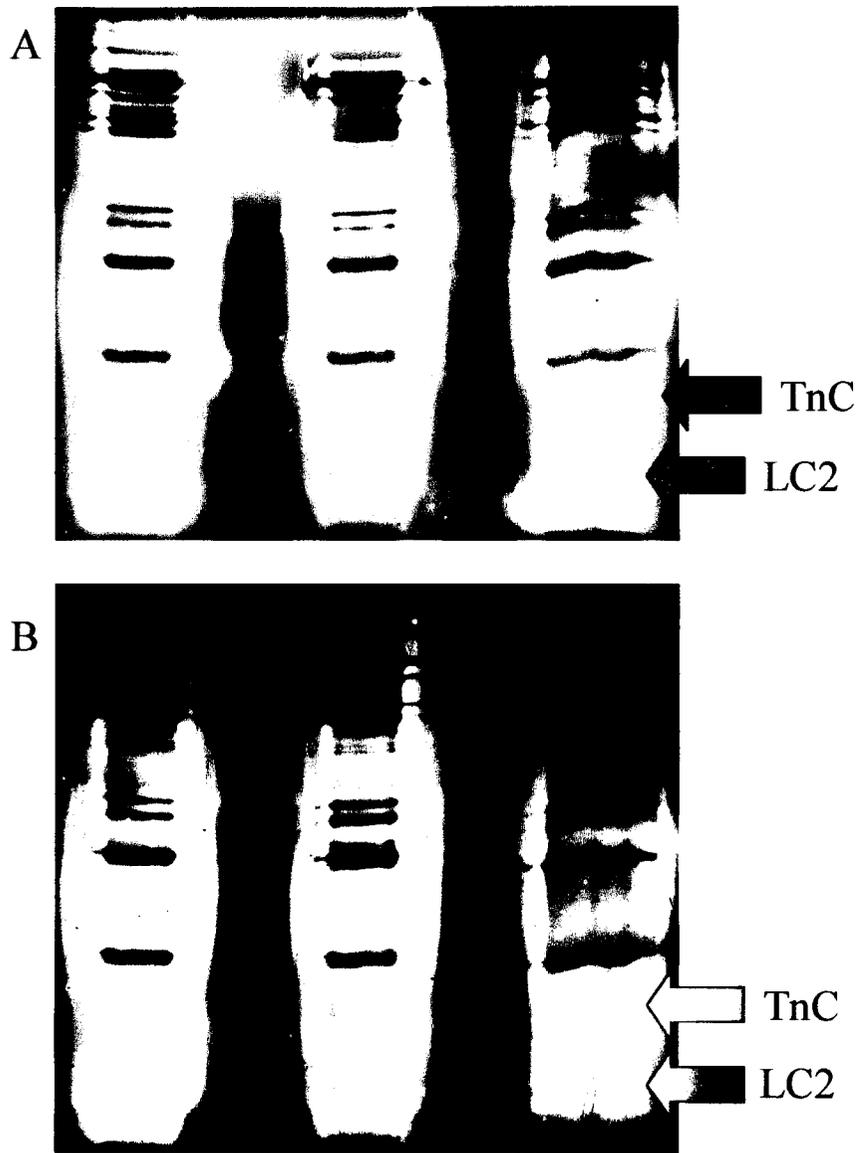
Figure 8. Gel electrophoresis of single fibres

SDS PAGE of 1.5mm segments of rabbit psoas muscle fibres run in every second lane on
a 0.5mm thick gel, stained with Silver Stain Plus (Biorad).

Panel A and B: Lane 1: TnC extraction with 0.5mM trifluoperazine; Lane 3: Control fibre;

Lane 5: Fibre reconstituted with valine mutant of TnC. Panel A is a lighter exposure to

verify the presence of the upper molecular weight bands. The darker exposure in B allows

the visualization of TnC and LC2.

Control Force-pCa Curves

SL 2.2	SL 2.6	SL 3.0
pK 5.96+.03	pK 6.05+.02	pK 6.08+.01
n 4.18+0.16	n 3.14+0.13	n 2.52+0.12

Serine Mutant of TnC
Force-pCa Curves

Sl 2.2	SL 2.6	SL 3.0
pK 5.92+.01	pK 5.92+.02	pK 5.93+.02
n 2.62+0.07	n 2.24+0.04	n 1.78+0.05

Valine Mutant of TnC
Force-pCa Curves

SL 2.2	SL 2.6	SL 3.0
pK 5.83+.05	pK 5.90+.05	pK 5.93+.02
n 3.07+0.07	n 2.42+0.06	n 2.18+0.09

Threonine Mutant of TnC
Force-pCa Curves

SL 2.2	SL 2.6	SL 3.0
pK 5.78+.02	pK 5.90+.02	pK 5.97+.01
n 2.95+0.08	n 2.49+0.09	n 2.05+0.09

Alanine Mutant of TnC
Average Force-pCa curve

SL 2.2	SL 2.6	SL 3.0
pK 5.68+.03	pK 5.76+.02	pK 5.83+.02
n 2.32+0.07	n 1.95+0.05	n 1.86+0.05

Figure 9. Force-pCa curves of fibres reconstituted with the serine, threonine, valine or alanine mutants of TnC at sarcomere lengths of 2.2, 2.6 and 3.0μm. Closed circles, 2.2μm; red triangles, 2.6μm; inverted green triangles, 3.0μm. Error bars represent standard error at each pCa value. The Hill coefficient printed at the top of each graph is the value fit to the average curve shown in each panel.

A

SL 2.2µm
pK 5.92+.003
n 4.34+0.12

SL 2.6µm
pK 6.03+.007
n 3.41+0.18

SL 3.0µm
pK 6.10+.01
n 2.80+0.18

Relative force

100

80

60

40

20

0

7   6   5   4

SL 2.2µm
pK 5.66+0.06
n 2.37+0.31

SL 2.6µm
pK 5.81+0.02
n 2.50+0.1

SL 3.0µm
pK 5.91+0.02
n 2.12+0.1

B

Relative force

100

80

60

40

20

0

7   6   5   4

pCa
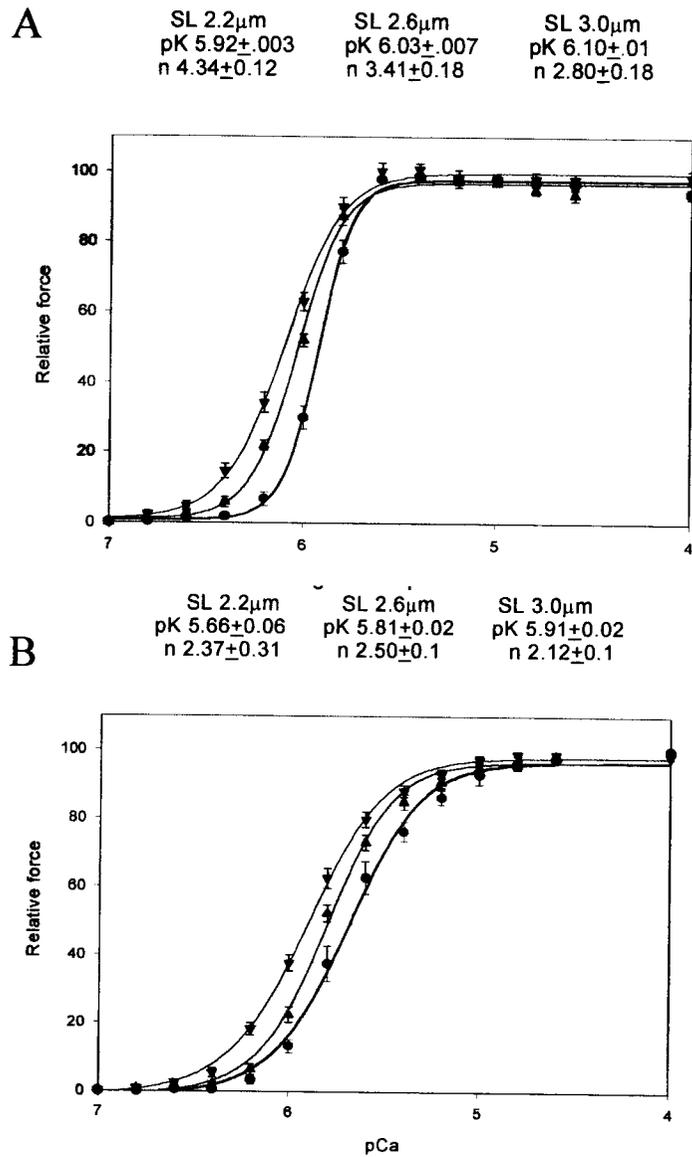
Figure 10. Force-pCa curves of fibres reconstituted with the glycine mutant of

TnC at sarcomere lengths of 2.2, 2.6 and 3.0µm.

Panel A: Average control curves. Panel B: Average glycine curves.

Closed circles, SL 2.2µm; red triangles, SL 2.6µm; inverted green triangles, SL 3.0µm.

Error bars represent standard error at each pCa value.

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Table 2. Force-pCa analysis of fibres reconstituted with the serine, threonine, valine and alanine mutants of TnC

TnC protein	SL 2.2 μm		SL 2.6 μm		SL 3.0 μm	
	pCa_{50}	n_H	pCa_{50}	n_H	pCa_{50}	n_H
Native TnC	5.96 ± 0.03	4.29 ± 0.22	6.05 ± 0.02	3.22 ± 0.12	6.08 ± 0.01	2.55 ± 0.15
T54S	5.92 ± 0.01‡	2.68 ± 0.25	5.92 ± 0.02	2.27 ± 0.13	5.93 ± 0.02	1.82 ± 0.21
T54	5.78 ± 0.02	2.99 ± 0.19	5.90 ± 0.02	2.52 ± 0.15	5.97 ± 0.01	2.13 ± 0.31‡
T54V	5.83 ± 0.05	3.45 ± 0.28	5.90 ± 0.05	2.75 ± 0.16	5.93 ± 0.02	2.19 ± 0.11‡
T54A	5.68 ± 0.03	2.42 ± 0.20	5.76 ± 0.02	1.97 ± 0.11	5.83 ± 0.02	1.87 ± 0.03

‡ Not statistically different from control fibres with native TnC using an Anova test with $p > .05$.

Table 3. Force-pCa analysis of fibres reconstituted with the glycine mutant of TnC

TnC protein	SL 2.2 μm		SL 2.6 μm		SL 3.0 μm	
	pCa_{50}	n_H	pCa_{50}	n_H	pCa_{50}	n_H
Native TnC	5.92 ± 0.03	4.34 ± 0.12	6.03 ± 0.01	3.41 ± 0.18	6.10 ± 0.01	2.80 ± 0.18
T54G	5.69 ± 0.01	2.26 ± 0.15‡	5.81 ± 0.01	2.33 ± 0.13	5.91 ± 0.03	2.09 ± 0.07

‡ The difference in the Hill coefficient at a sarcomere length of 2.2μm is not statistically different from the value at 2.6μm using an Anova test ($p > .05$).

A statistical analysis of the data using the method of analysis of variance (ANOVA) followed by a Post Hoc test where applicable (Newman Keuls test) showed there was no significant difference between the data collected from single fibres cut into 2 pieces (control and experimental) versus data collected from unpaired fibres (i.e. fibres randomly selected as control or experimental). Since there was no difference (Appendix 3), the simpler of the two methods, using unpaired fibres, was used for the remaining four

mutant proteins. The change in calcium sensitivity observed with the reconstitution of each variant TnC, is tabulated in table 4 below to allow the direct comparison of the ΔpCa_{50} values of all five mutant proteins, at each sarcomere length.

Table 4. Change in the length dependent calcium sensitivity of fibres reconstituted with the mutant TnC proteins relative to control fibres

TnC protein	SL 2.2µm ΔpCa_{50}	SL 2.6µm ΔpCa_{50}	SL 3.0µm ΔpCa_{50}
T54S	0.04‡	0.13	0.15
T54	0.18	0.15	0.15
T54V	0.13	0.15	0.15
T54A	0.28	0.29	0.25
T54G	0.26	0.22	0.19

All tabulated values represent a rightward shift to higher calcium concentrations indicating an overall decrease in calcium sensitivity of the fibres with respect to the native rabbit TnC with the exception of serine at 2.2µm. ‡ The ΔpCa_{50} between the control fibre and the serine mutant at a sarcomere length of 2.2µm is not statistically significant as determined by analysis of variance, with p > .05.

In general, increasing the sarcomere length of the fibre, whether control or reconstituted, resulted in an increase in calcium sensitivity accompanied by a decrease in cooperativity as indicated by the decreasing Hill coefficient (n_H). There was, however, one notable exception to this observation. The calcium sensitivity of fibres reconstituted with the serine mutant remained unchanged at all three sarcomere lengths. The fibres generally demonstrated the expected decrease in cooperativity with increasing sarcomere length so that fibres reconstituted with the remaining TnC variants retained their

increasing length dependent calcium sensitivity with a concomitant decrease in cooperativity as the sarcomere length increased.

The measure of cooperativity (n) or the Hill coefficients (n_H) as calculated with equation 2, for fibres reconstituted with the mutant TnC proteins were obviously different from the values calculated for the control fibres at a sarcomere length of 2.2μm and 2.6μm. However, at a sarcomere length of 3.0μm, only the serine and alanine mutants showed a significant decrease in cooperativity when compared to the control fibres (table 2).

The method of analysis of variance was used to compare and contrast the five mutant proteins in terms of their effect on length dependent cooperativity. Comparing the fibres reconstituted with the mutant proteins, the cooperativity decreased as the fibres were stretched from a sarcomere length of 2.2μm to 3.0μm with the exception of threonine, which showed no change in cooperativity with the change in sarcomere length. Comparing all of the mutant proteins at a single sarcomere length revealed very little difference between the mutants with the exception of alanine. A statistically significant difference between the alanine and valine mutants at 2.2μm and 2.6μm, and between alanine and threonine, as well as alanine and glycine at 2.6μm was noted. There was no significant difference between any of the mutants at a sarcomere length of 3.0μm.

The change in pCa_{50} values observed as the sarcomere length increased (table 5) clearly indicate that while there was some variability in the measured length dependent calcium sensitivity with each mutant, the total changes were similar with the exception of the serine mutant. The pCa_{50} value for serine remained the same as the sarcomere length was increased from 2.2 to 3.0μm.

Table 5. Change in the average length dependent calcium sensitivity with increasing sarcomere length of fibres reconstituted with the mutant TnC proteins

TnC protein	SL2.2 to 2.6μm ΔpCa_{50}	SL 2.6 to 3.0μm ΔpCa_{50}	Total Change ΔpCa_{50}
Native	0.09	0.03	0.12
T54S	0.00	0.01	0.01
T54	0.12	0.07	0.19
T54V	0.07	0.03	0.10
T54A	0.08	0.07	0.15
T54G	0.12	0.10	0.22

Similarly, the degree of cooperativity of activation along the length of the thin filament was compared (table 6). It was apparent that while calcium sensitivity was impaired with the serine substitution, the difference in the length dependent cooperativity was unremarkable once the fibres were stretched to a sarcomere length of 3.0μm.

Table 6. Change in the average length dependent cooperativity with sarcomere length

TnC protein	SL 2.2 to 2.6μm Δn	SL 2.6 to 3.0μm Δn	Total Change Δn
Native	-1.04	-0.62	-1.66
T54S	-0.38	-0.46	-0.84
T54	-0.65	-0.24	-0.89
T54V	-0.46	-0.44	-0.90
T54A	-0.37	-0.09	-0.46
T54G	+0.13	-0.38	-0.25

In figure 11a, b and c, the five mutants are presented in 3 panels, each representing the averaged data at a single sarcomere length. The control data (ie. from
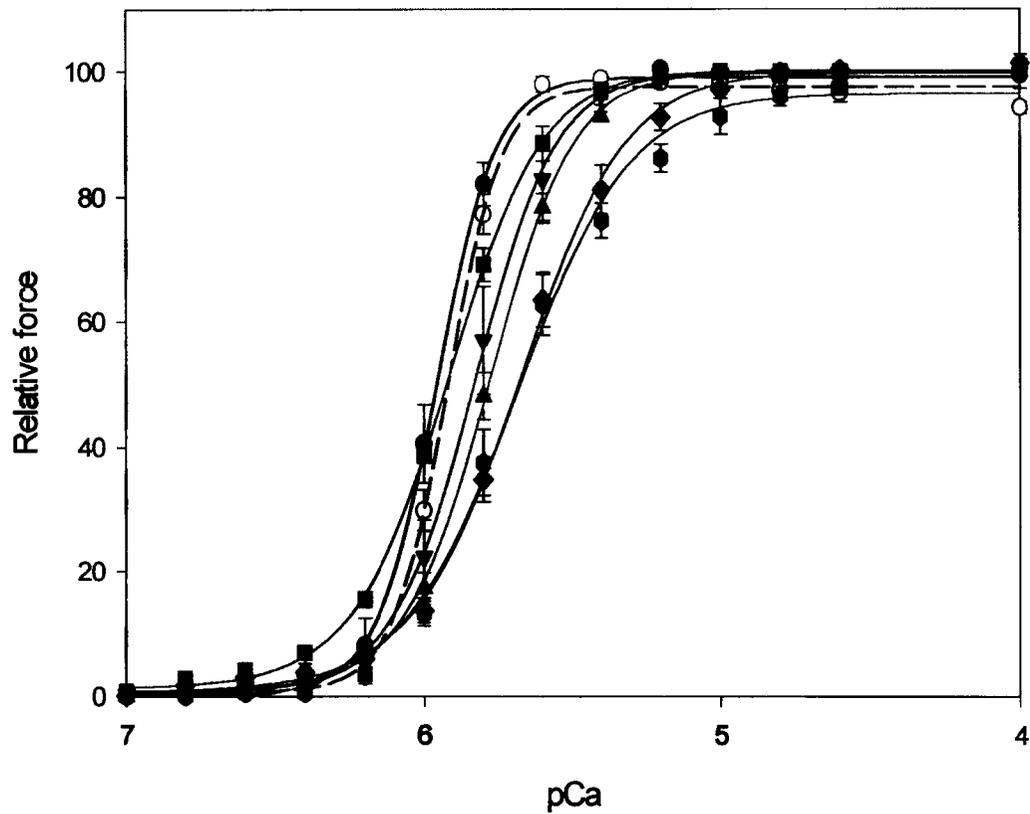
Figure 11A. Comparison of force-pCa curves at sarcomere length of 2.2μm.

The two sets of control fibres are shown. Open circles with the dashed fitted curve represents the control curve for the glycine mutant. The closed circles with the black fitted line represents the control data for the other 4 mutants. All other data points and curve fits are coloured as follows: red squares, serine; green triangles, threonine; dark blue inverted triangles, valine; pink diamonds, alanine; cyan hexagons, glycine.
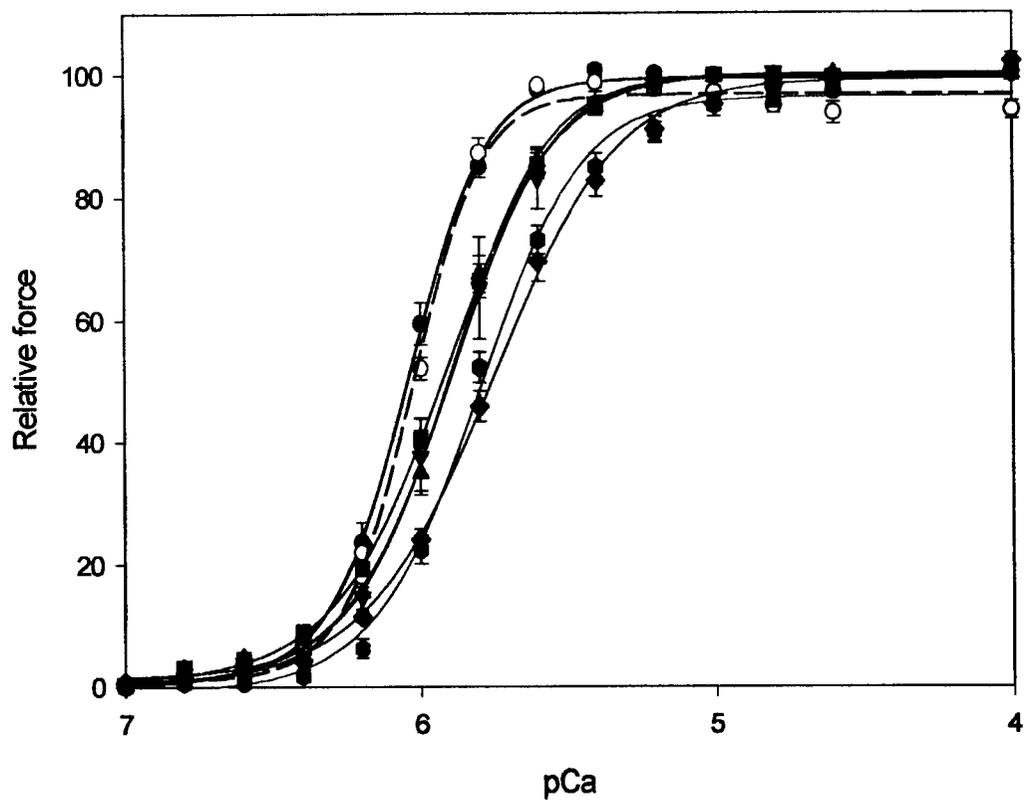
Figure 11B. Comparison of force-pCa curves at sarcomere length of 2.6μm.

The two sets of control fibres are shown. Open circles with the dashed fitted curve represents the control curve for the glycine mutant. The closed circles with the black fitted line represents the control data for the other 4 mutants. All other data points and curve fits are coloured as follows: red squares, serine; green triangles, threonine; dark blue inverted triangles, valine; pink diamonds, alanine; cyan hexagons, glycine.
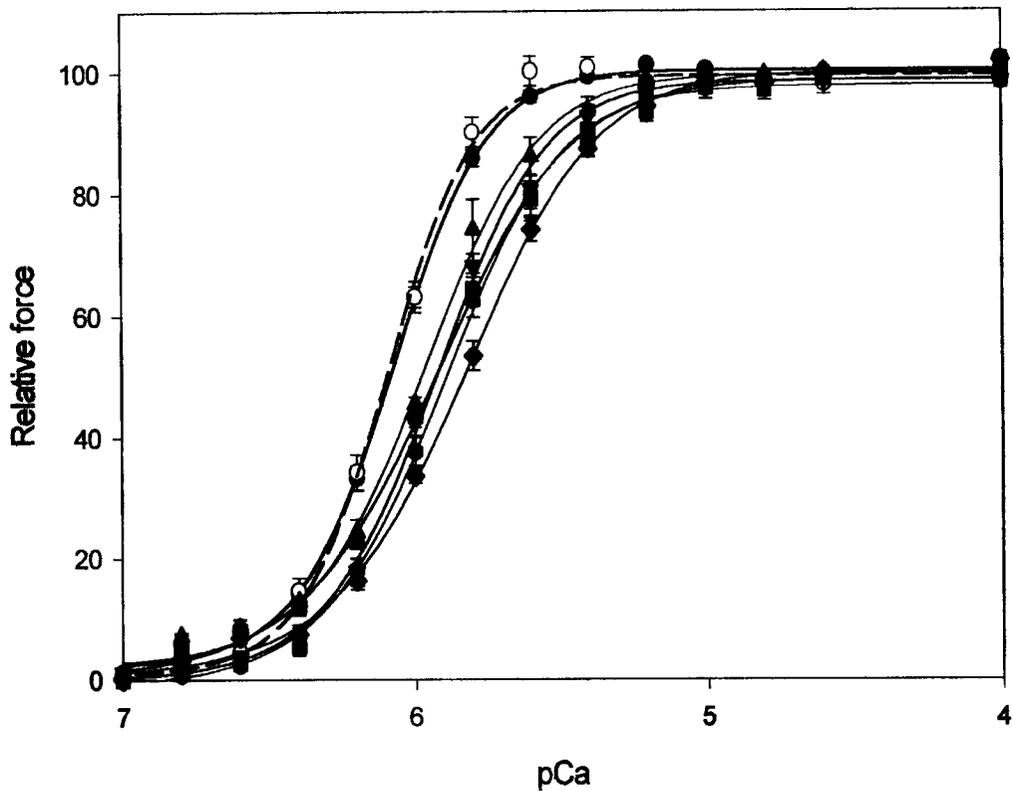
Figure 11C. Comparison of force-pCa curves at sarcomere length of 3.0μm.
The two sets of control fibres are shown. Open circles with the dashed fitted curve
represents the control curve for the glycine mutant. The closed circles with the black
fitted line represents the control data for the other 4 mutants. All other data points and
curve fits are coloured as follows: red squares, serine; green triangles, threonine; dark
blue inverted triangles, valine; pink diamonds, alanine; cyan hexagons, glycine.

untreated rabbit psoas fibres) was generated from 2 different tissue samples, and so the control data for the glycine mutant is included. The curves are all right-shifted to higher calcium concentrations with respect to the untreated rabbit fibre with the exception of the fibres reconstituted with the serine mutant at a sarcomere length of 2.2μm. In other words, the calcium sensitivity generally decreased with the introduction of the chicken skeletal TnC in the rabbit skeletal fibres. However, the amount by which it decreased was dependent on which of the mutant proteins was reconstituted into the rabbit fibres.

All fibres reconstituted with a mutant protein with the exception of serine showed a statistically significant shift to a higher pCa_{50} when compared to the untreated rabbit fibres at a sarcomere length of 2.2μm. At higher sarcomere lengths of 2.6μm and 3.0μm, all mutant TnC proteins showed a significant shift of the pCa_{50} to the right of the untreated fibres.

A comparison of the pCa_{50} values between the fibres reconstituted with the mutant chicken skeletal proteins reveals no statistical difference between the serine and valine mutants at all three sarcomere lengths, between the threonine and valine mutants and threonine and glycine mutants at all three sarcomere lengths, between the alanine and glycine mutants at 2.2μm and 2.6μm. The difference between valine and glycine mutants at 2.6μm and serine and glycine mutants at 3.0μm is also not statistically significant.

At a sarcomere length of 2.2μm (figure 11a) all of the mutants, except for serine, are shifted to the right with respect to the control fibres. At a sarcomere length of 2.6μm (figure 11b), the increase in calcium sensitivity of the threonine mutant and valine mutant shifts their traces to nearly overlap the serine mutant, which has not shifted, while the alanine mutant and the glycine mutant are both still shifted to the right of the serine,

threonine and valine mutants. The threonine, valine and glycine are similarly shifted to the left at a sarcomere length of 3.0μm. The calcium sensitivity of the alanine mutant does not shift to the left to the same degree as the glycine mutant and remains significantly different from the other four mutants at this length.

3.3 Rate of Force Redevelopment

The rate of force redevelopment was recorded without the use of sarcomere length control. In studies without sarcomere length control, k_{tr} is reduced by about 30% and there is often a small departure from a single exponential fit at high $[Ca^{2+}]$. Therefore, a rate constant should be calculated from the time to redevelop one-half of the maximum force (Metzger et al, 1989; Chase et al, 1994; Regnier et al, 1999). In this study, the rates were taken at the $t_{1/2}$ point and compared to the value obtained from a non-linear regression analysis (Sigma Plot version 5.1) using a single exponential fit. A rate of 10.8 sec^{-1} for skinned rabbit skeletal fibres with native TnC (Table 7) falls within the range for the expected value if a 30% decrease is anticipated without the use of sarcomere length control (Chase et al, 1994). While the re-stretch to the original fibre length and the use of sarcomere length control would eliminate any sarcomere length dependence of force production, there is however a linear correlation between k_{tr} with, and without, sarcomere length control (Chase et al, 1994).

If a correction factor of 30% is applied to the rates obtained without sarcomere length control, the k_{tr} values for the mutant TnC proteins fall within a calculated range of 2.5 to 6 sec^{-1}. Applying this same correction factor to the control value, the rate is

estimated at 14 sec^{-1} which approximates the literature values with sarcomere length control (Chase et al, 1994).

The actual values obtained by fitting a single exponential curve to the data are shown in Table 7. The single exponential curves fitted to the data are shown in figure 12. The fitted lines represent the averaged curves, normalized with respect to the maximum tension obtained with the mutant.

Table 7. Summary of rates of force redevelopment of fibres reconstituted with each TnC variant

TnC protein	Force Redevelopment (k_{tr})			
	Rate using $t_{1/2}$	Standard error	Single exponential fit	Standard error
Native rabbit			10.8	0.3590
T54S n=3	4.59	0.6941	4.43	0.1550
T54 n=5	3.41	0.3396	3.41	0.3131
T54V n=5	4.44	0.3131	4.62	0.3186
T54A n=6	3.56	0.2340	3.53	0.2340
T54G n=10	1.90	0.2895	1.83	0.2745
Native at pCa6	0.945	0.0711	0.962	0.0035

The standard error was calculated using Sigma Plot (95% confidence level). For control fibres at pCa 4, n=23 and at pCa 6, n=10.
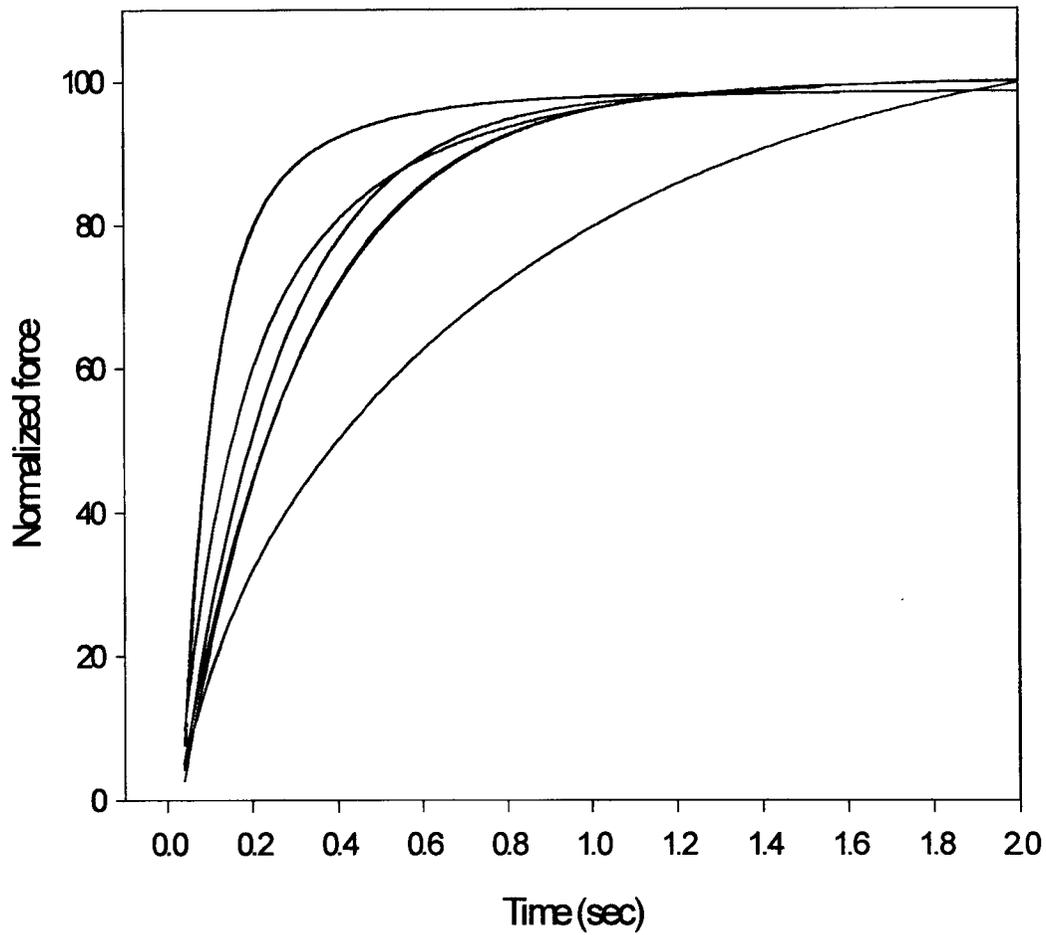
Figure 12. Averaged force redevelopment curves of fibres reconstituted with each of the mutant TnC proteins relative to the average control fibre with native rabbit TnC.
The data for each curve fit represents a single mutant normalized with respect to the maximum tension obtained for that mutant. Control, black; serine, red; threonine, green; valine, dark blue; alanine, pink; glycine, cyan. The threonine and alanine curves overlap.

Figure 13 shows the k_{tr} trace of a single fibre prior to extraction of native TnC and after reconstitution with the valine mutant of TnC. These traces have not been normalized with respect to maximum force to show the decrease in force that accompanies the decrease in k_{tr}.

Figure 14 represents the mean k_{tr} value at pCa 4.0 of fibres reconstituted with the mutant proteins normalized with respect to the control fibres with native rabbit TnC. The average is plotted for each mutant with the standard error bar shown. All of the mutants show a significantly reduced rate of force redevelopment.

The normalized k_{tr} values plotted against normalized tension relative to an untreated rabbit fibre (figure 15) fell within the early rise of the steep curvilinear relationship that is observed when the k_{tr} values of control fibres are plotted from $0.1P_o$ to P_o (Regnier et al, 1998). The maximum relative force values of the fibres reconstituted with the mutant TnC proteins ranged from a low of $0.59P_o$ for the glycine mutant to $0.85P_o$ for the serine mutant relative to control fibres (P_o).

The k_{tr} experiments were carried out at a single activating calcium concentration (pCa 4.0). To compare the k_{tr}-pCa relationship to the tension-pCa relationship of the mutant TnC proteins, the pCa value at which fibres reconstituted with mutant proteins achieved their maximum force was extrapolated from the control force-pCa curve (table 8). The maximum tension observed for each mutant TnC, represented by the red circles in figure 16, was plotted against the pCa value at which the untreated skinned fibres produced the same level of tension. By comparison the k_{tr}-pCa curve represented by the blue squares is right shifted relative to the force-pCa curve.
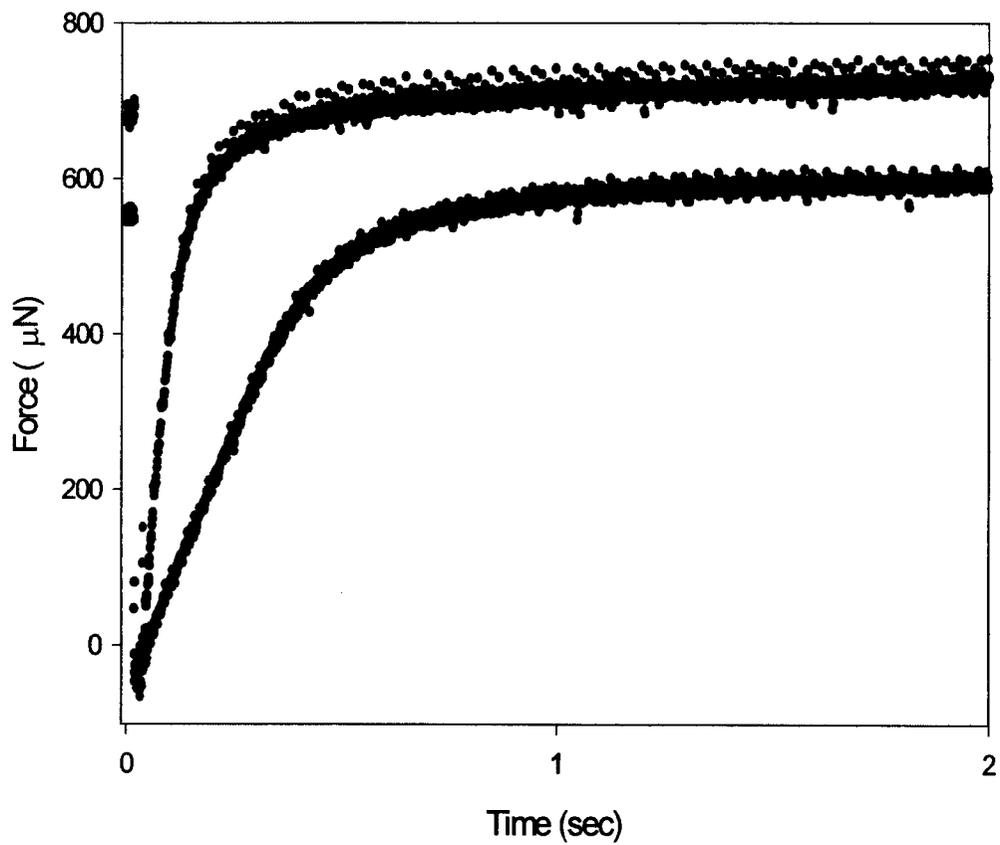
Figure 13. Force redevelopment traces from the data acquisition files.

Comparison of a fibre before extraction and after reconstitution with the valine mutant of

TnC. Data were not normalized to show the drop in force associated with the valine mutant.
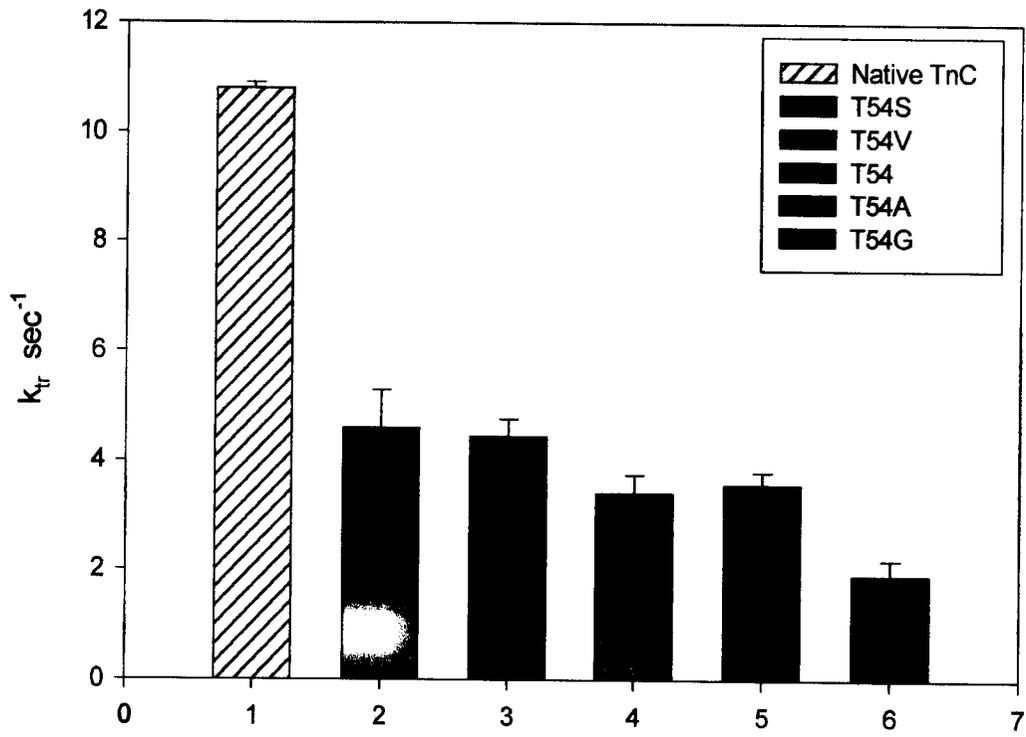
Figure 14. Mean rate of force redevelopment calculated from $t_{1/2}$ values.

Legend is shown on the graph. Standard errors are shown.
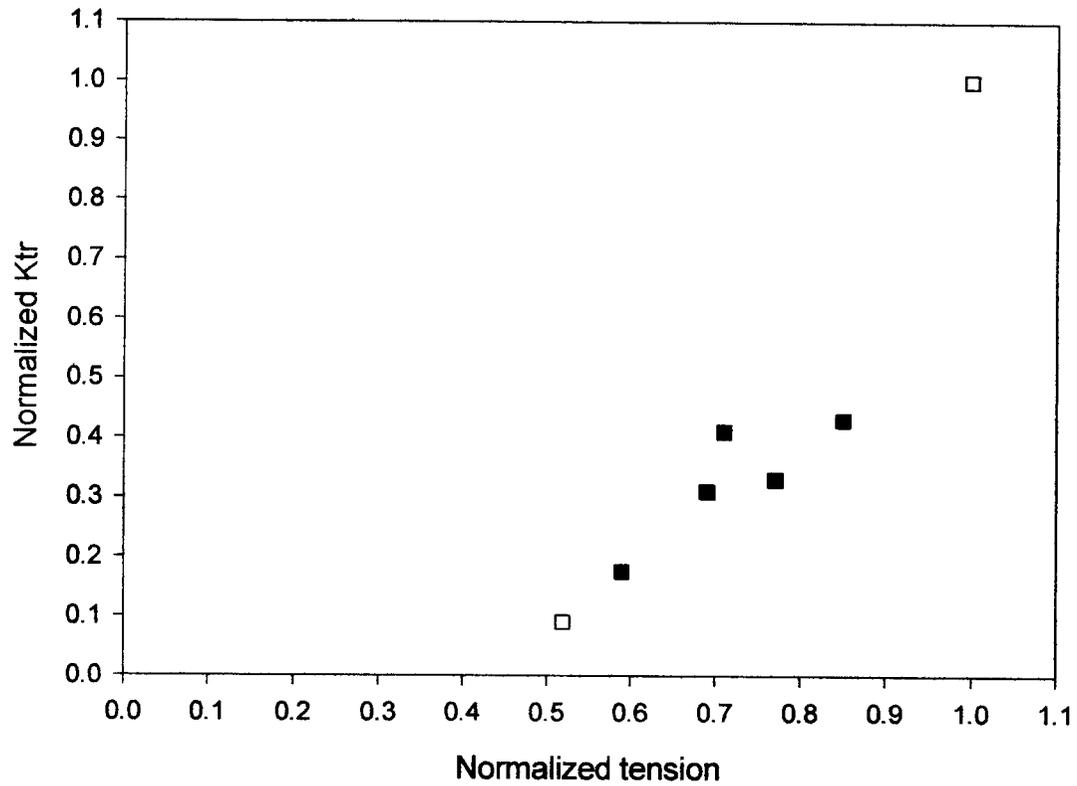
Figure 15. Normalized k_{tr} relative to the normalized tension produced by fibres reconstituted with each of the mutant proteins.

Values taken from table 2, represent the steep relationship between k_{tr} and pCa as force

rises above 50% of the maximum of control fibres. Open squares represent control fibres,

closed squares represent fibres reconstituted with mutant TnC proteins at both pCa 6.0 and

pCa 9.0.
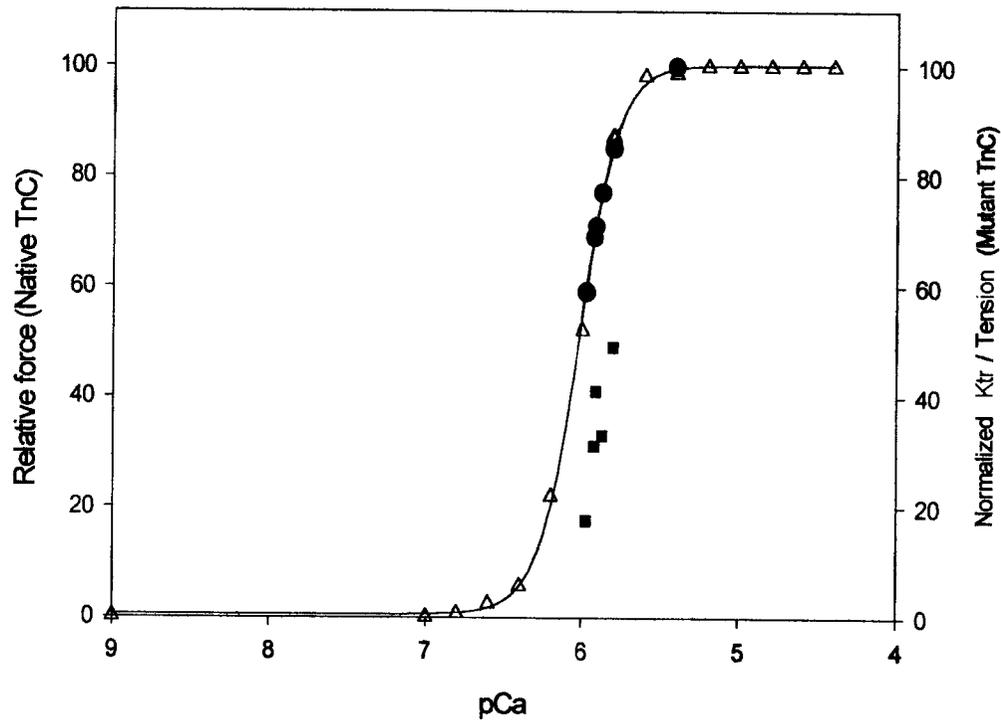
Figure 16. Normalized force-pCa and normalized k_{tr}-pCa relationship of fibres reconstituted with mutant Tnc proteins relative to control fibres with native rabbit TnC.

Open triangles represent the data points of the averaged force-pCa curve of the control fibres. The fitted line represents the fit to the data points using the modified Hill equation (equation 2). The red circles represent the relative pCa value relating to the maximum isometric tension observed with the mutant proteins reconstituted into skinned fibres. The blue squares represent the k_{tr} values for each mutant protein normalized with respect to the control fibres (table 8) and are plotted as a function of the pCa value extrapolated above, for each mutant protein. Note that the resulting k_{tr} versus pCa curve is right shifted with respect to the normalized tension versus pCa curve.

Force redevelopment curves of control fibres at pCa 6.0 were compared to fibres reconstituted with the glycine mutant of TnC. A mean k_{tr} value of $0.95sec^{-1}$ was observed (figure 17) for the control fibre compared to $1.90\ sec^{-1}$ for the glycine mutant (table 7).

Table 8. Rate of force redevelopment normalized with respect to fibres with native rabbit TnC

TnC protein	Relative k_{tr}	Relative pCa value
Native rabbit	1	5.6
T54S	0.43	5.86
T54V	0.41	5.87
T54	0.31	5.91
T54A	0.33	5.92
T54G	0.17	5.97
Native rabbit	0.09	6.00

The tabulated k_{tr} values are relative to the control fibre activated at pCa 4.0 (row 1). The last row represents a control fibre activated at pCa 6.0 for comparison with the glycine mutant (T54G).
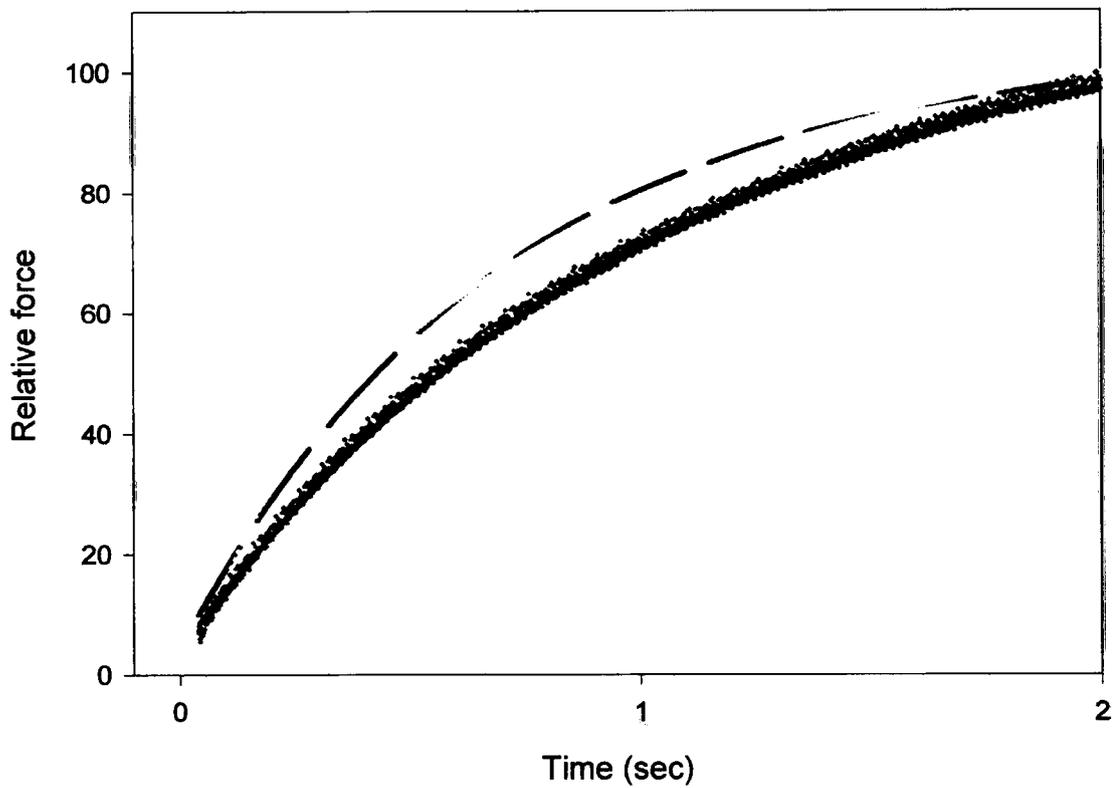
Figure 17. Average force redevelopment curves of control fibres at pCa 6.0 compared to fibres reconstituted with the glycine mutant of TnC activated at pCa 4.0.

The averaged force redevelopment curve with the glycine mutant of TnC at pCa 4.0 is shown in yellow. The dashed line represents the non-linear regression fit of a single exponential function through the data points. The averaged force redevelopment curve of the control fibres at pCa 6.0 is shown in blue with the solid line representing the single exponential fit to the data.

3.4 Maximum Unloaded Shortening Velocity (V_{max})

The maximum velocity of shortening was measured using the slack test method. This "slack time" plotted against the step size of the release was used to measure of the maximum unloaded shortening velocity of skinned skeletal fibres reconstituted with each of the mutant TnC proteins. An initial series of control experiments were performed to determine the smallest step size sufficient to slacken the fibre (figure 18). In all fibres tested, the minimum tension was observed with a release of 100nm/hs and above.

A series of traces in figure 19 describes the general protocol for the remaining experiments. An attempt was made to include a slack test release, followed by a release with restretch to measure force redevelopment as well as a third small release or stretch to obtain a transient. Few fibres could survive more than one activation before tearing. As a result a protocol that only included a slack test release followed by or preceded by a transient was used as it had been confirmed that the baselines were stable and the steps sufficiently large to drop the force to zero. Panels A through D also demonstrate that the change observed with the mutant TnC was reversible with re-extraction of the mutant and reconstitution with native rabbit TnC. This latter protocol was not followed with all fibres since it only allowed the acquisition of a single measurement per fibre. The fibres could not undergo several activations with the expectation that they would then recover completely upon re-extraction of the mutant TnC and reconstitution with the native rabbit TnC.

In a sample experiment, a series of images from the same fibre, at 20x magnification, were acquired using the Image Pro Plus software following each step of the extraction and reconstitution protocol (figure 20). While the striation pattern is not as
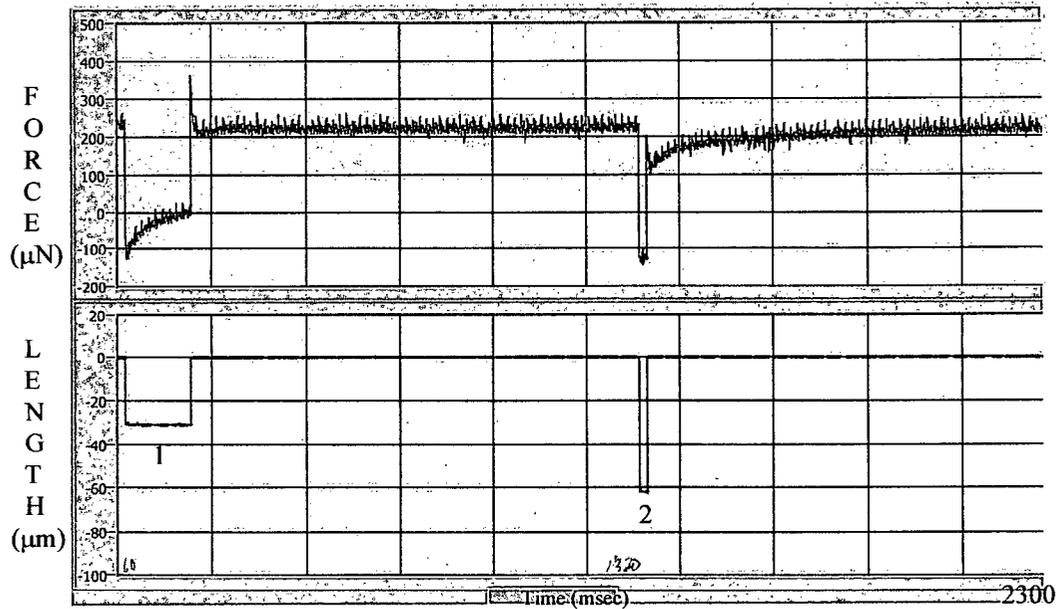
Figure 18. Sample trace to illustrate the slack test protocol

Comparison of minimum force values of a 100nm/hs release and a 200nm/hs release.

The smallest step release size used to acquire slack test data was 100nm/hs (1). To verify

that the control fibres did go slack with such a small release, a 200nm/hs release (2) with

restretch was introduced after full recovery of tension and the minimum force values were

compared. In this sample the minimum force value with the 100nm/hs step release was

$-127.7\mu N$. The average minimum value for the 20ms release of 200nm/hs was $-127.4\mu N$.
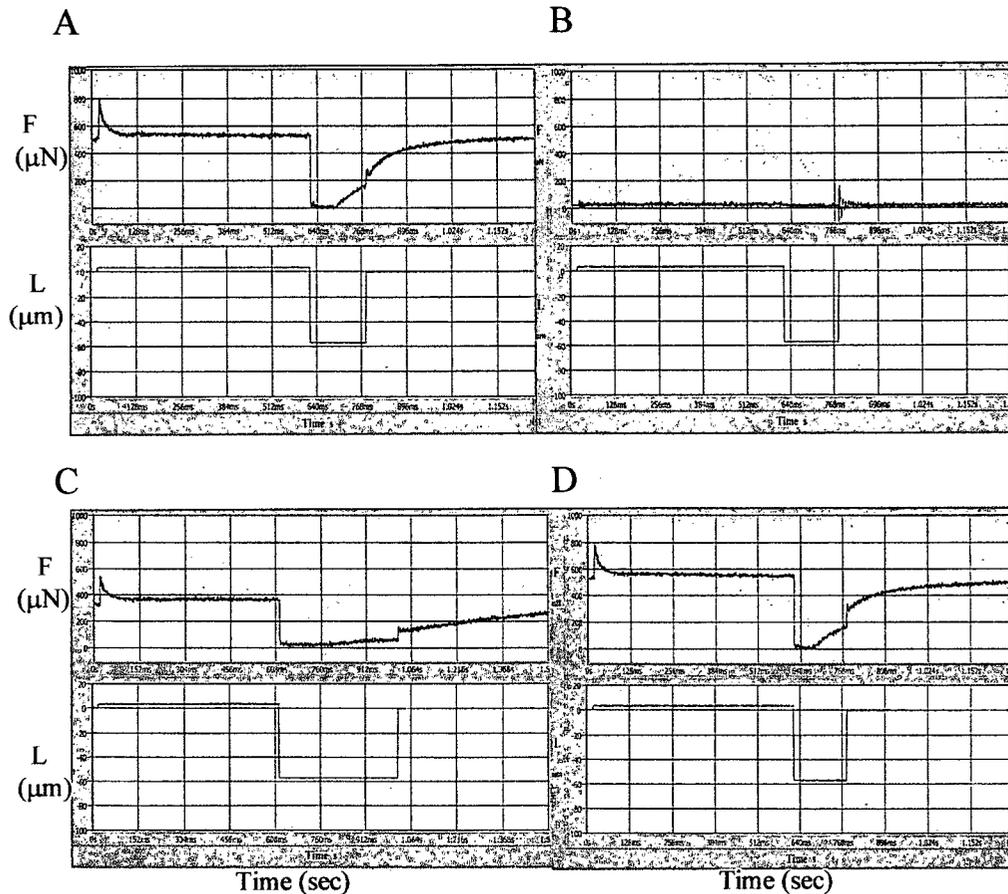
Figure 19. Representative traces from a single fibre at four different stages of a single slack test experiment.

The raw data, from four data acquisition traces, record the initial maximum force followed 20ms later by a small stretch. A recovery time of 590ms is followed by a release of variable size, from 100 to 300 nm/hs, to record the time required for the fibre to absorb the slack and begin to generate force. A final restretch to the original length records the recovery to the initial maximum force.

Panel A: untreated fibre;

Panel B: after extraction of TnC;

Panel C: after reconstitution with the glycine mutant of TnC;

Panel D: re-extraction of the mutant TnC followed by reconstitution with native rabbit TnC.

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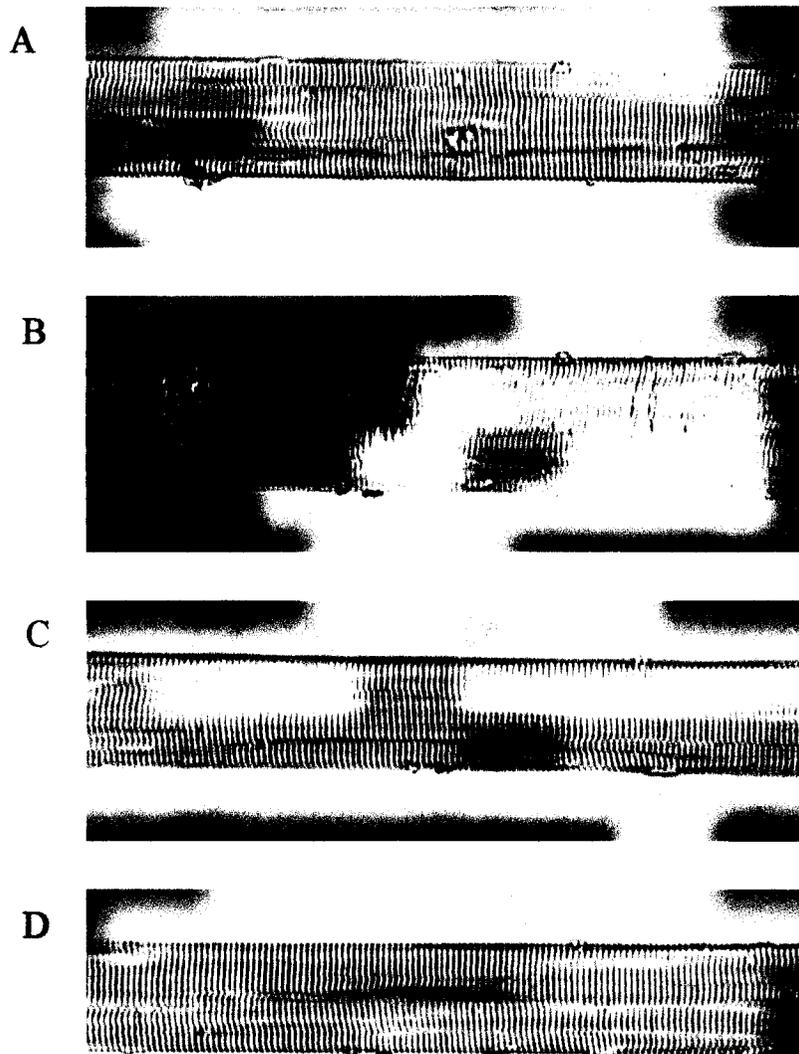
Figure 20. Images from a single fibre during the extraction and reconstitution protocol of a slack test experiment.

Panel A: Skinned rabbit psoas fibre set at a sarcomere length of 2.6μm.

Panel B: Fibre after extraction of the native rabbit TnC.

Panel C: Fibre after reconstitution with the glycine mutant of TnC.

Panel D: Fibre after re-extraction of the mutant TnC followed by reconstitution with native rabbit TnC. All images are 20x magnification.

distinct in the TnC extracted fibre, the striation pattern of the fibre reconstituted with the glycine mutant of TnC is distinct and the sarcomere length is easily set at 2.6μm so that it appears no different than the untreated fibre or the fibre after re-extraction and reconstitution with native rabbit TnC in panel D.

For each experiment, measurements were taken before TnC extraction and after reconstitution with one of the TnC mutant proteins. A plot for each of the mutant proteins (figure 21) represents the averaged data for each point on the graph. Each data point represents an average of n=6 with the exception of the glycine mutant with Pi where n=3. The maximum shortening velocity was determined by linear regression analysis using the correlation coefficient to position the break point between V_{o1} and V_{o2} (refer to Introduction, 1.2.3.1). The untreated rabbit fibres were best fit by a single straight line, both with and without 10mM P_i. The maximum shortening velocity of 5.44nm/hs/ms without added P_i is comparable to the average value of 4.26ML/sec (Hoffman et al, 1991). This value converts to 5.22nm/hs/ms using the average fibre length reported in their paper of 1,800 $\square$m with an average sarcomere length of 2.45$\square$m. With the addition of 10mM P_i a small increase in V_{max} to 5.81nm/hs/msec was observed with the control fibres. A slight increase in V_{max} has been reported elsewhere when P_i is added to the activating solution. (Metzger, 1996).

All of the fibres reconstituted with mutant TnC proteins exhibited a break in the shortening velocity plots resulting in a biphasic curve with an early fast rate of shortening (V_{o1}) and a later slow rate of shortening (V_{o2}) even at a maximally activating calcium concentration (Figure 22). The addition of 10mM P_i failed to produce a single-phased shortening velocity curve resembling V_{o1} (Figure 22).
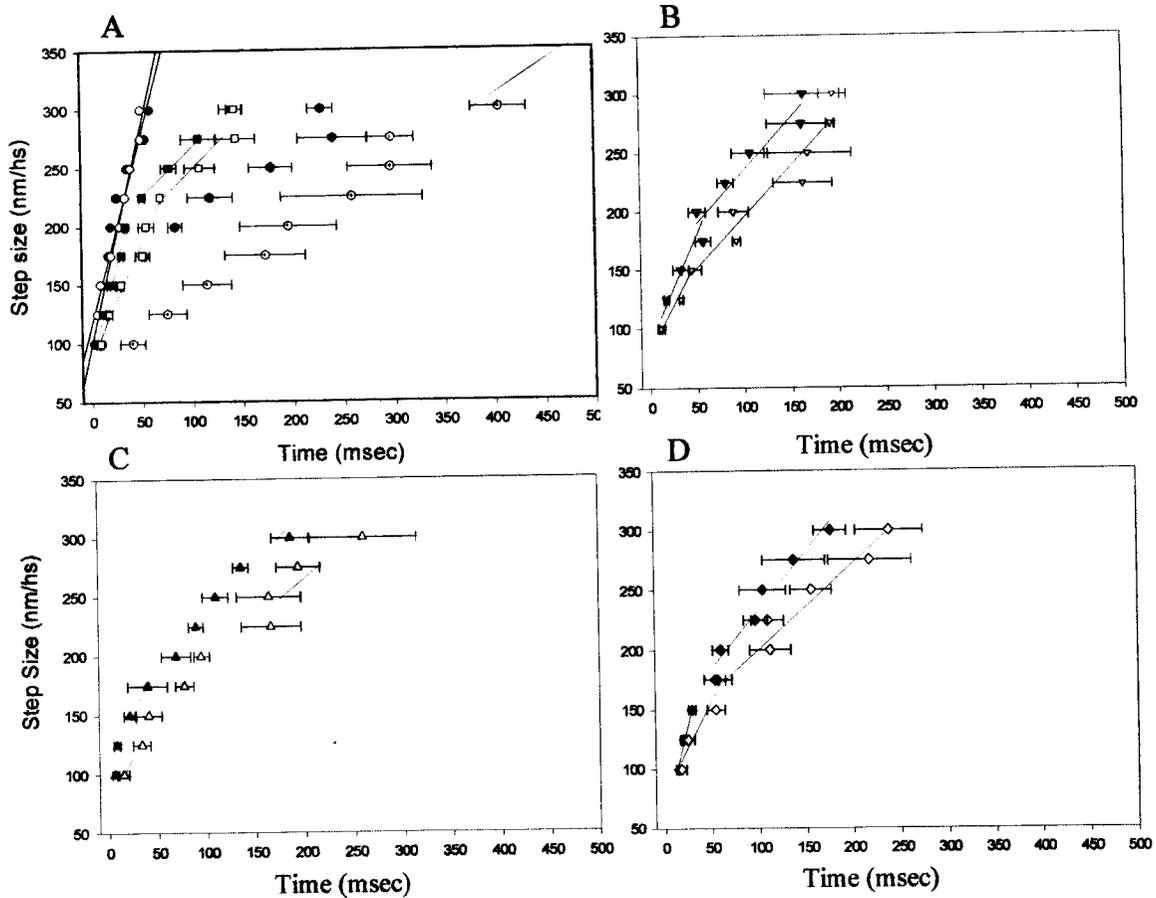
Figure 21. Maximum shortening velocity of control rabbit fibres contrasted with fibres reconstituted with a mutant TnC, and activated with and without 10mM Pi.

The maximum velocity of shortening was measured before extraction of TnC and after reconstitution with one of the mutant TnC proteins. Fibres were activated at pCa 4.0 in the presence (open symbols) or absence (closed symbols) of 10mM P_i. Standard error bars shown. The lines represent the linear regression fit to the data points: serine, red; glycine, cyan; threonine, green;valine, blue; alanine, pink.

Panel A: Control fibres, circles; serine, squares; glycine, dotted hexagons.

Panel B: Valine, inverted triangles.

Panel C: Threonine, triangles.

Panel D: Alanine, diamonds.
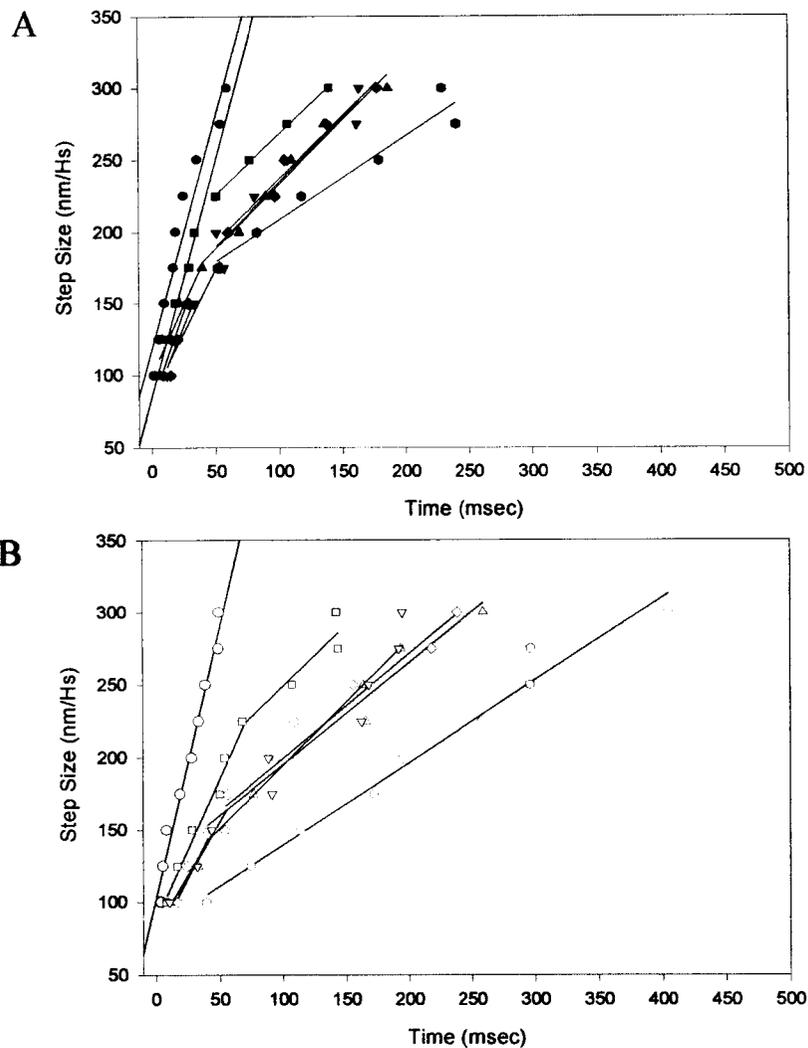
Figure 22. Maximum shortening velocity of fibres with and without 10mM P_i.

Vmax of control fibres with native TnC plot as a single phase at pCa 4.4 with or without added P_i. Reconstitution with a mutant TnC results in a bi-phasic plot with a fast and a slow component (panel A) that is not relieved by the addition of 10mM phosphate to the activating solution (panel B). The point of inflection is dependent on the mutant TnC present in the fibre and on the presence or absence of P_i. Legend: red squares, serine; green triangles, threonine; blue inverted triangles, valine; pink diamonds, alanine; cyan hexagons, glycine.

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The early fast phase, V_{o1}, was similar for all of the mutant proteins in the absence of phosphate, and comparable to the rate observed with the control fibres (table 9). The serine and threonine mutants of TnC were within 5% of the control value with the remaining mutants ranging from 7% to 12.5% slower. The glycine mutation caused the largest drop in force and in the absence of phosphate, resulted in the largest decrease in slow phase (V_{o2}) of the shortening velocity (table 9).

Table 9. Correlation between P_o and the maximum shortening velocity without added inorganic phosphate

Troponin C	*V_{o1}	r^2	V_{o2}	r^2	P/P_o
Native Rabbit	5.44	0.93			1.00
T54S	5.31	0.98	0.84	0.998	0.85±0.017
rTnC (T54)	5.25	0.91	0.72	0.97	0.77±0.041
T54V	4.76	0.92	0.68	0.91	0.71±0.021
T54A	5.05	0.90	0.96	0.97	0.69±0.020
T54G	4.81	0.96	0.58	0.95	0.59±0.015

* These values are not significantly different as determined using the Student's t-test.

While the control fibres show a small increase in the shortening velocity with the addition of inorganic phosphate, the addition of 10mM P_i does not increase the shortening velocity of the later, slow phase, V_{o2}, of the fibres reconstituted with the mutant TnC proteins. All plots remain biphasic. With the addition of 10mM P_i, V_{o1} decreases by 34% to 89% and appears to be dependent of which of mutant TnC proteins is present in the fibre (table 10). V_{o2} remains essentially unchanged.

Table 10. Correlation between P_o and the maximum shortening velocity with 10mM inorganic phosphate added to the activating solution.

Troponin C	V_{o1}	r^2	V_{o2}	r^2	P/P_o
Native Rabbit	5.81	0.98			1.00
T54S	3.26	0.99	0.85	0.88	0.54±0.019
rTnC (T54)	3.49	0.94	0.68	0.96	0.42±0.045
T54V	2.54	0.97	0.99	0.91	0.46±0.032
T54A	2.81	0.89	0.73	0.95	0.28±0.027
T54G	0.54	0.98	0.54	0.98	0.19±0.016

The degree of translation, that is, the shortening of the fibre before the break-point (table 11) in the shortening velocity rates, changed significantly for fibres reconstituted with the threonine mutant of TnC (15% decrease), the valine mutant (29% decrease) and the glycine mutant (100% decrease). However, the large decrease in V_{o1} for glycine with the addition of P_i can be attributed to an 81% drop in maximum force relative to the control fibres (table 10).

Table 11. The degree of translation before the break point in the plot of the maximum shortening velocity with and without the addition of 10mM inorganic phosphate

Mutant Troponin C (F29W)	Translation before break-point (nm/hs) No added P_i	Translation before break-point (nm/hs) with 10mM P_i
T54S	217	222
rTnC (T54)	189.5	160.5
T54V	200	142.1
T54A	171.7	168.3
T54G	172.2	0.00

For comparison, control fibres activated at submaximal calcium have a published value of 85.6 ±3.1 nm/hs for the break point to the slow phase of shortening (Hoffman et

al, 1991). The fibres reconstituted with the mutant TnC proteins and activated at pCa 4.0 appear to shorten further before the break point to a slower rate of shortening. With the addition of 10mM P_i, the degree of translation decreases for some of the mutants, with V_{o1} disappearing completely in fibres reconstituted with the glycine mutant. This loss of V_{o1} can be attributed to the large decrease in force, to below 20% P_o (Gordon et al, 2000), with the addition of phosphate.

A comparison of the fibres both with and without 10mM P_i in the activating solution (Figure 22) reveals that while the threonine, valine and alanine mutations appear to cluster as a group it is not because of similar rates for V_{o2}, but rather the result of the slower rate of V_{o2} combined with the different degree of translation.

The biphasic shortening plots observed with the mutant TnC proteins which are not relieved by the addition of 10mM P_i suggest that the break point observed with control fibres at submaximal calcium concentrations occur by a different mechanism than is observed in the current study since in the former studies P_i increased V_{o2} to a rate resembling V_{o1}. There appears to be some process that does not affect the time to break point to a great extent, but does change the rate of translation such that the degree of translation in that time is decreased. The notable exception is glycine after the addition of P_i.

A small increase in the time to break point is seen with the addition of P_i to fibres reconstituted with the serine mutant but may not be statistically relevant. Referring to figure 21, the standard error bars represent a statistical analysis of a pool (n=6) of measurements for each given step release size and not 6 complete traces on 6 fibres.

3.5 Tension Transients

Single skinned skeletal rabbit psoas fibres were mounted between two hooks and the sarcomere length was set at 2.6μm. The length of the fibre between the fixed areas was measured and recorded along with the sarcomere length as measured with the Image Pro Software using a live image of the fibre. In the course of each of the previous k_{tr} or slack test experiments a small stretch or release was added at the plateau of tension to record a tension transient representing a length change of 0.6% or 1.2%.

The analysis with respect to the maximum tension of each mutant (P_o') reveals a change in ΔT (Figure 23) that is unchanged from the value calculated for the control fibres (table 12). A comparison of this ratio using the Student's t-test reveals that there is no statistical difference within each step size between the mutants and the controls. This suggests that the decrease in force is only the result of a decrease in the number of cross-bridges attached for a given TnC protein.

Table 12. The change in relative stiffness of fibres normalized with respect to P_o'

Troponin C	R8 ΔT/P_o'	R16 ΔT/P_o'	S8 ΔT/P_o'	S16 ΔT/P_o'
Native	0.2800±0.0048	0.4964±0.0099	0.3688±0.0135	0.5677±0.0173
Serine	0.2540±0.0136	0.4600±0.0110	0.3333±0.0120	0.5200±0.0400
Threonine	0.2840±0.0128	0.4880±0.0218	0.3950±0.0350	0.6950±0.0350
Valine	0.2867±0.0063	0.5063±0.0207	0.3714±0.0215	0.6313±0.0122
Alanine	0.2838±0.0152	0.4811±0.0273	0.3200±0.0193	0.6771±0.0384
Glycine	0.3140±0.0114	0.5217±0.0265	0.3880±0.0246	0.5880±0.0545

R8 and R16 represent releases of 8 and 16nm/hs respectively. S8 and S16 represent stretches of 8 and 16nm/hs respectively. The small step size represents a length change of 0.6% while the larger step size represents a length change of 1.2%.

With the addition of 10mM Pi to the activating solution (pCa 4.0), the relative stiffness was the same for four of the mutant proteins with one exception (table 13).
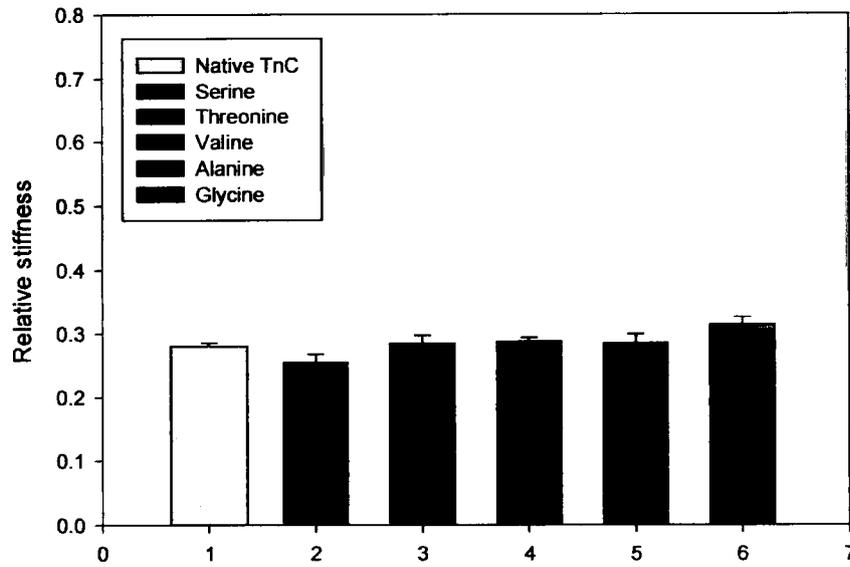
Figure 23. The change in relative stiffness of fibres reconstituted with a mutant TnC normalized with respect to Po'.

The $\Delta T/P_o$' values are plotted with standard error bars shown. The ratio of the change in stiffness with respect to force was measured after a release of 8nm/hs and found to be proportional to the ratio observed in control fibres. The legend is contained within the panel.

The glycine mutant of TnC showed a significant increase ($p < .05$) in stiffness relative to the decrease in force listed in table 10. This suggests that there is not only a decrease in the number of bridges but also a decrease in the force per bridge in the presence of phosphate (Figure 24).

Table 13. The change in relative stiffness of fibres normalized with respect to P_o' in the presence of 10mM phosphate.

Troponin C	R8 P_i $\Delta T/P_o''$	R16 P_i $\Delta T/P_o'$
Serine	0.3350±0.0132	0.5300±0.0167
Threonine	0.3720±0.0180	0.6300±0.1000
Valine	0.3629±0.0270	0.6200±0.0545
Alanine	0.3833±0.0080	0.4933±0.0328
Glycine	0.5164±0.0243	0.7183±0.0308

A comparison of the change in stiffness of fibres reconstituted with the mutant TnC proteins with respect to P_o (maximum force of control fibres with native TnC) shows that the decrease in stiffness was proportional to the decrease in force (Figure 25) using P_o'/P_o from table 1 to calculate the relative change (table 14).

Table 14. The change in relative stiffness of fibres normalized with respect to P_o of control fibres with native rabbit TnC.

Troponin C	R8 $\Delta T_m/P_o$	R16 $\Delta T_m/P_o$	S8 $\Delta T_m/P_o$	S16 $\Delta T_m/P_o$
Native	0.2800±0.0048	0.4964±0.0099	0.3688±0.0135	0.5677±0.0173
Serine	0.2070±0.0160	0.3860±0.0140	0.2833±0.0133	0.4150±0.0350
Threonine	0.2220±0.0076	0.3520±0.0218	0.2600±0.0400	0.5250±0.0450
Valine	0.2208±0.0094	0.3500±0.0215	0.2414±0.0150	0.4175±0.0193
Alanine	0.1825±0.0125	0.3400±0.0230	0.2550±0.0203	0.4329±0.0151
Glycine	0.1733±0.0079	0.2942±0.0204	0.2180±0.0227	0.3420±0.0256
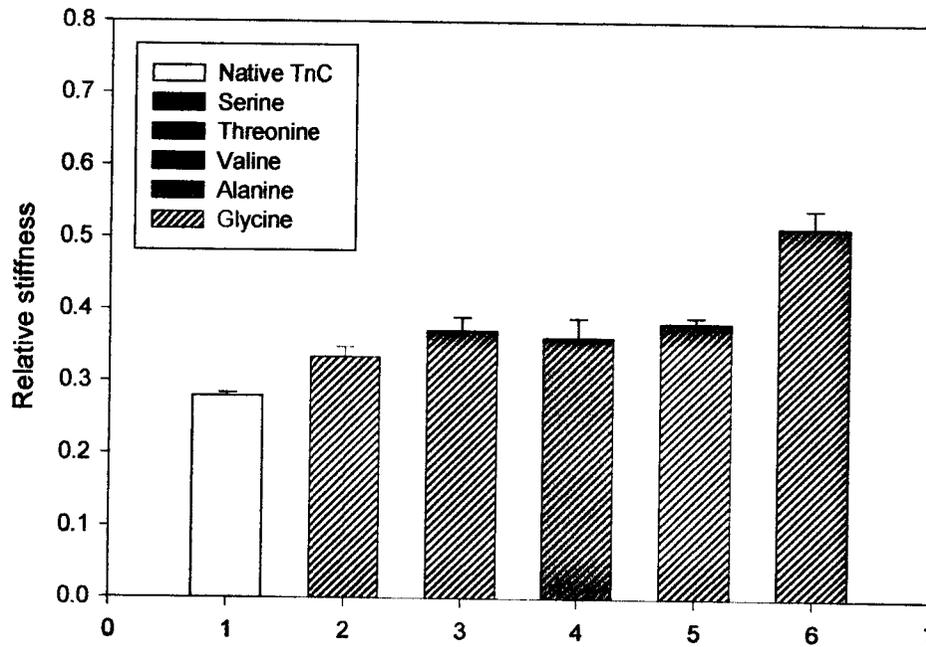
Figure 24. The change in relative stiffness of fibres reconstituted with mutant TnC in the presence of 10mM Pi, normalized with respect to P_o'. The $\Delta T/P_o$' values are plotted with standard error bars shown. The control fibre measurements are without added phosphate. The ratio of the change in stiffness with respect to force was measured after a release of 8nm/hs. It is apparent that with the glycine mutant, force decreased more than stiffness.
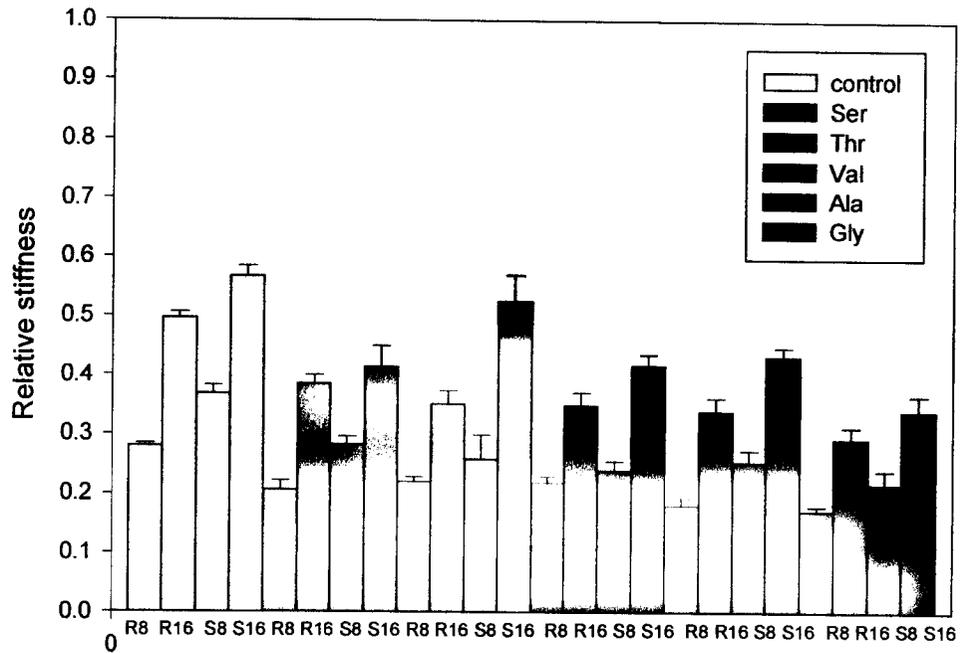
Figure 25. The change in stiffness relative to control fibres.

The ΔT values are taken as a ratio of the maximum tension of the averaged control

fibres. This plot demonstrates that the mutant TnC proteins cause a decrease in

stiffness relative to the control value.

However, in the presence of 10mM Pi the decrease in stiffness was not proportional to the measured decrease in force (table 15). The expected change in stiffness calculated using the ratio of P_o'/P_o in 10mM Pi was lower than the measured value. Compared to control fibres it becomes more apparent that while both force and stiffness decrease, the stiffness decreased less than the force.

Table 15. The change in relative stiffness of fibres normalized with respect to P_o of control fibres with native rabbit TnC in the presence of 10mM phosphate.

Troponin C	R8 P_i $\Delta T_m/P_o$	R16 $\Delta T_m/P_o$
Serine	0.1650±0.0233	0.2678±0.0120
Threonine	0.1660±0.0229	0.2350±0.0450
Valine	0.1886±0.0199	0.1550±0.0171
Alanine	0.1417±0.0079	0.1500±0.0173
Glycine	0.0945±0.0045	0.1600±0.0148

4. DISCUSSION

This research was designed to provide information that would contribute to our understanding of the fundamental mechanism of force production in striated muscle. It was hypothesized that the level of activation characteristics of skeletal muscle might rest with the calcium affinity of the regulatory domain of TnC. It was further hypothesized that since the sight of mutation shifted the equilibrium of the calcium-free and calcium-bound states that there might be a downstream effect on the kinetics of the cross-bridge cycle. The experimental plan focused specifically on the correlation between the calcium-binding properties of the regulatory domain of TnC, the level and rate of force development, and the shortening velocity.

To determine whether or not calcium binding to site II is the final determinant of the actual activation characteristics in skeletal muscle a series of mutant TnC proteins were used. These mutants of chicken skeletal TnC, prepared by serial substitution of the N-cap position of the C-helix (position 54), displayed a range of dissociation constants (Leblanc et al, 2000; Leblanc, 1996) providing a range of calcium affinities within the low-affinity domain (figure 9 and table 1) while minimizing changes in the global conformation of the regulatory domain of TnC as previously seen in the steady state fluorescence data (Leblanc et al, 2000).

The TnC mutants were previously characterized in terms of their calcium binding properties and calcium off-rates in solution using extensive biochemical and spectroscopic analyses (Leblanc et al, 2000). This study was designed to seek a correlation between the calcium-binding properties of TnC and the contractile properties of skinned single muscle cells reconstituted with the mutant proteins and in doing so,

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gain some insight into the molecular mechanism of calcium-TnC regulation of muscle activation. These experiments focused on the cross-bridge mechanism, using mechanical perturbations to produce tension changes that reflected cross-bridge behaviour. Because of the fast dissociation rate of calcium from TnC, it was hypothesized that the concentration of calcium-bound TnC, and therefore the degree of muscle activation, was a direct result of the equilibrium of calcium binding to and dissociating from TnC. The affinity of TnC for calcium reflected in the calculated "on" rates and measured "off" rates (Table 16) could underlie the physiological mechanism that determines the level of activation in muscle.

Table 16. Summary of binding parameters determined by the titration of the intrinsic protein fluorescence of the mutant TnC proteins in solution

Mutant	pCa ($-\log K_d$) (M)	Rate 1 (s^{-1})	Rate 2 (s^{-1})
T54S	5.866±0.008	N/A	N/A
T54	5.865±0.011	900±20	94±9
T54A	5.680±0.005	1190±500	100±22
T54V	5.643±0.020	1470±100	123±23
T54G	5.373±0.009	1260±100	100±14

Table adapted from Leblanc et al (2000). Rate 1 and rate 2 represent the calcium off-rates for the two calcium binding sites of the low-affinity domain of the troponin C mutant proteins at 21±1°C.

4.1 Maximum Tension at pCa 4.0

The first step was to measure the tension generating capabilities of skinned skeletal muscle fibres from rabbit psoas muscle after reconstitution with each of the chicken variants of TnC.

The proteins used in this study were all based on the recombinant chicken skeletal TnC with an unblocked Met at the first residue. In the native protein, there is no Met residue and the alanine residue is blocked by an acetyl group. Chicken TnC has 6 residues at the beginning of its sequence (M, A, S, M, T, D) that are not found in the rabbit sequence. From residue 7 for chicken, which is residue 3 in rabbits, the remainder of the N-terminal domain of the 2 proteins is 95% identical (Regnier et al, 1999). The sequences diverge at 8 residues, with the remaining differences mostly being conserved substitutions. The introduction of a single amino acid substitution at the N-cap residue, using site directed mutagenesis, resulted in a range of maximal isometric tension ranging from $0.85P_o$ to $0.59P_o$ with P_o being the maximum force observed in the untreated skinned rabbit psoas fibres (ie. control fibres). This decrease in tension is therefore not entirely due to the occurrence of species-specific isoforms of TnC. Figure 5 clearly demonstrates that the extraction protocol was not the reason for the variable tension levels observed with the chicken skeletal proteins.

The introduction of the reporter group (F29W) for the spectroscopic analyses has been reported not to affect the maximal tension of the recombinant protein (rTnC) (Chandra et al, 1994). The use of the rTnC protein to reconstitute skinned rabbit skeletal fibres has been demonstrated not to alter the k_{tr} versus tension relationship (Regnier et al, 1999) even though the expression of the cloned chicken skeletal TnC protein in bacterial system introduces an isoleucine residue (I130T) in the high affinity domain of TnC (Pearlstone et al, 1992; Trigo-Gonzalez et al, 1993). It was not expected that the introduction of an isoleucine residue would alter the function of the protein once introduced into skinned fibres since it was demonstrated (Szczesna et al, 1996) that the

ability of TnC to bind to the fibres depended on the activity of calcium binding loop III, and not the less stable loop IV. If loop III was active this was sufficient for binding of TnC to the thin filament even in the presence of magnesium rather than calcium.

Finally, it should be emphasized that a single amino acid difference within a series of five mutants of TnC is responsible for the range of calcium affinities that is observed.

4.2 Force-pCa Curves

The force pCa curves show that the calcium sensitivity of the reconstituted fibre was dependent on the amino acid substitution (figure 9 and 10). However, not all shifts in pCa_{50} could be solely attributed to the calcium affinity of the mutant protein as seen with the serine mutant of TnC.

An analysis of the two folded states of chicken skeletal TnC, the calcium-free state and the fully calcium-bound state (4 calcium ions bound) of 5 variants of the protein was undertaken in a previous study (Leblanc, 1996; Leblanc et al, 2000). Solution studies revealed a large decrease in the cooperativity of calcium binding with the mutants. However, flow dialysis using $^{45}Ca^{2+}$ confirmed the presence of 4 functional calcium binding sites (Leblanc, 1996; Leblanc et al, 2000). The force/pCa curves of fibres reconstituted with each of these mutants also showed a decrease in the Hill coefficient suggesting that the cooperative binding of calcium to TnC is translated to the fibre (figure 9 and 10, table 6).

Previous measurements using stop-flow fluorescence spectroscopy provided two calcium off-rates which presumably corresponded to the two calcium binding sites of the

low-affinity domain (Leblanc, et al, 2000); a constant rate of 100/sec, and a variable rate which ranges from 900/sec to 1470/sec. In solution, we also observed an increase in the dissociation constant that is not necessarily matched by the measured off-rate suggesting an interplay between both the on-rate and the off-rate (from the equilibrium data and the measured off-rates an on-rate was calculated for the low-affinity domain). This might account for the differences imparted to fibres reconstituted with alanine and valine. The calcium on-rate is diffusion limited and has been estimated to be the same for the Ala and Val mutants (Leblanc et al, 2000). The calcium affinity of the Val mutant in solution is slightly lower than for the Ala mutant and the calcium off-rate for valine is faster (table 16). The force-pCa curves show that the decrease in calcium sensitivity once reconstituted into the fibre was greater for the alanine mutant. However, the mechanical measurements suggest that the valine mutation inhibits function to a lesser extent less as will be discussed later.

The steady state fluorescence emission spectra of all of the F29W/54X proteins were found to be very similar in the calcium free state. The tryptophan probe was also reported to be in a similar environment at saturating calcium concentrations as well, suggesting that the mutation at the N-cap did not drastically alter the folded state of TnC (Leblanc et al, 2000). Circular dichroism used to analyze a change in the 2° structure of the whole protein did reveal an interesting difference for the serine mutation (figure 2). The change in the $[\theta]_{222nm}$, which is a measure of the change in the 2° structure of this predominantly helical protein, showed that the serine substitution caused a significant increase in the global fold of the protein in the absence of calcium (Leblanc, 1996). This suggests that there was less "fraying" of the end of the helices with serine in the N-cap

position of both regulatory calcium-binding loops. The change in the CD spectra, in the absence versus the presence of calcium, were similar for the remaining mutant TnC proteins (Leblanc, 1996).

Comparing the force-pCa curves, it would appear that the conformation of the serine mutant of TnC was optimized for calcium sensitivity and maximum tension at all sarcomere lengths tested as observed with the loss of the length dependent calcium sensitivity of the thin filament reconstituted with this mutant TnC. Fluctuations in the length dependent cooperativity along the length of the thin filament were generally negligible between the mutant TnC proteins but significantly different from the native TnC. This suggests that the calcium binding properties of TnC do not dominate the cooperativity measured with force-pCa curves but do however contribute to the overall cooperativity of thin filament activation. TnC does appear to play a significant role in the length dependent calcium sensitivity of muscle fibres. It is possible that a TnC-dependent conformational change occurs as the length of the fibre changes. This relationship to lattice spacing may be disrupted by the more folded structure of the serine mutant of TnC even in the absence of calcium which we observed acted to reduce the length dependent calcium sensitivity. This might account for nature not using the so called "best n-cap" for site II as in site I, providing evidence for site II as the main regulatory site in skeletal TnC, not unlike cardiac TnC with its non-functional site I. An experiment to test this theory would be to artificially alter the lattice spacing by increasing or decreasing the concentration of dextran, since it has been shown that the addition of dextran slows cross-bridge cycling (Metzger and Moss, 1988).

Figure 5 also shows that the rate at which maximum force was achieved was slowed compared to native TnC. To determine why, a series of mechanical experiments were designed to look at different steps in the cross-bridge cycle.

4.3 Correlation Between the Equilibrium Binding Constant of the N-Terminal Domain of TnC and the Regulation of Muscle Contraction

4.3.1 Level of Activation of Skinned Fibres in Response to the Calcium Affinity of Troponin C

In all of the mechanical experiments, the fibres were studied under maximum tetanic stimulation only to exploit the plateau region of the force-length relationship in which the crossbridge number is constant.

It has been argued that the large Hill coefficients obtained from tension-pCa curves indicate the presence of molecular cooperativity within the vertebrate thin filament (Brandt et al, 1984, Moss et al, 1985). In 1988 Brenner proposed that the position and steepness of the tension-pCa relation can be attributed to effects of calcium ions on the apparent forward rate constant (f_{app}) that limits formation of the force-bearing cross-bridge. As f_{app} increases with [Ca^{2+}] (where $f_{app} = k_{tr} - g_{app}$ and g_{app} is the apparent rate constant for dissociation of strongly bound cross-bridges) the proportion of cross-bridges that are strongly bound and force bearing also increases (Metzger & Moss, 1991). From this definition it is not unreasonable to expect that the mutant TnC proteins, with their decreased calcium affinity, variable length dependent calcium sensitivity and decreased cooperativity of thin filament activity compared to native rabbit TnC, would

attenuate the response of skinned skeletal muscle fibres to an imposed length change. The most obvious question to ask was whether the decrease in maximum tension and shift in calcium sensitivity was a simple matter of a decrease in the number of bound cross-bridges.

4.3.2 Tension Transients of Skinned Fibres Reconstituted with TnC Variants

Titration of the calcium specific sites of the TnC mutant proteins reveals a shift in the pCa_{50} values when compared to the wild-type TnC (Leblanc et al, 2000). The observed increase in the dissociation constant in the order Gly > Val > Ala > Thr/Ser reflects a change in the equilibrium between the calcium-free and calcium-bound state which was found in this study to correlate to a similar decrease in the calcium sensitivity of the skinned fibres. This is consistent with our initial hypothesis that the level of activation conferred on the thin filament by various isoforms of TnC can be predicted by the association and dissociation rates of calcium to the proteins. The mechanical manifestation of activation should correlate to the extent of calcium binding to TnC which in turn is determined by the molecular structure of the mutant proteins. Referring to figure 3, the calcium affinity of TnC is predicted to inhibit step 4, the binding of myosin to the actin filament.

The number of available ("unblocked") myosin binding sites on the thin filaments is regarded as an indicator of the level of activation. Since a direct measurement of these sites is not possible with our experimental set-up, an indirect method was used. Under some circumstances, isometric force can be used as an indicator of the degree of activation, because normally force is proportional to the number of active, attached,

cross-bridges (Huxley & Simmons, 1972, Bressler & Clinch, 1974). However, there are conditions under which force changes regardless of a constant activation level. One example is the effect of ADP in skinned fibres. ADP increases force substantially with little effect on the number of attached cross-bridges (Seow & Ford, 1997). The force increase is attributed to the shifting of low-force bridges into the high-force state. In general, if a redistribution of cross-bridges within the attached states occurred, then the force could change independently of level of activation. To use force as an indicator of activation, it was confirmed that no redistribution of the cross-bridges occurred within the attached states. The results confirmed that no redistribution of the attached bridges occurred despite a substantial (>40%) reduction in activation by the glycine mutant of TnC.

A measure of the stiffness of the fibres using a small stretch or release step was compared to the change in force for each mutant confirming that the decreased number of bound cross-bridges was dependent on which mutant was reconstituted into the fibre. In all cases however, the force to stiffness ratio upon activation at pCa 4.0 was the same. Relating the change in stiffness to the maximum tension measured for the control fibres prior to reconstitution, the decrease in stiffness at pCa 4.0 correlated well (within the S.E. values) with the measured decrease in force. We can therefore make the assumption that the level of activation is due to a decrease in the total number of attached cross-bridges.

With the addition of 10mM phosphate to the activating solution, the shift in the force to stiffness ratio observed with the glycine mutant was significantly higher than that observed with the native TnC (no Pi) and the remaining four mutant TnC proteins (table 13). When compared to the control fibres without Pi, the addition of 10mM phosphate

actually produced a larger decrease in force relative to stiffness for the valine, alanine and glycine mutants of TnC.

The two possibilities for the observed ratios are that either there was an increase in the number of bridges in a non-force producing state or the same number of bridges are attached but there is a shift to a low-force state such that the sum of both the strongly bound and weakly bound bridges produces a lower average force per bridge. The large decrease in force with the glycine mutant suggests that fewer force-producing cross-bridges are attached. The decrease in the force to stiffness ratio suggests that there has been a decrease in the force per bridge rather than a decrease in the total number of bridges. It is not expected that the introduction of the mutant TnC proteins would cause an increase in the total number of bound bridges in the presence of phosphate since a decrease in the number of bound cross-bridges was noted in the absence of phosphate. It is likely that the mutant proteins would affect the ability of the fibre to cooperatively induce the binding of strongly bound cross-bridges since there are fewer strongly bound bridges to begin with. Cross-bridges that are stalled in a strongly bound state might be expected to induce the binding of additional cross-bridges however each step of the cross-bridge cycle is still in equilibrium with the previous step with the detachment rate limiting the attachment rate.

Force can be expected to decrease more than stiffness in the presence of >10mM phosphate due to a shift from strongly bound force producing cross-bridges to weakly bound cross-bridges or strongly bound non-force producing cross-bridges (Martyn and Gordon, 1992). It was observed in this study when the mutant TnC proteins showed a greater than 25% decrease in force (table 15). Since the activating solution was

maintained at 200mM ionic strength it is expected that the number of weakly attached non-force producing cross-bridges was minimized. This means that the bridges are more likely stalled in a strongly bound, non-force producing state which has been previously reported with the addition of phosphate to rabbit psoas fibres (Martyn and Gordon, 1992) providing evidence that the relative distribution of the strongly attached states between force producing and non-force producing states may be controlled by calcium-bound TnC. The reversible equilibrium between these two states is illustrated in figure 26, step six (6).
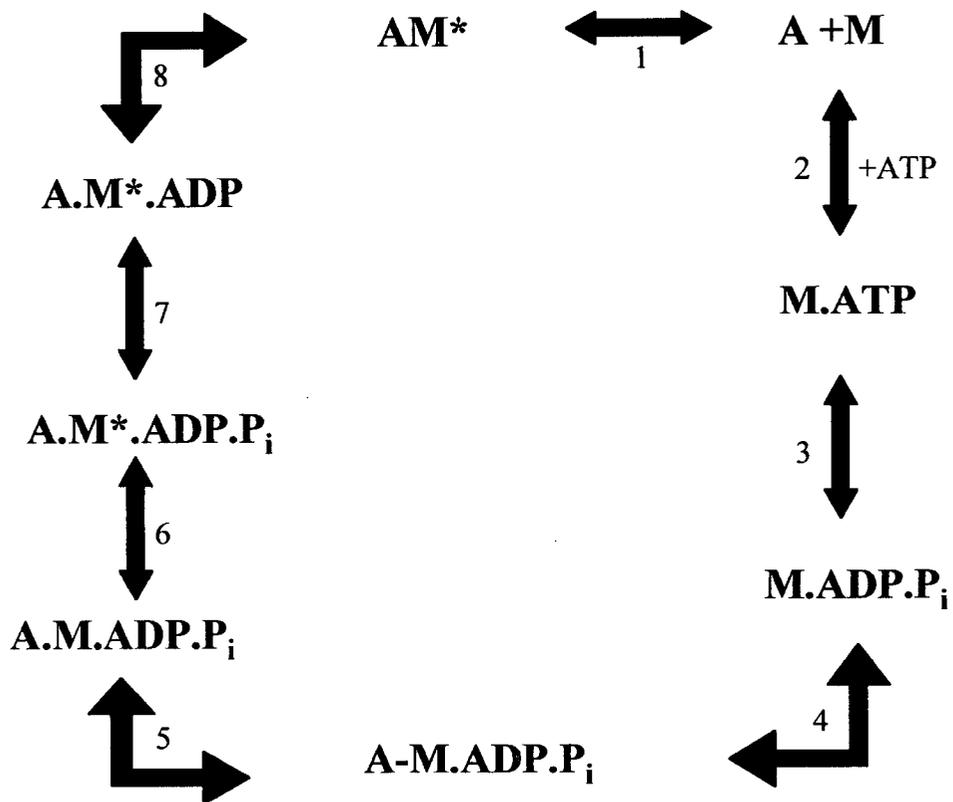
Figure 26. The cross-bridge cycle modified to include a strongly bound non-force producing step

This diagrammatic representation of the cross-bridge cycle illustrates the cyclic attachment and detachment of myosin (M) to the actin (A) filament. For clarity, the figure only includes the major steps in the pathway as they relate to the current findings. An asterix is used to identify force producing states. Included in this diagram is a strongly-bound, non-force producing (shown in blue) step prior to phosphate release (step 5). This step is proposed to be dependent on the calcium binding properties of TnC and therefore limit the rate at which the cross-bridges proceed to the strongly-bound force-producing state.

4.3.3 Force Redevelopment Kinetics (k_{tr})

The rate of force redevelopment provides a measure of the rate at which bridges attach and progress through to strongly attached bridges (Brenner, 1988). Brenner and Eisenberg (1986) proposed that the maximal actin-activated ATPase rate was equal to the rate of force redevelopment. In later studies, k_{tr} was shown to vary with the level of calcium and therefore the level of tension (Brenner, 1988; Metzger, 1989; Metzger and Moss, 1990). However, partial extraction of TnC to decrease force did not produce a decrease in k_{tr} (Metzger and Moss, 1991). It was therefore proposed that calcium directly affected some rate-limiting step in the transition from a non- or low-force bearing cross-bridge state to a force-producing state, excluding an active role for TnC beyond thin filament activation (Metzger and Moss, 1991).

It is interesting to note that nature has selected a threonine residue as the n-cap of the C-helix of chicken skeletal TnC but we have observed that the maximum tension with a serine residue at this position is higher than with threonine. It has been proposed that not all potential myosin-binding sites are open even at maximum tension levels (Seow and Ford, 1997). Therefore it is expected that fibres could still have the potential to be up-regulated. It has also been reported that the decrease in the level of activation in relation to TnC extraction is not a linear one (Moss, 1992). With that in mind, one possible explanation for the difference between a partially TnC extracted fibre and a fibre reconstituted with a mutant TnC protein is that the kinetics of calcium binding to native TnC remains unchanged whereas with the mutant TnC in place, they have. It is likely that the rate of cross-bridge cycling is not significantly reduced by extraction of TnC with tension levels remaining greater than 50% of P_o. Below pCa 6.0, as force decreases to

less than 50% of P_o, k_{tr} drops to a value between 1 - 2 sec^{-1} (Regnier et al, 1998). But in the case of the mutant TnC proteins, even a 15% decrease in force, as seen with the serine mutant of TnC, decreased k_{tr} by half despite activation at maximum $[Ca^{2+}]$. In other studies, the use of a calcium-insensitive TnC has shown that k_{tr} is primarily effected by calcium binding to TnC and not the calcium concentration of the activating solution (Hannon et al, 1993; Chase et al, 1994). Similarly, k_{tr} was increased by the addition of calmidazolium to the activating solution, reducing the calcium dissociation rate of TnC in submaximally activated fibres (Regnier et al, 1996).

In our current study, k_{tr} was always measured at pCa 4.0 so that $[Ca^{2+}]$ was not rate limiting. The decrease in calcium affinity was translated into a variable rate of force redevelopment that decreased in the order S > V > T > A > G. A plot of force versus k_{tr} was curvilinear (figure 15) and placed all of the mutant proteins in a range of force values equivalent to those of a control fibre activated at pCa levels between 5.97 and 5.87 (figure 16). To confirm that k_{tr} with the mutant TnC does vary in a manner equivalent to the change in the calcium, a control fibre was activated at pCa 6.0 for comparison to the glycine mutant (figure 17).

It has been demonstrated that in maximally activated fibres, the cross-bridge cycling rate determines k_{tr} while at low levels of calcium, the level of thin filament activation controls k_{tr} (Regnier et al, 1998). However, recruitment of cross-bridges and thin filament activation alone are not necessarily the only factors that increase the rate of force redevelopment at low calcium (figure 26, step 4) as seen when tension was reduced with low ATP (Regnier, 1998). At maximally activating calcium levels, changes in shortening velocity (figure 26, step 9) were found to correlate to changes in k_r while at

submaximum $[Ca^{2+}]$ they were not necessarily similar suggesting that they have different rate-limiting steps at submaximal activation. Interestingly, the mutant TnC proteins, activated at pCa 4.0 provide a range of activation levels from 85% P_o (maximum control tension) down to 59% P_o. While both k_{tr} and V_{max} decreased relative to control, the change in the rate of force redevelopment was not in the same sequence observed for the decrease in force or shortening velocity. The rate of force redevelopment with the valine mutant of TnC was faster than with the threonine mutant. The answer may lie with an earlier *in vitro* study of the TnC mutants at decreasing temperatures. In measurements using stopped-flow fluorescence analysis of calcium release from the threonine and valine mutants of TnC, it was found that as the temperature of the bath was first cooled to $16°C$ from $21°C$ (Leblanc, 1996; Leblanc et al, 2000) the faster of the two measured calcium off-rates decreased by approximately half of the original rate for both proteins with a slight increase in the fluorescence intensity of the slower rate. However, when the temperature was decreased further, first to $12°C$ then $9°C$, which more closely matches the temperature of the mechanical experiments of this study ($10°C$), the calcium off-rates of the valine mutant stabilized at 490 ± 65 sec^{-1} and 170 ± 10 (n=5) while the fast rate for threonine unexpectedly doubled to 1030 ± 100 sec^{-1} and the slower second rate remained essentially unchanged (n=5). The two rates are assumed to represent the dissociation of two calcium ions from the regulatory domain of TnC however the order of dissociation could not be assigned to a specific loop using this method of measurement. The relevance of the second rate, while still unclear, does bear further study since an increase in the amplitude of the fluorescence signal of both mutant TnC proteins was associated with the slower rate as the temperature was decreased .

The change in the calcium off-rate for the other mutants at the lower temperature is not known, but a direct comparison of the two mutants for which temperature data is available demonstrates that the change in the calcium off-rates plays an important role in the activation of the thin filament and subsequent rate of force redevelopment such that at 10°C the valine mutant with the slower rate of calcium dissociation has a faster k_{tr} than the threonine mutant.

Since other steps in the cross-bridge cycle can affect k_{tr} including the tension generating isomerization step (Regnier et al, 1995, 1998; Wahr et al, 1997) and the cross-bridge dissociation step as seen at low [ATP] (Regnier et al, 1998) it is still possible that the effect of the mutant proteins on k_{tr} is indirect.

With the addition of 10mM P_i to the maximally activating calcium solution (pCa 4.0), tension is decreased even more than that observed without Pi, but k_{tr} is increased (preliminary experiments with the valine (Figure 27) and glycine mutants of TnC). Others have reported similar increases in k_{tr} with decreased tension upon addition of phosphate to the activating solution (Metzger, 1996; Metzger and Moss, 1991). In a sample experiment to study the effect of Pi on k_{tr}, a control fibre was first activated at pCa 6.0 and k_{tr} was observed to decrease by an amount proportional to that observed in fibres that had been reconstituted with the glycine mutant of TnC. These latter fibres generated a maximum tension equivalent to control fibres activated at pCa 5.97, sufficiently close to pCa 6.0 to be able to make a direct comparison of k_{tr} relative to the maximum tension of control fibres (figure 17). An attempt was made to measure k_{tr} with control fibres at pCa 6.0 with 10mM P_i added. It was not possible to measure force redevelopment since there was no measurable force even after several minutes in the
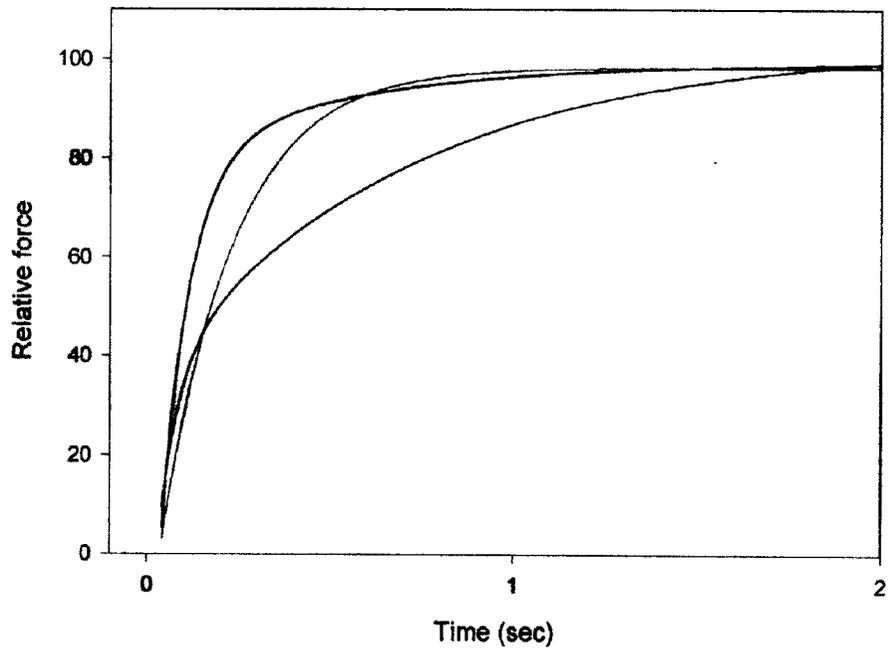
Figure 27. Force redevelopment of a fibre reconstituted with the valine mutant of TnC, activated with and without 10mM Pi.

The lines represent the double exponential fits to the data points (not shown).

Black: Control fibre prior to extraction of the native TnC.

Red: Fibre reconstituted with valine mutant and activated at pCa 4.0.

Blue: Fibre reconstituted with valine mutant with 10mM Pi added to activating solution.

activating solution. Conversely, fibres reconstituted with the glycine mutant generated an equivalent level of force to control fibres at pCa 6.0, with a measurable rate of tension redevelopment in the presence of 10mM P_i. Therefore, the mechanism by which force was decreased in both of these fibres differs such that the mutant TnC proteins not only influence thin filament upon calcium binding but must also affect one or more later steps in the cross-bridge cycle. Extraction or phosphorylation of LC2 is known to increase both the calcium sensitivity of tension and k_{tr} but is not an issue (figure 8). In the absence of added Pi, both tension and k_{tr} decrease. However when 10mM P_i was added to fibres with the mutant TnC (Figure 27), tension decreased while k_{tr} increased. The k_{tr} traces without added Pi could be fit with either a single or a double exponential function but a double exponential fit has been proposed to be an artifact (Brenner and Eisenberg, 1986). The k_{tr} traces with added Pi could only be fit with a double exponential function suggesting the possibility that there are two distinct phases reporting on two separate steps in the rate of force redevelopment.

It has been suggested that since k_{tr} can be accelerated by increasing [Pi] at all calcium concentrations that the calcium sensitivity of k_{tr} may stem from the calcium sensitivity of the phosphate release step itself or a step that is in equilibrium with it. In rabbit psoas fibres the decrease in maximum Ca^{2+}-activated tension seen with increased Pi is accompanied by an increase in k_{tr}, which leads to the prediction that the Pi release step is likely coupled to the rate-limiting step of k_{tr}. Further increases in Pi cause the effect to become saturated indicating that when excess Pi is added, a different, earlier step in the cross-bridge cycle limits k_{tr} (Wahr et al, 1997). However there is much disagreement in the literature about the calcium sensitivity of phosphate release (Walker

et al, 1992, 1997; Millar and Homsher, 1990) since the step(s) that precede Pi release are thought to be in rapid equilibrium with weak binding states.

Therefore, even though fibres reconstituted with the mutant TnC proteins appear to mimic a low level of calcium activation in the presence of maximum $[Ca^{2+}]$, they do not respond in the same manner as fibres activated at a corresponding low $[Ca^{2+}]$ in the presence of phosphate precluding the idea that it is simply a reduction in thin filament activation.. The rate of force redevelopment can be affected by several steps at the beginning of the cross-bridge cycle, as seen by others (Regnier et al, 1995, 1998; Wahr et al, 1997) including activation at low [ATP] (Regnier et al, 1998). From our results it is apparent that in the presence of phosphate, that at least two different steps in the cross-bridge cycle are controlled by calcium binding to TnC since the rates are variable and dependent on the amino acid substitution.

4.3.4 Unloaded Velocity of Shortening

The observed change in the kinetics of the cross-bridge cycle suggested the need to investigate the rate of detachment of the cross-bridges. The slack test measurements compared the shortening velocity of skinned skeletal muscle fibres with native TnC to fibres reconstituted with each of the mutant TnC proteins. Shortening velocity plots of fibres with native TnC were well fit with a single straight line. However, reconstitution with the mutant TnC proteins produced bi-phasic plots at pCa 4.0, with an early fast phase that did not vary greatly from one mutant to the next and so appeared to be independent of calcium activation as reported by others (Moss, 1986, Metzger and Moss, 1988; Martyn et al, 1994). The later slow phase of shortening did vary with activation,

which has also been previously reported (Metzger and Moss, 1988; Hofmann et al, 1991; Martyn et al, 1994).

A loss of LC2 might be suggested to have occurred during the extraction protocol to account for the decrease in V_{max} (Hofmann et al, 1991) however the calcium sensitivity of the fibres is right shifted, not left-shifted as would be observed if LC2 had been extracted.

It was originally proposed that the isotonic shortening of an activated fibre itself induced the loss of strong, force producing bridges and that the shortened sarcomere length with a reduced number of attached cross-bridges deactivated the thin filament as calcium was released from TnC (Ridgway and Gordon, 1984; Gordon and Ridgway, 1987). X-ray diffraction studies (Kress et al, 1986) showed the position of tropomyosin to be maintained by calcium alone, independent of the number of attached cross-bridges. This meant that a decrease in the number of attached bridges would not be sufficient to de-activate the thin filament during a release or during shortening. If this were the case, then even maximally activated fibres would become deactivated as the fibre shortened and biphasic shortening would be observed, but it is not. The shortening velocity of maximally activated control fibres is observed as a single fast phase (figure 21). Recent work has confirmed that in skeletal muscle fibres, calcium is not released from TnC as the fibre shortens (Martyn and Gordon, 2001). The use of a rhodamine labeled TnC demonstrated that the thin filament of skeletal muscle (unlike cardiac muscle) is not deactivated since Ca^{2+} is not released from TnC due to shortening of the fibre nor does it produce a conformational change in TnC to cause deactivation (Martyn and Gordon, 2001, Martyn et al, 2001).

Biphasic shortening is observed under several conditions. The shortening velocity of skinned skeletal fibres becomes biphasic when fibres are activated at submaximal $[Ca^{2+}]$ (Moss, 1986; Metzger and Moss, 1988; Martyn et al, 1994), by partial extraction of native TnC(Moss 1986) or by the addition of excess MgADP (Metzger, 1996) to the activating solution. At low calcium, the bi-phasic shortening has been characterized as having a relatively fast, calcium-insensitive shortening phase, followed by a slower, calcium-sensitive phase. With partial extraction of TnC the same two phases appear. For both conditions, the addition of phosphate increases the shortening velocity of the slow phase to produce a single rate equivalent to the fast phase of shortening. This suggests that the rate limiting step causing the slow phase occurs before the power stroke and the release of Pi. The addition of excess MgADP also produces bi-phasic shortening. Under these conditions fibres exhibit an apparent increase in calcium sensitivity with a leftward shift of the force-pCa curve as well as an increase in the maximum tension due to the formation of long lasting rigor bridges as ATP and ADP compete for the same binding site on myosin (Metzger, 1996). The addition of Pi has no effect, which means that under conditions of high [MgADP], the rate-limiting step follows the release of Pi in the cross bridge cycle.

In the current experiments, a slight increase in the shortening velocity was observed with the addition of 10mM Pi to the control fibres. The rate of the slow phase in reconstituted fibres however was mutant dependent and in all cases the biphasic nature of the plots was not relieved by the addition of 10mM Pi. We have observed a lowered activation of the thin filament by the mutant TnC proteins due to the reduction of the number of attached cross-bridges as measured by the tension transients, in the same way

activation is decreased as [Ca^{2+}] decreases. However this is where the similarity ends since the addition of 10mM P$_i$ does not relieve the biphasic shortening of fibres reconstituted with the mutant TnC proteins.

Metzger (1996) observed biphasic plots when partial extraction of TnC was used to decrease the maximum tension of the fibres for comparison to fibres activated at low calcium concentrations. The inability of phosphate to relieve the bi-phasic nature of the V$_{max}$ plots of the mutant TnC proteins means that the mutants do not mimic a simple calcium effect with their reduced calcium affinity. The simple explanation of incomplete reconstitution parallels partial extraction of TnC so that the decrease in V$_{max}$ would have been reversed by the addition of phosphate. Elevated Pi does not affect calcium binding to troponin-C which means that the decrease in force is not due to the calcium deactivation of the fibre (Kentish and Palmer, 1989; Palmer and Kentish, 1994).

The addition of phosphate should shift the population of attached cross-bridges toward low force-generating states (Hibberd et al, 1985). The observation that elevated Pi causes a greater decrease of force than stiffness (Hibberd et al, 1985; Kawai et al, 1987; Brozovich et al, 1988; Martyn and Gordon, 1992) is consistent with the idea that Pi release is coupled to a transition between two strongly attached states, one of which does not produce force (figure 26, step 6). By mass action, the higher Pi concentration would also increase the number of phosphate bound bridges, decrease the rate of release from A-M.ADP.Pi (figure 26, step 5) and as a result reduce the steady-state isometric tension. The limited progression of the cycle through to force generation and the power stroke would result in a build-up of weakly bound, non-force producing cross-bridges (A-M, A-M.ATP and A-M.ADP.Pi) not usually seen at ionic strengths of 200mM. According to

Pate and Cooke (1979), the decrease in the available free energy of hydrolysis of ATP would account for the decrease in force. As a result a decrease in the fast phase of shortening velocity is observed.

It has been proposed that a branch pathway (Kawai, 1987, 1988) within the cross bridge cycle allows the release of cross-bridges without the production of force in the presence of excess Pi. A decrease in force is observed but these cross-bridges still contribute to the stiffness measurement so that the stiffness decreases less than the force. Even though k_{tr} decreases as a result of the slowing of the main pathway through the cross bridge cycle the rate is accelerated through the branch pathway such that the measured k_{tr} actually increases. This is speculated to be the mechanism by which TnC extracted, or low-calcium activated fibres, demonstrate an increase in shortening velocity.

In a recent study using NEM-S1 (Swartz & Moss, 2001) the calcium dependence of V_{max} was altered such that slack test plots at submaximal $[Ca^{2+}]$ that were biphasic became linear. However, V_{max} was only about 75% of the velocity measured at maximal calcium levels. Swartz and Moss (2001) describe the effect of NEM-S1 in terms of these non-force producing but strongly bound bridges activating the thin filament to a state that allows the rate of cross-bridge detachment to be increased.

However, with the mutant TnC proteins in the fibre, the rate of the slow phase of shortening does not increase, suggesting that the addition of excess phosphate may limit both the forward progression through the cross-bridge cycle as well as the proposed branch pathway, perhaps as a result of a newly imposed rate-limiting step. The result is a decrease in force because the progression from the weakly bound state through to a force producing state is inhibited. Alternatively, if there was an increase in the number of

weakly bound cross-bridges, stiffness would increase due to an increase in pre-power stroke bridges that cannot proceed through the cycle through to the power stroke, and slow the shortening velocity.

At low levels of calcium activation, the use of NEM-S1 increases the number of strongly bound cross-bridges in a calcium independent manner, to increase the effective level of thin filament activation (Swartz & Moss, 2001). The resulting increase in the rate of cross-bridge detachment observed with NEM-S1 may be indicative of the need for a thin filament state that can only be acquired with strongly bound cross-bridges. If the low-velocity phase arises as a result of a decrease in the critical number of strongly bound cross-bridges, then the decrease in the number of bound cross-bridges with the mutant TnC proteins provides further evidence for this model.

Phosphate release is reported to be modulated by $[Ca^{2+}]$ (Walker et al, 1992). although it is not believed to be the main point in the cycle where Ca^{2+} regulation occurs (Millar and Homsher, 1990). I am proposing that the calcium regulation of phosphate release occurs via calcium binding to troponin C and its effect on the transition from a strongly-bound non-force producing state to a force producing state (figure 26, step 6).

The mutant TnC proteins, with an attenuated calcium affinity, appear to play a pivotal role in regulating the rate at which cross-bridges detach. It is apparent that the rate is variable and dependent on the mutant present in the fibre and unchanged by the addition of phosphate. This suggests that the rate of shortening velocity is controlled in part by the properties of TnC present in the fibres. If the bound cross-bridges are stalled in a pre-force producing state this suggests that TnC may also inhibit the cooperative binding of additional strongly bound cross-bridges.

Since the decrease in the slow phase of the shortening velocity with the mutant TnC proteins is insensitive to Pi the possibility of a rate-limiting step occurring after the release of Pi also exists. That leaves the release of ADP since [ATP], at 5mM, is not rate limiting. If ATP had been rate-limiting, the control fibres would also have demonstrated biphasic shortening as [MgADP] increased as well as an increase in force due to the accumulation of rigor cross-bridges (Pate and Cooke, 1989). We can however consider ADP release or isomerization as the rate-limiting step in ATP hydrolysis (Dantzig et al, 1991; Siemankowski, 1986) since these bridges are unavailable for re-attachment to the thin filament until they are first released from actin.

Using the Eisenberg-Greene model (1980) the release of ADP is strain dependent. The cross-bridge in the 90° conformation detaches from actin more rapidly than the post-sliding, positively strained, 45° conformation. Rather than each step being linked to a separate biochemical step, the processivity of the cross-bridges through the cycle of attachment and detachment can be viewed as an oscillation between weak- and strong-binding states as the interaction with actin changes. This simple alternating cycle performs mechanical work because the rate constants in the cycle are assumed to be sensitive to the mechanical strain in the cross-bridge (Eisenberg and Greene, 1980).

Since the maximum tension observed with the mutant TnC reconstituted fibres is actually decreased, and not increased by the accumulation of rigor bridges, this would suggest that the strain on the bridges following Pi release is counterproductive. That is, it is either not relieved to allow sliding of the thick and thin filament relative to each other and the bridges are delayed in their positively strained 45° orientation, or they do slide but are constrained by the lattice in the 90° negatively strained orientation. According to

the Eisenberg-Greene model however, the exchange of ADP for ATP would be very rapid and prevent the occurrence of negative work. Again, since [ATP] is not rate limiting, there is no reason to believe that the bridges would be remain negatively constrained.

The basis of the induced biphasic shortening of fibres in general is not known but it has been suggested that axially compressed cross-bridges are formed and become strained. This increased strain could then oppose the sliding of the thick and thin filaments during shortening. (Moss, 1986; Metzger and Moss, 1988). A study of the effects of phosphate and ADP on the shortening velocity at both submaximal and maximal calcium activation suggested that the biphasic plots are relieved because added Pi inhibits the formation of cross-bridges that bear a compressive strain by preferentially binding to them (Metzger, 1996). On the other hand MgADP detains cross-bridges in strong binding states and cause an increase in the population of cross-bridges bearing a compressive strain. It is therefore possible to alter the populations of strained cross-bridges by adding Pi or MgADP and alter the internal load within the fibre to affect the shortening velocity (Metzger, 1996).

If the low velocity phase is controlled by the accumulation of cross-bridges bearing compressive strain such that g_2 is decreased, and the affinity of this AM'.ADP state for Pi is increased with increasing strain, then the addition of excess Pi should decrease the formation of these strained cross-bridges and V_{max} should increase (Webb et al, 1986). This however has not been consistently observed with fibres during maximal calcium activation where added Pi has no effect on shortening velocity despite the hypothesized formation of negatively strained cross-bridges under these conditions

(Huxley, 1957). In our current study the addition of Pi did result in a very small increase in the shortening velocity of control fibres but not in fibres reconstituted with the mutant proteins. Metzger hypothesized that there is a qualitative difference in the negatively strained cross-bridges that arise during maximal compared to submaximal activation and that phosphate preferentially inhibits formation of the long-lived-type cross-bridges thought to arise during submaximal activation alone. As seen with k_{tr}, the mutant TnC proteins mimic submaximal activation, but the response of the fibres cannot simply be explained as low $[Ca^{2+}]$ effect. Our results show that either the formation of long-lived bridges is not inhibited by phosphate or that the slow phase of shortening is not simply the result of long-lived bridges.

With the addition of phosphate to the activating solution the initial velocity of shortening also begins to decrease in a mutant dependent manner. This result differs from fibres activated at submaximal $[Ca^{2+}]$ where added phosphate had no effect on the high velocity phase of shortening. It would seem that rather than relieving the load, the apparent load increases with Pi and the fast phase of the biphasic shortening, Vo_1, decreases as well. If the fast phase of shortening represents the ATPase rate which is myosin dependent, this result generates an apparent contradiction if V_{o1} and k_{tr} both represent the rate of ATP hydrolysis. It is more likely that they represent different kinetic steps in the cycling of bridges between the low force states and the high force states. In other work (Metzger, 1996) the addition of 5mM MgADP also slowed the fast phase of shortening velocity, speculated to be the result of competition between ADP and ATP for the nucleotide-binding domain of myosin. There is no reason to expect a direct effect of the mutant TnC on the ATPase rate, therefore the decrease in V_{o1} is likely a reflection of

a decrease in the overall processivity of the cross-bridge cycle due to the accumulation of products of the ATPase cycle.

A decrease in the rate of the ATPase cycle could decrease the rate of shortening and decrease the distance shortened before an internal load, originating with TnC, decreases the shortening velocity further. It has been suggested (Metzger, 1996) that the break point is a length dependent change in calcium sensitivity related to the duration of the sliding of the filaments. This condition of decreased calcium sensitivity exists sooner with the mutant TnC proteins and is enhanced as the fibre shortens. It is interesting to note that in the case of the serine mutant, the break point to the slow phase of shortening does not change with P_i since fibres reconstituted with the serine mutant do not demonstrate a length dependent calcium sensitivity.

5. CONCLUSION

If TnC functioned as a simple switch during initial activation of the thin filament, it would be expected to influence calcium sensitivity but not cross bridge cycling. Even though the calcium off-rates of the low-affinity calcium-binding sites of TnC are much faster than the time scale for the cross-bridge cycle, the overall equilibrium for calcium binding is shifted such that calcium is bound less often with the mutant than it is with the native TnC.

The mutant TnC causes a decrease in thin filament activation and decreases the progression through the cross bridge cycle so that both k_{tr} and V_{max} decrease. In the absence of Pi, k_{tr} is decreased with the same curvilinear response seen with native TnC at submaximal calcium activation. However, unlike the response at submaximal calcium where V_{max} and k_{tr} increase with Pi, with the mutant TnC, k_{tr} increases without an increase in V_{max} when 10mM Pi is added, showing that V_{max} and k_{tr} have different rate limiting processes.

Phosphate release is the energy transferal step for filament sliding to occur, but by simple mass action the excess Pi backs up the rate of cycling from the attachment of bridges to the strongly bound non-force producing state as demonstrated by the greater decrease in force than stiffness. This decreases the energy available creating an internal resistance to shortening. Since the bridges do not progress through Pi release they are not available for re-attachment, slowing V_{max}. If ADP is not released then ATP cannot bind to myosin, slowing the rate of ATP hydrolysis and we see a decrease in V_{ol}. Since V_{ol}

decreases while k_{tr} increases they are reporting on different rate limiting steps in the ATPase cycle.

It is possible that the formation of strongly attached, non-force producing bridges are inhibited from proceeding through some rate limiting isomerization to the high force state which stalls ADP release and decreases V_{max}. The maximum shortening velocity. would be expected to increase if a branch pathway existed to bypass the rate limiting step. The cross-bridges appear to be cycling more rapidly from the weakly bound state to a strongly bound state as evidenced by the increase in k_{tr}. However, fewer myosin-binding sites are available because of the presence of the mutant TnC so that even when they do detach the rate of re-attachment is slowed.

It is apparent that TnC plays a role not only in thin filament activation but also in the kinetics of cross-bridge cycling. TnC proteins not only regulate thin filament activation but must also have a downstream effect on one or more steps in the cross-bridge cycle which may provide some insight into isoform dependent rates across fibre types.

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Appendix 1. Measurement of Resonance Frequency of the Force Transducer

The resonant frequency of the force transducers used for the mechanical experiments was measured as follows. A stiff member made of 3/1000" stainless steel wire was attached between the force transducer and the servo-motor, ensuring that there was no compliance at the ends. A sine wave of adjustable amplitude was fed into the servo-motor using a function generator. The response of the force transducer was measured and compared to the input signal and output response of the servo-motor. The maximum response of the force transducer was the maximum frequency before signal degradation of the force transducer (figure 28 to 30).
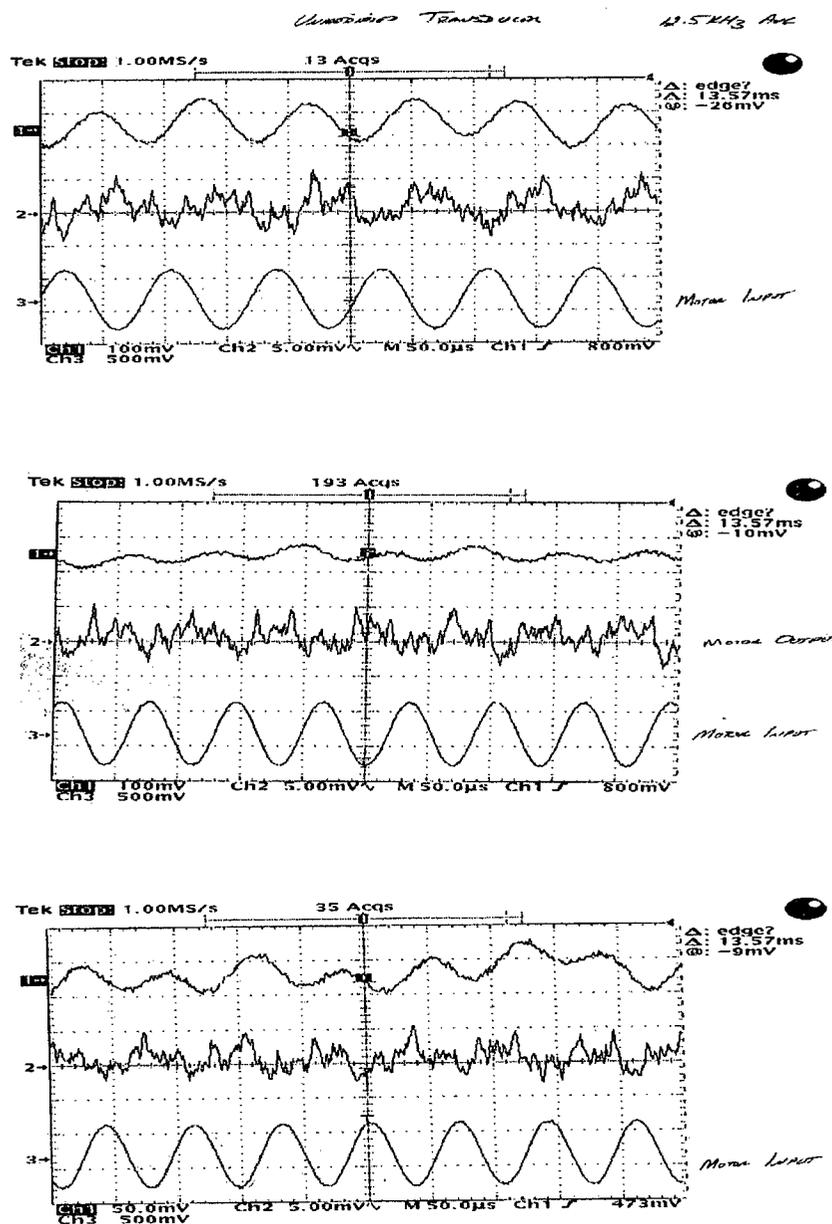
Figure 28. Measuring the resonant frequency of the force transducer with the unmodified beam.

The average resonant frequency was measured to be 12.5kHz.
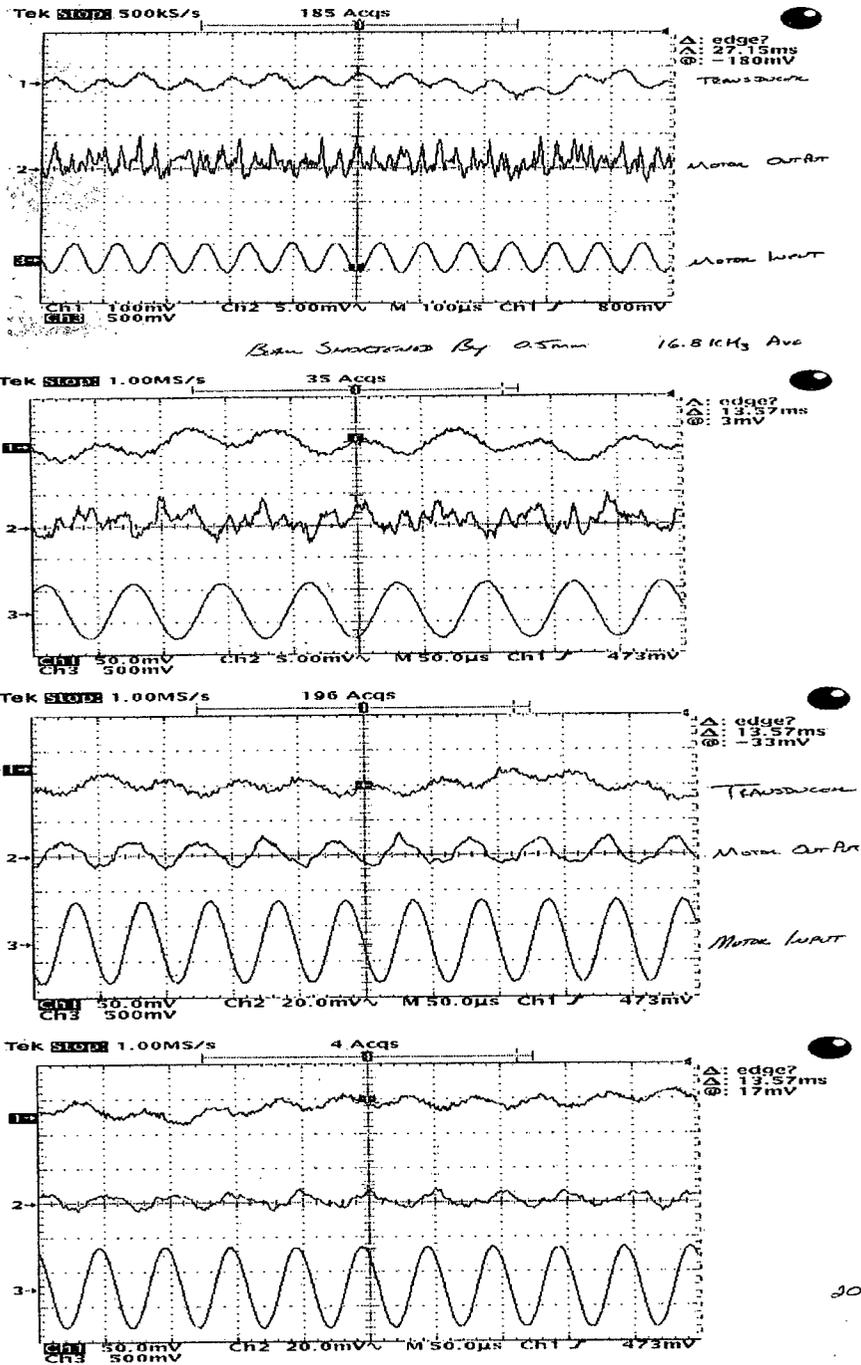
Figure 29. Measuring the resonant frequency of the force transducer with the beam shortened by 0.5mm

The average resonance frequency was measured to be 16.8kHz.
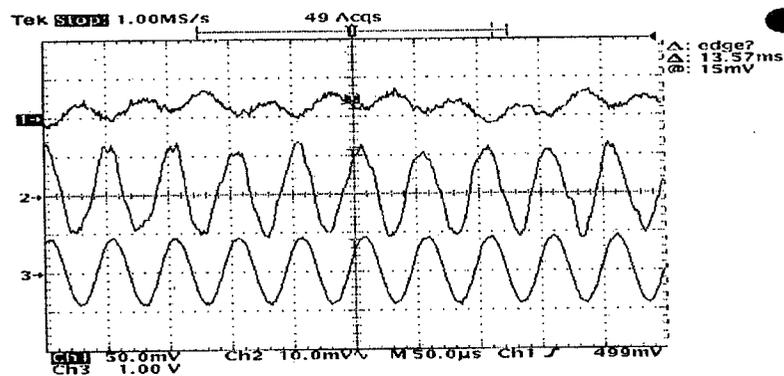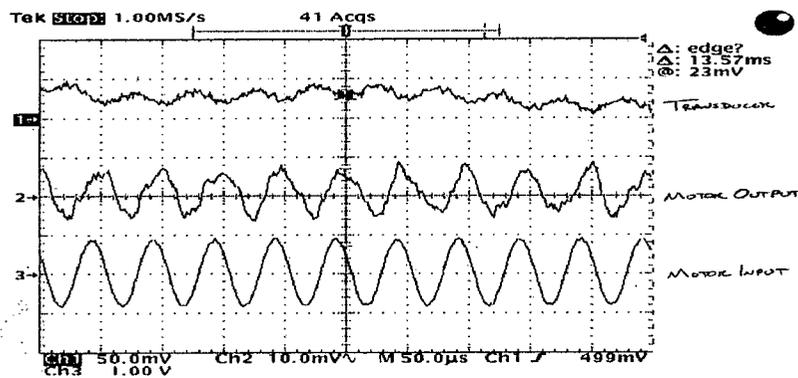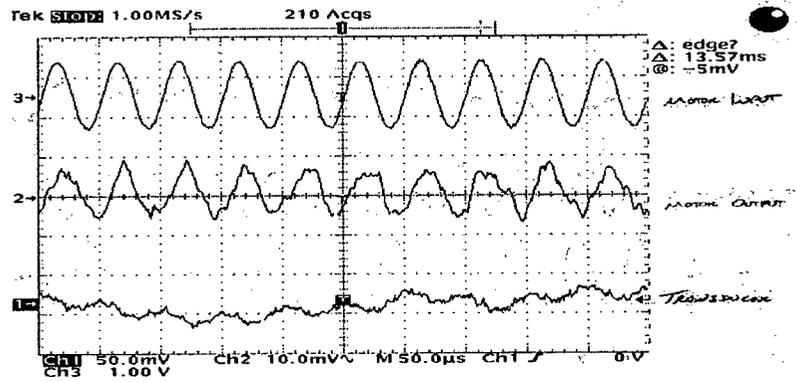
Figure 30. Measuring the resonant frequency of the force transducer with the beam shortened by 1mm.

The average resonance frequency was measured to be 24 kHz.

Appendix 2. Control Measurements Without a Fibre and With a Relaxed Fibre.

Traces were collected without a fibre mounted between the force transducer and the servo-motor as well as with a resting fibre in a bath with pCa 9.0 relaxing solution (Figure 31). A steady baseline was observed in both cases. It was confirmed that the experimental traces did not include artifactual fluctuations of the force signal.

A

B
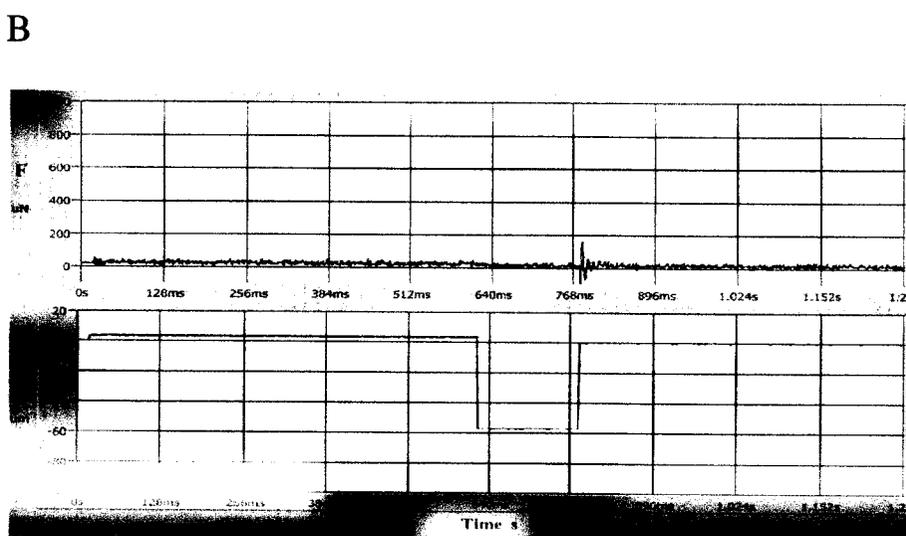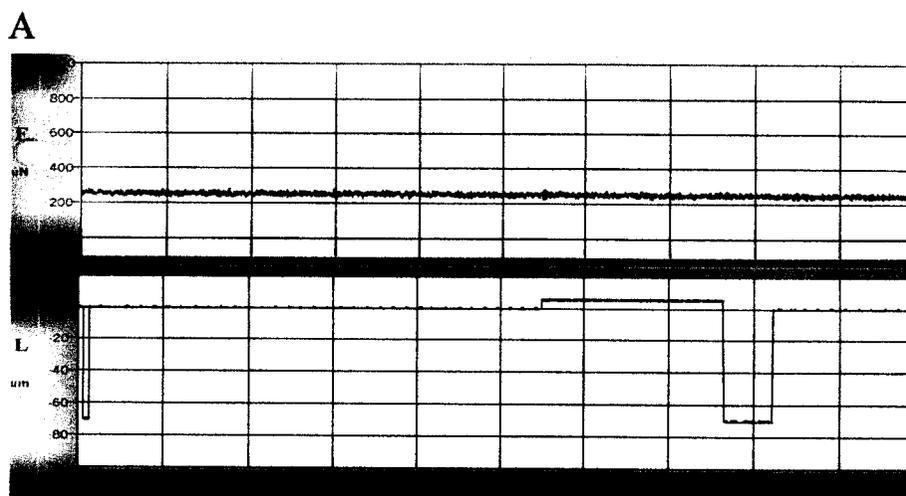
Figure 31. Control force measurements.

A sequence of releases was programmed to verify that a force signal is not generated without an activated fibre present between the motor and force transducer. Note the steady baseline throughout each trace.

Panel A: no fibre

Panel B: fixed and clipped fibre mounted in a bath at pCa 9.0. The restretch with the second large step does produce a small measurable oscillation in a stiff fibre.

Appendix 3. Results of Statistical Analysis of Force-pCa Curves with the Glycine Mutant of TnC.

Method A – Paired fibres

S.L. (μm)	Native TnC pCa_{50}	n_H	Glycine Mutant pCa_{50}	n_H
2.2	5.92 ± 0.007	6.07 ± 0.43	5.73 ± 0.03	2.28 ± 0.27
2.6	6.04 ± 0.01	3.81 ± 0.39	5.78 ± 0.02	2.47 ± 0.22
3.0	6.12 ± 0.01	2.76 ± 0.16	5.92 ± 0.01	2.01 ± 0.08

Method B – Unpaired fibres

S.L (μm)	Native TnC pCa_{50}	n_H	Glycine Mutant pCa_{50}	n_H
2.2	5.93 ± 0.006	5.01 ± 0.26	5.59 ± 0.01	2.09 ± 0.11
2.6	6.03 ± 0.008	3.42 ± 0.21	5.82 ± 0.01	2.54 ± 0.13
3.0	6.11 ± 0.01	2.79 ± 0.18	5.88 ± 0.009	2.14 ± 0.08

Average of all fibres

S.L. (μm)	Native TnC pCa_{50}	n_H	Glycine Mutant pCa_{50}	n_H
2.2	5.92 ± 0.003	4.34 ± 0.12	5.69 ± 0.01	2.26 ± 0.15
2.6	6.04 ± 0.007	3.41 ± 0.18	5.81 ± 0.01	2.33 ± 0.13
3.0	6.11 ± 0.01	2.80 ± 0.18	5.90 ± 0.008	2.09 ± 0.07

The method of analysis of variance was used to analyze the statistical significance ($p < .05$) of each pool of data. A statistical difference in the pCa_{50} value at different sarcomere lengths for both the native and glycine reconstituted fibres as well as between the control and mutant fibres at the same sarcomere length was noted. The Anova test (data shown on the next page) showed no statistical difference in the measured pCa_{50} values for the two pools of data at the same sarcomere length (ie. method A versus method B). Therefore, the measurement of the pCa_{50} values is independent of the method used.

Effect	Univariate Tests of Significance for SL2.2 (Spreadsheet1 in Workbook1) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	186.5197	1	186.5197	53007.37	0.000000
controls	0.0010	1	0.0010	0.29	0.620809
Error	0.0141	4	0.0035		

Cell No.	Tukey HSD test; variable SL2.2 (Spreadsheet1 in Workbook1) Probabilities for Post Hoc Tests Error: Between MS = .00352, df = 4.0000		
	controls	{1} 5.9000	{2} 5.9275
1	CP		0.621003
2	CU	0.621003	

Effect	Univariate Tests of Significance for 2.6 (Spreadsheet1 in Workbook1) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	291.3698	1	291.3698	174821.9	0.000000
controls	0.0002	1	0.0002	0.1	0.740859
Error	0.0100	6	0.0017		

Cell No.	Tukey HSD test; variable 2.6 (Spreadsheet1 in Workbook1) Probabilities for Post Hoc Tests Error: Between MS = .00167, df = 6.0000		
	controls	{1} 6.0400	{2} 6.0300
1	CP		0.741039
2	CU	0.741039	

Effect	Univariate Tests of Significance for 3.0 (Spreadsheet1 in Workbook1) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	358.2904	1	358.2904	101343.2	0.000000
controls	0.0000	1	0.0000	0.0	0.966428
Error	0.0283	8	0.0035		

Cell No.	Tukey HSD test; variable 3.0 (Spreadsheet1 in Workbook1) Probabilities for Post Hoc Tests Error: Between MS = .00354, df = 8.0000		
	controls	{1} 6.1100	{2} 6.1083
1	CP		0.966548
2	CU	0.966548	