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SUBSTITUTION OF CARDIAC TROPONIN C INTO RABBIT MUSCLE DOES NOT ALTER THE LENGTH DEPENDENCE OF Ca^{2+} SENSITIVITY OF TENSION

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SUMMARY

1. The isometric length–tension relationship for cardiac muscle is generally steeper than for skeletal muscle in the physiological range of sarcomere lengths. Recent studies suggest that cardiac troponin C (cTnC) may have intrinsic properties that confer greater length-dependent changes in Ca^{2+} sensitivity of tension than for skeletal troponin C (sTnC). We tested this hypothesis by characterizing tension–pCa (pCa is $-\log[Ca^{2+}]$) relationships in rabbit skinned psoas muscle fibres at mean sarcomere lengths of 2·32 and 1·87 μm both before and after partial replacement of endogenous sTnC with cTnC.

2. In untreated control fibres, the mid-point (pCa_{50}) of the tension–pCa relationship shifted to lower pCa by $0{\cdot}15 \pm 0{\cdot}02$ pCa units, i.e. became less sensitive to Ca^{2+}, when sarcomere length was reduced, and the relationship became steeper.

3. Partial extraction of endogenous sTnC and reconstitution with cTnC resulted in no change in the length-dependent shift of pCa_{50} when reconstitution with cTnC was more than 95% complete; however, when reconstitution was less than 95% complete, there were significant increases in the length-dependent shift in pCa_{50}.

4. An increase in the length-dependent shift of pCa_{50} was also observed in fibres from which sTnC was partially extracted, but no cTnC was subsequently re-added.

5. We conclude that differences in type of TnC alone are not sufficient to explain differences between skeletal and cardiac muscles in the length dependence of Ca^{2+} sensitivity of tension.

INTRODUCTION

The relationship between isometric tension and sarcomere length has been well characterized in tetanically stimulated skeletal muscle fibres in which sarcomere length was servo-controlled (Gordon, Huxley & Julian, 1966*a*, *b*). At lengths above the optimum for tension development, tension decreased due to a reduction in amount of overlap of thick and thin filaments and a consequent decrease in the number of myosin cross-bridges interacting with actin. At lengths below optimum,

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tension fell due to overlap of thin filaments with cross-bridges on the opposite end of the thick filament and at still shorter lengths due to a restoring force resulting from the compression of thick filaments against the Z-lines.

With only a few variations, similar length–tension relationships have been obtained in maximally Ca^{2+}-activated skinned preparations of both skeletal (Schoenberg & Podolsky, 1972; Moss, 1979; Julian & Moss, 1980; Allen & Moss, 1987) and cardiac (Fabiato & Fabiato, 1978; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986) muscles. However, the shape of the relationship changes considerably when Ca^{2+} is reduced to submaximal levels in skinned skeletal (Endo, 1973; Stephenson & Williams, 1982; Moss, Swinford & Greaser, 1983) and cardiac muscles (Fabiato & Fabiato, 1978; Gordon & Pollack, 1980; Kentish *et al.* 1986). At low concentrations of Ca^{2+}, tension at each sarcomere length decreases due to reduced activation of thin filaments, and the sarcomere length for optimum tension development is displaced to longer lengths. Tension has actually been observed to increase as sarcomere length is increased within a range in which the amount of thick and thin filament overlap is decreasing, indicating that Ca^{2+} sensitivity of tension is greater at long lengths. Supporting this idea, the mid-point of the relative tension–pCa (pCa is $-\log[Ca^{2+}]$) relationships in both cardiac and skeletal muscles shifts to higher pCa when sarcomere length is increased (reviewed by Allen & Kentish, 1985), perhaps as a consequence of reduced lateral separation of thick and thin filaments (Maughan & Godt, 1981; Moss *et al.* 1983; Allen & Moss, 1987).

When maximum tension at each submaximal Ca^{2+} concentration is normalized to 1·0, the ascending limb at a given level of Ca^{2+} activation appears to be steeper in cardiac than in skeletal muscle (Allen & Kentish, 1985), but the basis for this difference in not yet known. Recently, Babu, Sonnenblick & Gulati (1988) suggested that cardiac troponin C (cTnC) has intrinsic properties that give rise to greater length-dependent changes in Ca^{2+} sensitivity of tension than does skeletal troponin C (sTnC). This idea was based on a reduction of the length-dependent shift in the tension–pCa relationship of skinned cardiac muscle following replacement of endogenous cTnC with sTnC. We have tested whether cTnC alone is sufficient to account for greater length dependence of Ca^{2+} sensitivity of tension by assessing length-dependent changes in Ca^{2+} sensitivity in rabbit skinned psoas muscle fibres both before and after replacement of sTnC with cTnC. Thus, our experiment is the reverse of the one done by Babu *et al.* (1988). We find that skeletal muscle fibres containing cTnC undergo length-dependent changes in Ca^{2+} sensitivity that are similar to control fibres containing sTnC.

METHODS

Preparation

Psoas muscles were obtained from adult male New Zealand rabbits which were killed by cervical dislocation. Bundles of approximately fifty fibres were stripped free while in relaxing solution and were then stored for up to 21 days at −22 °C in relaxing solution containing 50% (v/v) glycerol (Moss, Giulian & Greaser, 1985). Individual fibres were pulled from the end of a bundle and mounted in an experimental chamber containing relaxing solution, so that a 2–3 mm segment was exposed between the connectors. Sarcomere length of the relaxed fibre segment was adjusted to 2·5–2·6 μm by changing overall segment length. Sarcomere length in the relaxed fibre was

determined from light photomicrographs of a central region of the fibre. The force transducer, motor and solution-changing device have been described previously (Moss, Giulian & Greaser, 1982).

Solutions

Fibre segments were activated in solutions containing concentrations of free Ca^{2+} between pCa 6·50 and pCa 4·50. The solutions contained 7 mM-EGTA, 1 mM-free Mg^{2+}, 20 mM-imidazole (pH 7·00), 4·42 mM-Na_2-ATP, 14·5 mM-creatine phosphate, various free Ca^{2+} concentrations, and KCl to adjust ionic strength to 180 mM. The pCa of relaxing solution was 9·0. The calculator program of Fabiato & Fabiato (1979) was used to calculate the final concentrations of each metal, ligand and metal–ligand complex based on the stability constants listed by Godt & Lindley (1982). The apparent stability constant for Ca^{2+}–EGTA was $2{\cdot}39 \times 10^6$ M^{-1} at 15 °C, pH 7·00, and an ionic strength of 180 mM.

Proteins

Skeletal TnC was purified from rabbit muscle (Greaser & Gergely, 1973). Cardiac TnC was obtained from bovine hearts using the procedure of Szynkiewicz, Stepkowski, Brzeska & Drabikowski (1985). Proteins were de-salted, freeze-dried and stored at −80 °C before use.

Measurement of tension

At each pCa, steady tension was allowed to develop, upon which the segment was rapidly (within 1 ms) slackened and then relaxed. The difference between steady tension and the tension baseline immediately after the slack step was measured as total tension. Active tension was determined as the difference between total tension and resting tension (< 1 mg) measured in relaxing solution at the same length. To facilitate rapid activation of the fibre segments and maintain striation uniformity during repeated activations, the fibres were bathed in a solution containing 6·9 mM-HDTA (1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid) and 0·1 mM-EGTA just prior to activation, a technique modified from Moisescu (1976). Tensions (P_r) at submaximally activating pCa are expressed as a fraction of P_0, the active tension at pCa 4·50 measured at the same length and under the same condition (i.e. control, extracted or cTnC recombined). Every third or fourth contraction was performed at pCa 4·50 in order to assess any decline in fibre performance (Moss *et al.* 1985).

The form and mid-point (pCa_{50}) of tension–pCa relationships were determined by Hill plot analysis of the data (Shiner & Solaro, 1984). Data were generally not well fitted by a single Hill equation, so that instead separate straight lines were fitted to tension data above and below $0{\cdot}5P_0$, as previously described (Moss *et al.* 1983). Slopes of the phases of the Hill plot were calculated as n, which is analogous to the Hill coefficient. pCa_{50} was determined as the lesser of the abscissal intercepts of the two straight lines fitted to the data.

Since the numbers of mechanical measurements in the experimental protocols were extensive, data points were obtained at only four or five submaximal Ca^{2+} concentrations so that the fibres maintained structural integrity throughout an entire experiment. Even so, failure of fibres due to sarcomere length non-uniformity or sudden tearing sometime during the protocol exceeded 60%. pCa values of submaximally activating solutions were selected to emphasize tensions around $0{\cdot}5P_0$, thereby permitting accurate characterization of the mid-points of the tension–pCa relationships (i.e. pCa_{50}). This together with the relatively small number of data points collected did not permit accurate characterization of slopes of the two phases of the Hill plots.

Experimental protocols

One of two protocols was used. In each of these, tension–pCa relationships were first measured at 15 °C in the untreated control segments at sarcomere lengths of approximately 2·3 and 1·9 μm, which were assessed by photomicrographs of central regions of the fibre segments during steady activation (Moss, 1979). The relationship at 2·3 μm was always the first to be measured.

Protocol 1. Most fibres were then subjected to a protocol to partially extract endogenous sTnC and subsequently re-add cTnC. Extraction of sTnC was by published methods (Cox, Comte & Stein, 1981; Zot & Potter, 1982) in which the fibre was bathed at 15 °C in 20 mM-imidazole (pH 7·85) and 5 mM-EDTA, with the modification that 500 μM-trifluoperazine was added thereby reducing extraction time to 2–3 min (Metzger, Greaser & Moss, 1989). Following extraction, fibres were

bathed 3 × 5 min, in relaxing solution to wash them free of trifluoperazine. Similar results were obtained in fibres extracted for 60–120 min in solution that contained no trifluoperazine. We generally avoided complete extraction of endogenous sTnC since these fibres did not recover maximum tension with recombination of cTnC. Even then, only three of fifteen fibres on which the

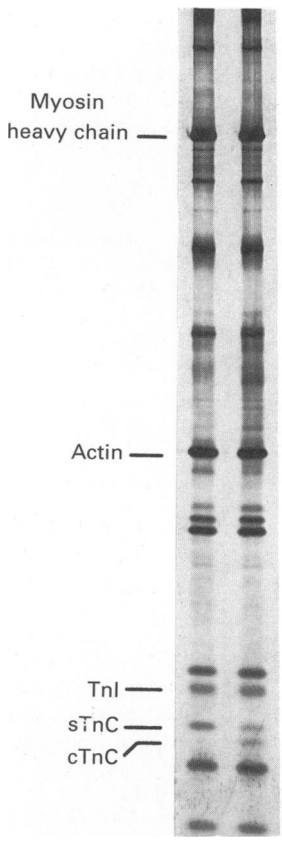

Fig. 1. SDS–polyacrylamide gel of segments taken from the same single fibre both before (lane 1) and after (lane 2) partial extraction of sTnC and recombination of cTnC. From top to bottom, horizontal lines indicate myosin heavy chain, actin, troponin-I, skeletal troponin-C (sTnC) and cardiac troponin-C (cTnC).

complete experimental protocol was performed fully recovered maximum tension upon recombination. The cTnC was recombined into extracted fibres by bathing the fibres in relaxing solution containing 0·4–0·6 mg bovine cTnC per millilitre. Fibres were subjected to repeated 10 s soaks, usually three or four, until additional soaks resulted in no further increase in P_0. This method of recombination was used in order to minimize non-specific binding of cTnC to the fibres and because we generally obtain more nearly stoichiometric recombinations than with prolonged soaks (Metzger *et al.* 1989).

Extraction of sTnC and recombination of cTnC were verified in many but not all fibres by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of segments taken from the same fibre both before and after the extraction–recombination protocols (Moss, Lauer, Giulian & Greaser, 1986). The gels were subsequently silver stained to visualize the protein bands (Fig. 1). The amounts of sTnC extracted and cTnC recombined were inferred directly from changes in

maximal Ca^{2+}-activated tension following each of these protocols (Tables 2 and 3), which assumes that the relationship between maximum tension and cTnC content is similar to the one determined previously for sTnC (Moss *et al.* 1985). The relationship between maximum tension as percentage P_0 and percentage sTnC remaining following extraction is linear down to about $0{\cdot}7P_0$, but at lower values decreases in P_0 systematically overestimate the amount of TnC extracted. Still, we chose to use percentage P_0 because gels tend to overestimate the amount of TnC re-added due to non-specific binding within the fibres, which was evident on some gels.

Tension–pCa relationships were measured in the cTnC-recombined fibres at the same long and short sarcomere lengths as before extraction.

Protocol 2. Some fibres were subjected to partial extraction of sTnC without subsequent re-addition of cTnC. Extraction was verified by SDS–PAGE of fibre segments obtained before and after extraction, and the extent of extraction was quantified based on the decrease in P_0 following extraction. Tension–pCa relationships were measured in the extracted fibres at sarcomere lengths of approximately 2·3 and 1·9 μm.

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether there were significant changes in pCa_{50} of the tension–pCa relationship or n values from Hill plots as functions of changes in sarcomere length or TnC content. When ANOVA showed significant variations, a Bonferroni t test was used to determine P values. A level of $P < 0{\cdot}05$ was chosen as indicating significance. All values are reported as mean ± standard error of the mean unless otherwise noted.

RESULTS

Length dependence of the tension–pCa relationship in untreated control fibres

Length-dependent changes in the tension–pCa relationship measured in control fibres were similar to previous results from this laboratory (Allen & Moss, 1987). The P_0 and Ca^{2+} sensitivity of tension, assessed as the pCa_{50} of the tension–pCa relationship, decreased in untreated control fibres when sarcomere length was reduced from an average of $2{\cdot}32 \pm 0{\cdot}02$ to $1{\cdot}87 \pm 0{\cdot}02$ μm (Table 1). Photomicrographs of a central region of an activated single fibre segment at long and short sarcomere lengths are shown in Fig. 2. At both lengths, Hill plots of the tension–pCa relationships could be fitted by two straight lines, which can be seen in the control data of Figs 3–5. The slope (n_1) of the line fitted to data at $P/P_0 > 0{\cdot}5$ increased when sarcomere length was reduced, while the slope (n_2) for $P/P_0 < 0{\cdot}5$ decreased (Table 1).

Effects on length dependence of Ca^{2+} sensitivity due to replacement of sTnC with cTnC

Effects of recombining cTnC into extracted fibres varied depending on the extent of reconstitution with cTnC. Based on maximum Ca^{2+}-activated tensions, reconstitution was nearly complete in some fibres but was incomplete in others despite nearly identical extraction–recombination protocols. Similar variability has been observed previously in attempts to recombine both sTnC (Moss *et al.* 1985) and cTnC (Moss *et al.* 1986) into fast-twitch fibres. With some exceptions (see Table 3), reconstitution was more likely to be complete in fibres from which less sTnC was initially extracted, assessed by a smaller reduction in P_0 following the extraction procedure. Only three extracted fibres bound enough cTnC to yield tensions greater than 95% control P_0, and in the early stages of this work many fibres were discarded when it was discovered that recombination was incomplete.

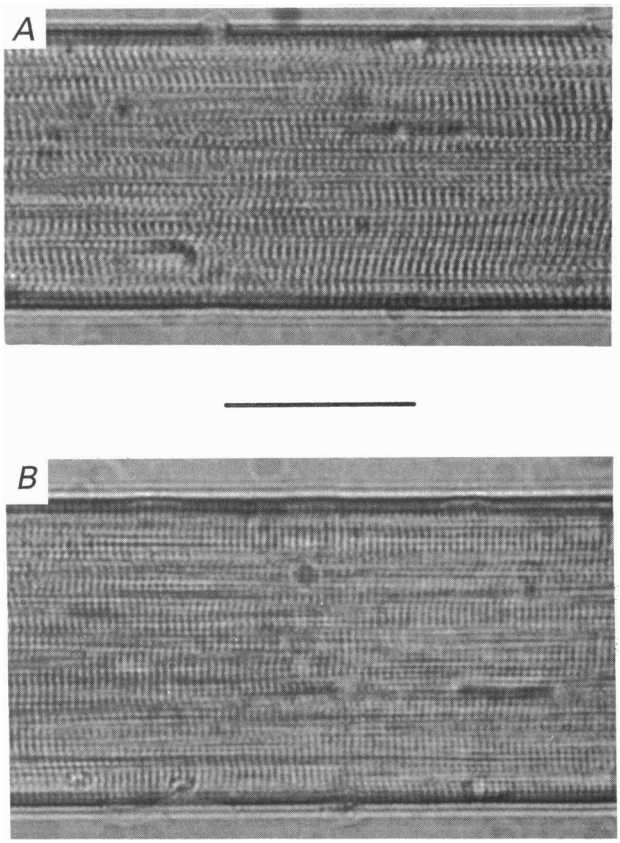

Fig. 2. Light photomicrographs of a single-fibre segment during maximal activations at long and short sarcomere lengths. Mean sarcomere lengths are 2·45 μm in part *A* and 1·95 μm in part *B*. Calibration bar indicates 50 μm.

TABLE 1. Characteristics of control fibres at long and short sarcomere lengths

	Sarcomere length (μm)	pCa_{50}	n_1	n_2	P/P_0
Control long ($n = 23$)	2·32 ± 0·02	6·03 ± 0·01	1·81 ± 0·07	11·5 ± 0·7	1·00
Control short ($n = 23$)	1·87 ± 0·02	5·88 ± 0·01***	2·16 ± 0·08**	9·4 ± 0·5*	0·80 ± 0·01

Asterisks indicate significant differences between corresponding values at long *versus* short lengths: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

In fibres in which recombination with cTnC was greater than 95% complete, the length-dependent decrease in pCa_{50} of the tension–pCa relationship was unchanged (Tables 2A and 3A). The pCa_{50} for one fibre (Fig. 3) decreased by 0·14 pCa units when sarcomere length was reduced from 2·27 to 1·76 μm. In this case, partial extraction

of sTnC reduced P_0 to 15% of the control value; however, reconstitution with cTnC resulted in recovery of P_0 to 96% of control. The length-dependent shift in pCa_{50} following reconstitution with cTnC was 0·12, which is similar to the shift observed in the control. Analysis of the form of tension–pCa relationships from fibres in this group indicates that on average the only significant change in the slopes of the

TABLE 2. Effects of sTnC extraction and cTnC recombination on length dependence of Ca^{2+}-activated tension

	Sarcomere length (μm)		pCa_{50}	n_1	n_2	P_0 after extraction	Maximum tension (P/P_0)
A. cTnC recombination to > 0·95 P_0 (n = 3)							
Long	2·26±0·03	Control	6·06±0·02	1·77±0·05	11·0±1·7	—	1·00
		Recombined	6·06±0·02	1·84±0·55	8·7±2·8	0·35±0·10	0·99±0·02
Short	1·86±0·05	Control	5·91±0·03	2·52±0·09	11·8±1·5	—	0·86±0·01
		Recombined	5·91±0·03	2·18±0·14	11·3±1·0	—	0·84±0·02
B. cTnC recombination to < 0·89 P_0 (n = 12)							
Long	2·35±0·02	Control	6·03±0·02	1·89±0·12	11·5±1·1	—	1·00
		Recombined	5·91±0·04	1·68±0·15	9·7±0·8	0·31±0·04	0·75±0·04*
Short	1·87±0·02	Control	5·88±0·02	2·18±0·11	9·6±0·5	—	0·78±0·02
		Recombined	5·65±0·04**	2·02±0·23	6·4±0·8	—	0·43±0·04***
C. Partial extraction of sTnC (n = 7)							
Long	2·31±0·04	Control	6·01±0·03	1·72±0·07	11·9±0·7	—	1·00
		Extracted	5·86±0·08	1·59±0·15	8·8±2·3	0·61±0·06	0·61±0·06
Short	1·86±0·03	Control	5·88±0·04	1·99±0·15	8·6±1·0	—	0·81±0·02
		Extracted	5·63±0·05*	1·58±0·14	3·1±0·3**	—	0·33±0·05***

Asterisks indicate significant differences between corresponding values from control and extracted or extracted–recombined fibres at the same length.

relationships following reconstitution was a decrease in n_1 at the shorter sarcomere length. Earlier work (Moss *et al.* 1986; Gulati, Scordilis & Babu, 1988) showed that partial replacement of endogenous sTnC in skeletal muscle fibres resulted in a decrease in the steepness of the tension–pCa relationship at lengths corresponding to the long lengths in this study. The apparent lack of effect of cTnC on steepness in the present study is presumably due to the fact that the small number of data points required to describe the mid-region of the tension–pCa relationship was insufficient to accurately characterize the slope of either phase of a Hill plot. Thus, since in many cases only two data points defined a phase of the Hill plot, there was considerable variability in determinations of n values.

Fibres reconstituted with cTnC to maximum tensions less than 95% P_0 consistently showed an increase (0·26 *vs.* 0·15 pCa unit) in the length-dependent shift in pCa_{50} of the tension–pCa relationship (Tables 2B and 3B). Data from one fibre, in Fig. 4, show that pCa_{50} decreased by 0·14 pCa units when sarcomere length was reduced from 2·38 to 1·89 μm. Partial extraction of the sTnC reduced maximum tension to 23% of control P_0, but reconstitution with cTnC was incomplete, yielding a maximum tension of only 76% P_0. In this case, the length-dependent shift in pCa_{50} was 0·31 pCa units, substantially greater than the shift observed before extraction and partial recombination with cTnC.

It is important to note that the extent of reconstitution with TnC required to achieve full recovery of Ca^{2+} sensitivity of tension may actually be less than to 95% P_0. However, it is unlikely to be as low as 88% which is the next lowest value for which we have data. Recovery to 88% P_0 resulted in a large increase in the length-dependent shift in Ca^{2+} sensitivity of tension.

TABLE 3. Effects of sTnC extraction and cTnC re-addition on length-dependent shift of pCa_{50}

Fibre	Δ Sarcomere length (μm)	Control† ΔpCa_{50}	Experimental‡ ΔpCa_{50}	P_0 after extraction	P_0 after Re-addition
		A. cTnC recombination to $> 0{\cdot}95P_0$			
504	0·48	0·24	0·24	0·44	0·98
522	0·30	0·10	0·09	0·47	1·03
524	0·51	0·14	0·12	0·15	0·96
Means ± S.E.M.	0·43 ± 0·07	0·16 ± 0·04	0·15 ± 0·05	0·35 ± 0·10	0·99 ± 0·02
		B. cTnC recombination to $< 0{\cdot}89P_0$			
407	0·52	0·22	0·26	0·57	0·68
410	0·48	0·12	0·42	0·18	0·55
411	0·45	0·12	0·26	0·26	0·65
417	0·49	0·14	0·31	0·23	0·76
418	0·47	0·09	0·18	0·35	0·85
420	0·45	0·14	0·21	0·46	0·82
426	0·61	0·27	0·23	0·36	0·85
428	0·49	0·15	0·24	0·38	0·88
505	0·46	0·15	0·24	0·33	0·84
621	0·51	0·11	0·26	0·10	0·45
731	0·42	0·13	0·17	0·18	0·87
816	0·53	0·21	0·29	0·32	0·74
Means ± S.E.M.	0·49 ± 0·01	0·15 ± 0·02	0·26 ± 0·02*	0·31 ± 0·04	0·75 ± 0·04
		C. Partial extraction of sTnC			
503	0·63	0·21	0·35	0·84	—
516	0·52	0·12	0·17	0·62	—
526	0·43	0·17	0·25	0·63	—
531	0·26	0·04	0·23	0·66	—
615	0·48	0·17	0·19	0·70	—
623	0·55	0·15	0·37	0·51	—
811	0·27	0·05	0·08	0·31	—
Means ± S.E.M.	0·45 ± 0·05	0·13 ± 0·02	0·23 ± 0·04*	0·61 ± 0·06	—

Asterisks indicate significant differences ($P < 0{\cdot}05$) between mean values of ΔpCa_{50} obtained from control and experimental fibres.

† Difference between pCa_{50} at long *vs.* short lengths before extraction of TnC.

‡ Difference between pCa_{50} at long *vs.* short lengths after extraction of sTnC (part C) or after extraction of sTnC and subsequent re-addition of cTnC.

Effects on length dependence of Ca^{2+} sensitivity due solely to partial extraction of sTnC

Results from fibres in which reconstitution with cTnC was incomplete suggest that changes in length dependence of pCa_{50} of the tension–pCa relationship may be due to a deficiency in TnC content of the fibres. To test this possibility, the length

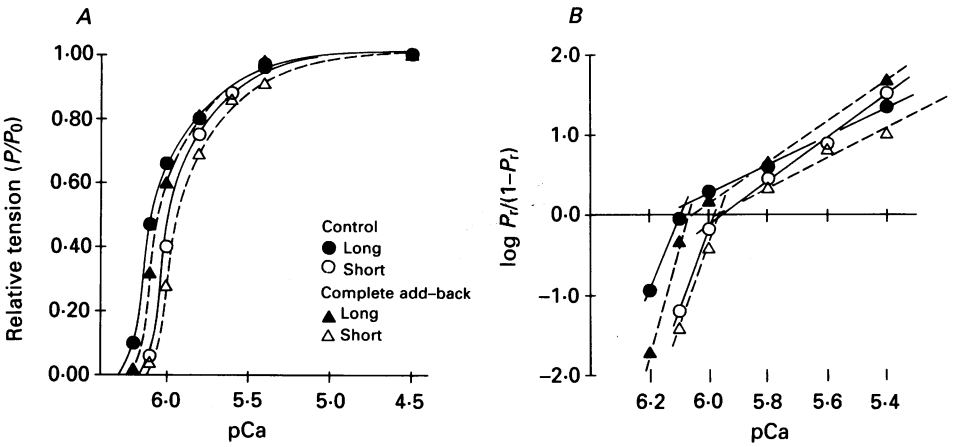

Fig. 3. Tension–pCa relationships at long and short lengths from a fibre in which add-back of cTnC was nearly complete. Relationships were obtained both before extraction (control) and following partial extraction of sTnC and add-back of cTnC. Sarcomere length at long length was 2·27 μm; that at short length was 1·76 μm. P_r = relative tension = P/P_0. Fibre no. 524 in Table 3.

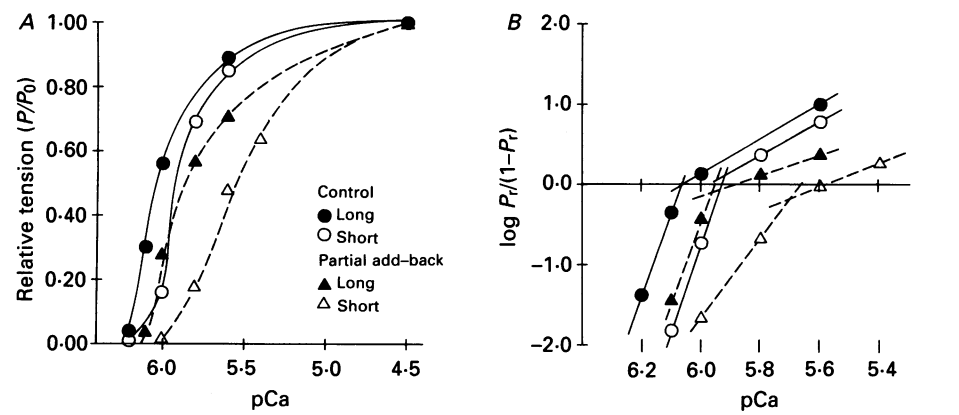

Fig. 4. Tension–pCa relationships at long and short lengths from a fibre in which add-back of cTnC was incomplete. Relationships were obtained both before extraction (control) and following partial extraction of sTnC and add-back of cTnC. Sarcomere length at long length was 2·38 μm; that at short length was 1·89 μm. Fibre no. 417 in Table 3.

dependence of pCa_{50} was assessed before and after partial extraction of sTnC, with no subsequent recombination with cTnC. Partial extraction of endogenous sTnC resulted in a decrease of pCa_{50} of the tension–pCa relationship at long lengths (Table 2C). Previous studies (Brandt, Diamond & Schachat, 1984; Moss *et al.* 1985; Babu, Scordilis, Sonnenblick & Gulati, 1987; Brandt, Diamond, Rutchik & Schachat, 1987) have shown that this effect is reversed completely by recombination of sTnC into extracted skeletal muscle fibres. The extent of extraction in the present study was purposely kept small in order to approximate the maximum tensions observed in the

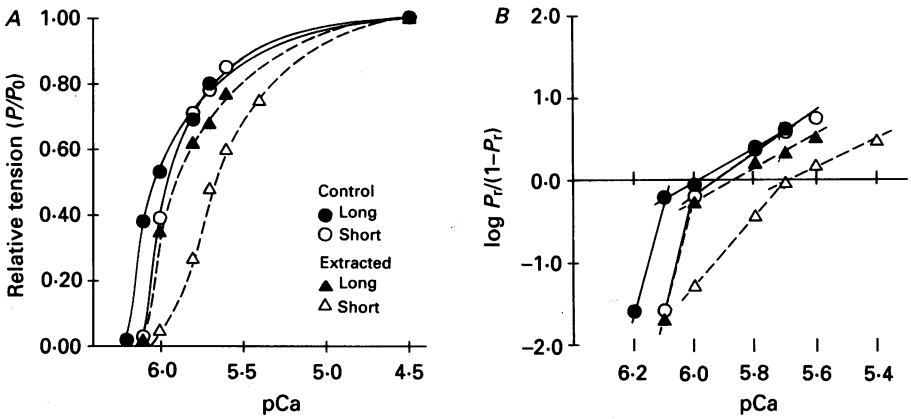

Fig. 5. Tension–pCa relationships at long and short lengths from a fibre in which sTnC was extracted but no cTnC was added back. Relationships were obtained both before and after partial extraction of sTnC. Sarcomere length at long length was 2·17 μm; that at short length was 1·91 μm. Fibre no. 531 in Table 3.

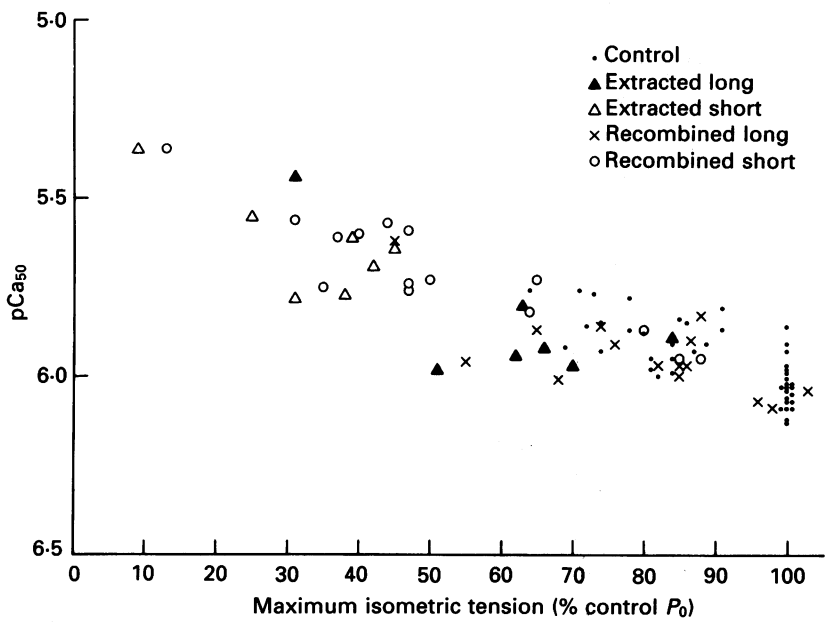

Fig. 6. Relationship between pCa_{50} and maximum tension-gathering capability. pCa_{50} was measured in control, sTnC-extracted and cTnC-recombined fibres. Maximum isometric tension for each condition is expressed as percentage control tension (i.e. P_0) measured in the same fibre at long length and pCa 4·5.

group of fibres reconstituted with cTnC to tensions less than 0·95 control P_0. For the fibre represented in Fig. 5, pCa_{50} decreased by 0·04 pCa units when sarcomere length was reduced from 2·17 to 1·91 μm, a relatively small change in length. Partial

extraction of sTnC reduced maximum tension to 66% of control P_0, and the length-dependent decrease in pCa_{50} was 0·23 pCa units, which is significantly greater than control. In every fibre tested, partial extraction of sTnC resulted in an increased length-dependent shift in pCa_{50} (Table 3C).

The length-dependent shift in pCa_{50} was analysed further by plotting pCa_{50} from control, extracted and cTnC-recombined fibres *versus* the maximum tension-generating capability under each condition, as percentage control P_0 in the same fibre (Fig. 6). The independent variable in this plot, i.e. relative tension, is assumed to be a measure of maximum possible activation of the thin filament, which varies with sarcomere length (e.g. Allen & Moss, 1987) and TnC content (Brandt *et al.* 1984, 1987; Moss *et al.* 1985). From Fig. 6, pCa_{50} decreases, i.e. Ca^{2+} sensitivity of tension decreases, as maximum isometric tension-generating capability is reduced by decreasing sarcomere length or TnC content. Linear regression analysis of the data indicated that 75% of the variation in pCa_{50} can be accounted for by coincident variation in tension-developing capability, which is similar to the findings of a recent study in which temperature was used to vary tension (Sweitzer & Moss, 1990).

DISCUSSION

sTnC and cTnC confer similar length dependence of Ca^{2+} sensitivity of tension in skinned skeletal muscle fibres

The main hypothesis addressed by this study is whether cTnC alone confers unique length dependence to the Ca^{2+} sensitivity of tension, and thereby accounts for the steeper length–tension relation in cardiac muscle compared with skeletal. Our results suggest that this is not the case, in that the length dependence of pCa_{50} in skeletal muscle fibres is similar with both cTnC and sTnC, at least within the range of sarcomere lengths studied. Extraction of endogenous sTnC to yield maximum tensions as low as 0·15 P_0 and reconstitution with cTnC to 96–103% of control P_0 resulted in no significant change in the shift in pCa_{50} when sarcomere length was reduced from about 2·3 μm by an average of 0·4 μm. However, in extracted fibres in which reconstitution was less than 95% complete, assessed from maximum tension-generating capability, the change in pCa_{50} upon reducing sarcomere length was significantly greater than the change seen in control fibres or in fibres that were reconstituted to greater than 95% of control P_0. Further experiments in which endogenous sTnC was extracted from fibres, with no re-addition of cTnC, strongly suggest that the greater length-dependent shift of pCa_{50} in incompletely cTnC-reconstituted fibres was a result of TnC deficiency and was not characteristic of cTnC re-addition.

Babu *et al.* (1988) reported a decrease in the length-dependent shift of pCa_{50} over a similar range of sarcomere lengths when endogenous cTnC of chemically skinned myocardium from hamster was extracted and replaced with sTnC. This result is not consistent with our findings; however, our results do not exclude the possibility that cTnC confers special sensitivity to length only in combination with cardiac regulatory and contractile proteins. Still, there may be other explanations for qualitatively different results in the two studies. From tension records in Babu *et al.* (1988), it is evident that reconstitution of myocardium with sTnC was incomplete,

since maximum tension was reduced following reconstitution. However, this does not explain the differences between the studies, since from present findings we would expect incomplete recombination to actually increase length-dependent shifts in pCa_{50}. Relevant to this point, we have previously shown that extractions of small amounts of endogenous cTnC from skinned cardiac myocytes caused no change in pCa_{50} at a given sarcomere length (Sweitzer & Moss, 1990).

Two procedural points deserve particular attention when considering results of experiments of this type. First, effects of TnC substitution on length-dependent changes in pCa_{50} should be determined from comparisons of tension–pCa relationships obtained in the *same* fibres both before and after extraction of endogenous sTnC and re-addition of cTnC. Variability in pCa_{50} values between fibres can result from factors such as differences in sarcomere length during activation, amounts of sTnC extracted and efficacy of cTnC re-addition. Had we compared pCa_{50} values from various fibres at different stages of the protocols for TnC replacement, our conclusions could very well have differed from the ones actually reached based on paired control and experimental data from the same fibres. In this regard, Babu *et al.* (1988) made their conclusions from comparisons of data from different fibres at various stages of their extraction and recombination protocols (see their Fig. 2). Indeed, the pCa_{50} of tension–pCa relationships obtained at their long length changed more as a result of extraction and recombination with cTnC than the actual length-dependent change in pCa_{50} following replacement of cTnC with sTnC.

Secondly, sarcomere length should be monitored during assessment of the length dependence of pCa_{50} (see Fig. 1), since Ca^{2+} sensitivity of isometric tension decreases as sarcomere length is reduced (Table 1). Undetected changes in sarcomere length during activation could lead to erroneous conclusions with regard to length-dependent effects. For example, if attachments at the ends of a fibre segment are compliant, incomplete recombination with TnC would result in a smaller length-dependent shift of pCa_{50}, since sarcomere length during steady contraction would be longer at each pCa due to a decrease in tension-generating capability.

Possible mechanisms of greater length-dependent shifts of pCa_{50} in TnC-deficient fibres

The length-dependent shift of pCa_{50} in TnC-deficient fibres increased as a function of the amount of TnC extracted. The maximum length-dependent shift in pCa_{50} was 0·37 pCa units, observed in a fibre in which maximum tension was 0·51 P_0 after partial extraction of sTnC. Previously, Ca^{2+} sensitivity of tension was shown to vary with changes in length (Endo, 1973; Stephenson & Williams, 1982; Moss *et al.* 1983; Allen & Moss, 1987; reviewed by Allen & Kentish, 1985) or TnC content (Brandt *et al.* 1984, 1987; Moss *et al.* 1985). In Fig. 5 the reduction in Ca^{2+} sensitivity due to coincident decreases in sarcomere length and TnC content was greater than the sum of the reductions when sarcomere length and TnC content were decreased independently. Thus, sarcomere length and TnC content appear to be interactive in determining Ca^{2+} sensitivity of tension. The greater length-dependent change in pCa_{50} following extraction of sTnC may be due to a reduction in co-operative activation of the thin filaments by attached cross-bridges resulting from the combined effects of decreased likelihood of cross-bridge attachment at shorter lengths (Allen & Moss, 1987) and

disruption of near-neighbour co-operativity between functional groups of the thin filament (Brandt *et al.* 1984, 1987; Moss *et al.* 1985).

Another factor that may contribute to altered length dependence of Ca^{2+}-activated tension in TnC-deficient fibres is a decrease in overall activation at short lengths due to greater extraction of sTnC from regions of non-overlap of thick and thin filaments. Gordon, Ridgway, Yates & Allen (1988) showed that sTnC is extracted at a greater rate in fibres that are stretched to long lengths. In the present study, extractions of sTnC were usually done at sarcomere lengths of about 2·5 μm or slightly greater. Assuming an I–Z–I length of 2·24 μm, thin filament length of 1·1 μm, and thick filament length of 1·65 μm in rabbit muscle (Huxley, 1963), a 0·40 μm segment of each thin filament was not overlapped by thick filaments during extraction. Reducing sarcomere length by about 0·6 μm to achieve the shorter sarcomere length would then result in overlap of 0·3 μm of this previously non-overlapped zone. If it is assumed that the terminal 0·73 μm, i.e. (1·65 μm − 0·20 μm bare zone)/2, of each end of the thick filament contains cross-bridges, total extraction of sTnC from the initial zone of non-overlap could account for a 41 %, i.e. (0·30 μm/0·73 μm) × 100, reduction in maximum tension at short lengths in fibres that were sTnC extracted but to which no TnC was re-added. However, maximum tension at short lengths decreased by an average of 59 %, i.e. $[(0{\cdot}81P_0 - 0{\cdot}33P_0)/0{\cdot}81P_0] \times 100$, in these fibres. Thus, even if preferential extraction of TnC from the ends of the thin filament was complete, it would still be insufficient to explain all of the observed reduction in maximum tension.

With regard to the Ca^{2+} sensitivity of tension, preferential loss of TnC from the original zone of non-overlap could certainly contribute to decreased Ca^{2+} sensitivity of tension at short lengths in partially TnC-extracted fibres and in fibres that were incompletely reconstituted with cTnC, since Ca^{2+} sensitivity of tension decreases with extent of TnC extraction (Brandt *et al.* 1984, 1987; Moss *et al.* 1985). In these cases, overall Ca^{2+} sensitivity would presumably be determined by a combination of reduced TnC content in the thin filament zones of original overlap and non-overlap and the physical factors discussed above.

With regard to fibres that completely recovered P_0 upon re-addition of cTnC, these fibres also recovered maximum tensions at the shorter length. Therefore, if there was preferential extraction of TnC from the zone of initial non-overlap, the thin filaments in these fibres would contain even greater amounts of cTnC than we would infer from changes in P_0 during the extraction and reconstitution procedures. Thus, the finding that there was no change in length dependence of Ca^{2+} sensitivity of tension in these fibres would reinforce our conclusion that cTnC alone is not sufficient to confer enhanced sensitivity to sarcomere length.

Mechanisms for possible differences in length–tension relationships of living skeletal and cardiac muscles

Since cardiac muscle cannot be tetanized under physiological conditions, comparisons of length–tension relationships between skeletal and cardiac muscles should be considered in terms of twitch contractions. When this is done, differences between the two muscles are less pronounced than when either relationship is compared to the relationship from tetanically stimulated skeletal muscle fibres.

Starting at optimum length, twitch tension in both muscles decreases rather rapidly when muscle length is reduced between contractions, although the fall-off of tension appears to be more rapid in cardiac (Allen, Jewell & Murray, 1974; Krueger & Pollack, 1975; Julian, Sollins & Moss, 1976; Allen & Kentyish 1985; Kentish *et al.* 1986) than in skeletal (Close, 1972) muscle. Thus, in both muscles, the length–twitch tension relationship is steeper than the length–tetanic tension relationship of frog skeletal muscle.

Two mechanisms common to both muscles probably account for most of the greater steepness of the length–twitch tension relationship. First, since skeletal and cardiac muscles do not ordinarily assume a sarcomere length much less than 1·8–2·0 μm at rest, measuring of twitch tensions at short lengths requires that the resting muscle be slackened in order to achieve short sarcomere lengths during a subsequent twitch. In these cases, much of the duration of myoplasmic Ca^{2+} transient is consumed by shortening to the desired length as the muscle takes up slack, so that the remainder of the Ca^{2+} transient is insufficient to achieve a steady-state distribution of force-generating cross-bridges at the isometric length. As sarcomere length is reduced, relatively fewer cross-bridges will attach and develop force during the truly isometric phase of contraction and peak tension will decrease. In tetanic contractions, the necessity of imposing slack in order to achieve short lengths during activation does not give rise to this problem, since a steady-state distribution of force-generating cross-bridges can be achieved. A second mechanism that appears to contribute to the fall-off of twitch tension at short lengths is shortening-induced dissociation of Ca^{2+} from TnC as the slackened muscle shortens to the shorter isometric length. In both barnacle muscle (Ridgeway & Gordon, 1984) and mammalian myocardium (Allen, Nichols & Smith, 1988), extra Ca^{2+} appears in the myoplasm when length is suddenly reduced during twitch contractions. Thus, at the beginning of the isometric phase of a twitch at short length, the level of activation of thin filaments is likely to be less than would be predicted on the basis of the Ca^{2+} transient alone.

There are several factors that may contribute to a steeper length–twitch tension relationship in cardiac muscle, some of which are discussed in detail by Allen & Kentish (1985). Recent work indicates that the Ca^{2+} transient during a twitch is reduced at short lengths in ferret ventricular muscle, which correlates with reductions in twitch tension (Allen & Kurihara, 1982; Allen *et al.* 1988). Another possibility, not previously raised, is that substantially slower cross-bridge turnover kinetics in cardiac muscle contribute to its steeper length–tension relationship. That turnover kinetics are slower appears to be the case from measurements of actin-activated ATPase activities of skeletal and cardiac myosins (Solaro & Shiner, 1976), the rates of relaxation from rigor of skeletal (Ferenczi, Homsher & Trentham, 1984; Goldman, Hibber & Trentham, 1984*a*) and cardiac (Barsotti & Ferenczi, 1988) muscles following photolytic release of ATP from caged ATP, and the rate of force development in skeletal (Goldman, Hibberd & Trentham, 1984*b*) and cardiac (Barsotti & Ferenczi, 1988) muscles following photolysis of caged ATP in the presence of Ca^{2+}. Slower cycling rates in cardiac muscle will result in the formation of fewer cross-bridges than in skeletal muscle during a given Ca^{2+} transient, and peak twitch tension will consequently be less. Also, if cross-bridges cycle more slowly, a

greater part of the myoplasmic Ca^{2+} transient will be occupied by shortening from slack length to various sarcomere lengths on the ascending limb.

Differing length–tension relationships could also arise from differences in length dependence of Ca^{2+} binding to TnC in the two muscles (Allen & Kentish, 1985). For example, Hofmann & Fuchs (1988) showed that Ca^{2+} bound to cTnC varies with sarcomere length in cardiac muscle, but this is not the case in skeletal muscle (Fuchs, 1978). From the present results, it is unlikely that cTnC is the sole mediator of this effect since the length dependence of pCa_{50} did not change when endogenous sTnC was replaced with cTnC. Additional work is required to determine whether cTnC in the presence of cardiac thin filament proteins exhibits enhanced sensitivity to length in terms of its ability to bind Ca^{2+}, which could be consistent with the results of Babu *et al.* (1988), or whether length-dependent Ca^{2+} binding is secondary to other length-dependent factors such as probability of cross-bridge binding.

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