Unloaded Shortening of Skinned Muscle Fibers from Rabbit Activated with and without Ca^{2+}

Donald A. Martyn,* P. Bryant Chase,‡§ James D. Hannon,§ Lee L. Huntsman,* Martin J. Kushmerick,*‡§ and Albert M. Gordon§

*Center for Bioengineering, ‡Department of Radiology, and §Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195 USA

ABSTRACT Unloaded shortening velocity (V_{US}) was determined by the slack method and measured at both maximal and submaximal levels of activation in glycerinated fibers from rabbit psoas muscle. Graded activation was achieved by two methods. First, [Ca^{2+}] was varied in fibers with endogenous skeletal troponin C (sTnC) and after replacement of endogenous TnC with either purified cardiac troponin C (cTnC) or sTnC. Alternatively, fibers were either partially or fully reconstituted with a modified form of cTnC (aTnC) that enables force generation and shortening in the absence of Ca^{2+}. Uniformity of the distribution of reconstituted TnC across the fiber radius was evaluated using fluorescently labeled sTnC and laser scanning fluorescence confocal microscopy. Fiber shortening was nonlinear under all conditions tested and was characterized by an early rapid phase (V_E) followed by a slower late phase (V_L). In fibers with endogenous sTnC, both V_E and V_L varied with [Ca^{2+}], but V_E was less affected than V_L. Similar results were obtained after extraction of TnC and reconstitution with either sTnC or cTnC, except for a small increase in the apparent activation dependence of V_E. Partial activation with aTnC was obtained by fully extracting endogenous sTnC followed by reconstitution with a mixture of aTnC and cTnC (aTnC:cTnC molar ratio 1:8.5). At pCa 9.2, V_E and V_L were similar to those obtained in fibers reconstituted with sTnC or cTnC at equivalent force levels. In these fibers, which contained aTnC and cTnC, V_E and V_L increased with isometric force when [Ca^{2+}] was increased from pCa 9.2 to 4.0. Fibers that contained a mixture of aTnC and cTnC were then extracted a second time to selectively remove cTnC. In fibers containing aTnC only, V_E and V_L were proportional to the resulting submaximal isometric force compared with maximum Ca^{2+}-activated control. With aTnC alone, force, V_E, and V_L were not affected by changes in [Ca^{2+}]. The similarity of activation dependence of V_{US} whether fibers were activated in a Ca^{2+}-sensitive or -insensitive manner implies that V_{US} is determined by the average level of thin filament activation and that, with sTnC or cTnC, V_{US} is affected by Ca^{2+} binding to TnC only.

INTRODUCTION

Calcium binding to the thin filament regulatory protein troponin C (TnC) initiates a series of events that ultimately allows actomyosin cross-bridge cycling in vertebrate striated muscle. Beyond simply affecting the availability of myosin-binding sites on actin, it has been suggested that Ca^{2+} regulates contraction by a mechanism that includes the modulation of cross-bridge cycling kinetics. Ca^{2+}-regulation of kinetics is suggested by reports that the rate of isometric tension redevelopment (k_{TR}) is strongly influenced by the level of Ca^{2+} activation in skinned skeletal fibers (Brenner, 1988). Another mechanical parameter, unloaded shortening velocity (V_{US}), characterizes the kinetics of the actomyosin interaction during filament sliding. In intact fibers, V_{US} is relatively insensitive to changes in filament overlap (Claflin et al., 1989; Edman, 1979; Gordon et al., 1966) and, thus, to the number of cycling cross-bridges, or the level of isometric force during steady-state activations (Edman, 1979; Gulati and Babu, 1985). Studies on skinned fibers, which allow direct control of activation level by Ca^{2+}, have led to the conflicting conclusions that V_{US} either does (Farrow et al., 1988; Julian, 1971; Julian and Moss, 1981; Julian et al., 1986; Metzger and Moss, 1988; Moss, 1986; Wise et al., 1971) or does not (Brenner, 1980, 1986; Podolin and Ford, 1986; Podolsky and Teichholz, 1970) depend on the degree of thin filament activation. Although different interpretations were made in these studies, in each case skinned fiber shortening was nonlinear and velocity decreased with time and the extent of shortening; the nonlinearity of shortening was exaggerated at low force levels.

The observation that unloaded shortening in skinned skeletal fibers occurs in an initial high velocity phase followed by slower shortening suggests a resolution to these conflicting conclusions (Moss, 1986); the initial high velocity phase of shortening, measured exclusively in several of the earlier studies, varied less with activation than the low velocity phase (Moss, 1986, 1992). If, like k_{TR}, V_{US} does vary with activation, it suggests that the rate of cross-bridge detachment during shortening is altered (Wise et al., 1971), which could appear as an activation-dependent internal load (Moss, 1986). An equally likely alternative is that the apparent activation dependence of V_{US} results from a passive internal resistance to shortening (Brenner, 1980, 1986; Wise et al., 1971).

Interpretation of experiments that used Ca^{2+} to alter activation level is now known to be complicated by the observed feedback between Ca^{2+} binding and cross-bridge attachment, which itself is altered by shortening (Gordon and

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Address reprint requests to Donald A. Martyn, Center for Bioengineering, WD-12, University of Washington, Seattle, WA 98195.

Dr. Hannon's current address: Department of Anesthesiology, Mayo Clinic, Rochester, MN 55905.

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Ridgway, 1987). Therefore, alternate approaches would be helpful to dissociate the degree of thin filament activation from $[Ca^{2+}]$. Cationic peptides, which bind to actin and thus inhibit myosin attachment (Cartoux et al., 1992), were found to inhibit V_{US} as well as force in skinned fibers during maximum Ca^{2+} activation (Chase et al., 1991). Another approach is to extract TnC partially, which results in decreased activation and lower V_{US} at a given $[Ca^{2+}]$, leading to the suggestion that V_{US} depends on the level of thin filament activation rather than on $[Ca^{2+}]$ per se (Moss, 1986, 1992). However, both of these methods require Ca^{2+} to initiate force generation. An additional complication to understanding the role of Ca^{2+} is the possibility that Ca^{2+} could modulate V_{US} via binding to myosin light chain 2 (LC_2) on the thick filament (Hofmann et al., 1990). To test for modulation of cross-bridge kinetics by Ca^{2+} binding to sites other than TnC (such as LC_2) requires one to remove the Ca^{2+}-sensitive component of thin filament activation and yet allow active cross-bridge cycling.

In this study, we have used an activating form of cTnC (aTnC) (Hannon et al., 1993) to achieve force production and shortening of skinned rabbit psoas fibers without Ca^{2+}. It has been suggested that an intramolecular disulfide bond in aTnC effectively locks the molecule into a conformation analogous to that in which Ca^{2+} is bound (Putkey et al., 1993). Results obtained with fibers activated with aTnC were compared with those obtained with fibers activated by Ca^{2+} binding to either sTnC or cTnC. We found that, in agreement with Brenner (1986) and Moss (1986), shortening was nonlinear and varied with isometric force in both control fibers and fibers reconstituted with either sTnC or cTnC. In aTnC-reconstituted fibers, velocity also depended on activation level and neither force nor shortening were affected by changes in $[Ca^{2+}]$, which indicates that Ca^{2+} binding to sites other than TnC are not involved in regulation of V_{US}.

A preliminary report of these data has appeared in abstract form (Martyn et al., 1993).

MATERIALS AND METHODS

Single fiber segments were prepared from glycerinated rabbit psoas muscle as previously described (Chase and Kushmerick, 1988). Animals were euthanized with pentobarbital (120 mg·kg^{-1} administered through the marginal ear vein). End compliance was minimized by chemical fixation of the fiber segment ends using localized microapplication of glutaraldehyde (Chase and Kushmerick, 1988). Individual fiber segments were treated with 1% Triton X-100 in pCa 9.2 solution for 10 min. Fiber segments were attached to the apparatus with aluminium foil clips. The total length of the fixed regions was determined at the end of each experiment and subtracted from the overall length to obtain the unfixed fiber length (L_F). At a relaxed sarcomere length (L_S) of 2.51 ± 0.04 μm (mean ± SD; n = 22), L_F was 1.55 ± 0.10 mm (mean ± SD; n = 22) and the diameter was 61 ± 10 μm (mean ± SD; n = 22). At pCa 4.0, L_S decreased to 2.32 ± 0.09 μm (mean ± SD; n = 22); the diameter was unchanged, being 99 ± 4% (mean ± SD; n = 19; determinations were not made on 3 fibers) of that at pCa 9.2, because of the inclusion of Dextran T-500 in the bathing solutions (Chase et al., 1993).

Force was measured with a Cambridge Technology Model 400A force transducer (resonant frequency 2.2 kHz) and L_F was controlled with a Cambridge Technology (Watertown, MA) model 300 servo motor (300 μs step response). L_S was continuously monitored by helium-neon laser diffraction as described (Chase et al., 1993). Steady-state L_S and fiber diameter were also measured from photomicrographs (400X) obtained in each condition. Analog signals were low-pass-filtered to avoid aliasing. Signals were recorded by digitizing 1024 points with 12-bit resolution at a per channel rate of 1.3–10 kHz (V_{US} measurements) or 30 kHz (measurements of elastic modulus).

Because of the Ca^{2+} insensitivity of aTnC-mediated force (Hannon et al., 1993), these experiments involved long continuous activations (e.g., Figs. 1 and 4). Therefore, we maintained fiber mechanical properties and structure by using Brenner's method (Brenner, 1983; Chase and Kushmerick, 1988; Sweeney et al., 1987). The fiber segment was shortened transiently every 5 s at a rate equal to or greater than the maximum shortening velocity (Brenner, 1983), which reduced force to the baseline (Fig. 1). Measurements of isometric force, elastic modulus, and V_{US} were made during the steady-state period between the transient releases (Sweeney et al., 1987). The force baseline was unambiguously determined by a large amplitude release that caused the fiber to slacken. Fiber force was normalized to cross sectional area as calculated from the diameter assuming circular geometry. In maximum activating Ca^{2+} (pCa 4.0), the control force was 288 ± 83 mN·mm^{-2} (mean ± SD; n = 22); relaxed force (pCa 9.2) was 2.3 ± 0.8% (mean ± SD; n = 22) of the maximum Ca^{2+}-activated force.

V_{US} was measured using the slack method (Edman, 1979). Length steps (ΔL_F) for slack tests ranged from 3.5 to 12% L_F and were applied nonsequentially. Slack time was defined as the time between the completion of the length step and the moment at which force began to redevelop. The latter was chosen as the intersection point between the force baseline and the least-squares regression of the initial linear rise of force (Fig. 2 *A*, *inset*). This method of defining the slack time was less arbitrary than estimating by eye the first point at which force increased above the noise in the force baseline, although in a subset of data analysis by both methods yielded similar results. Velocity (in FL·s^{-1}) over a range of ΔL_F was determined as the slope of the linear least-squares regression of ΔL_F versus slack time. To pool data from several fibers, force, velocity, and elastic modulus were normalized to the initial control at pCa 4.0. Normalization to initial control was chosen because of the irreversible nature of aTnC activation and likely resulted in a small (on the order of 10–15%) underestimation of normalized force and velocity for data acquired late in an experiment.

The elastic modulus was monitored during steady-state contractions using 1 kHz sinusoidal oscillations (~0.15% L_F peak-to-peak) as a measure of cross-bridge attachment (Ford et al., 1981). The digitized force and fiber length signals were Fourier-transformed, and the elastic modulus was obtained from the ratio of the amplitudes of the 1-kHz components. In maximum activating Ca^{2+} (pCa 4.0), the control elastic modulus was 30 × 10^6 ± 11 × 10^6 N·m^{-2} (mean ± SD; n = 15; determinations were not made on 7 fibers); relaxed elastic modulus (pCa 9.2) was 6.1 ± 2.0% (mean ± SD; n = 15; determinations were not made on 7 fibers) of the maximum Ca^{2+}-activated elastic modulus.

Solutions were prepared as described previously (Martyn and Gordon, 1988). Relaxing and activating solutions contained (in mM): 5 Mg^{2+}-adenosine 5′-triphosphate (MgATP), 15 phosphocreatine (PCr), 1 orthophosphate (Pi), 15 [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA), at least 40 3-[*N*-morpholino]propanesulfonic acid (MOPS), 135 Na^+ + K^+, 1 Mg^{2+}, pH 7.0, 250 units·ml^{-1} creatine phosphokinase (CK), and Dextran T-500 (4% w/v; Pharmacia, Piscataway, NJ). Solution $[Ca^{2+}]$ was altered by adding varying amounts of Ca(propionate)$_2$ as determined with a computer program that took into account the desired free $[Ca^{2+}]$ and the binding constants of all solution constituents for Ca^{2+}; to maintain ionic strength constant at 200 mM, [MOPS] was varied appropriately at each pCa. The experimental temperature was 12–13°C.

To extract sTnC fully, fibers were exposed for 20 min at 12°C to a solution containing (in mM): 5 ethylenedinitrilotetraacetic acid (EDTA), 20 tris(hydroxymethyl)aminomethane (Tris), pH 7.2 (Cox et al., 1981), with 0.5 trifluoperazine dihydrochloride (TFP; Aldrich Chemical Co., Milwaukee, WI) (Metzger et al., 1989). After full sTnC extraction, fibers exhibited no Ca^{2+}-activated force, indicating functionally complete extraction of sTnC (Hannon et al., 1993). Fibers were reconstituted at pCa 9.2 with purified TnC (0.2–2.0 mg·ml^{-1} sTnC, cTnC, aTnC, or a 1:8.5 mixture of aTnC and cTnC).

Rabbit sTnC and cTnC were prepared according to Potter (1982), and aTnC was prepared as previously described (Hannon et al., 1993). For sTnC or cTnC, reconstitution of TnC into fibers was allowed to proceed for 20 min. With aTnC, force increased during incubation in pCa 9.2 solution containing 0.22 mg·ml^{-1} (~12 μM) aTnC and was allowed to attain a steady level (10–20 min; Fig. 4). At the end of the experiment, fibers were analyzed for protein composition by SDS-PAGE as previously described (Hannon et al., 1993). Relative protein content of Ag-stained gels were analyzed by laser scanning densitometry (Ultroscan XL, LKB, Bromma, Sweden).

For experiments involving laser scanning epifluorescence confocal microscopy (Bio-Rad MRC-600), fibers were reconstituted with purified sTnC labeled with rhodamine at Cys 98 (Rhod-sTnC) (Gordon et al., 1988; Yates et al., 1985). During and subsequent to reconstitution with Rhod-sTnC, fibers were manipulated under minimal light conditions (red filter). Digital fluorescence images (768 × 512 pixels; fluorescence intensity resolution was 8 bits per pixel) were obtained by averaging six to seven individual scans of the viewing field. No fluorescence was detected from unlabeled fibers. Radial intensity distribution was obtained by averaging a 25-pixel-wide region of the fiber image perpendicular to the fiber axis.

RESULTS

Ca^{2+} activation of fibers with Ca^{2+}-sensitive TnCs

Before extraction and reconstitution of fibers with aTnC, we first determined the dependence of isometric force, isometric elastic modulus, and V_{US} upon [Ca^{2+}] in unextracted control fibers (Fig. 1 *A*). For comparison, because aTnC is a modified cTnC, we likewise examined the activation dependence of V_{US} after full TnC extraction (Materials and Methods) and reconstitution with cTnC (Fig. 1 *B*). In control fibers with endogenous sTnC, steady-state activations were achieved at submaximal [Ca^{2+}] (Fig. 1 *A*), which resulted in forces between 17.7 ± 3.4% (pCa 6.6; mean ± SD; n = 13 determinations on 12 fibers) and 58.1 ± 8.7% (pCa 6.4; mean ± SD; n = 13 determinations on 12 fibers) of the maximum obtained at pCa 4.0. Corresponding values of normalized elastic modulus were 26.6 ± 6.5% (mean ± SD; n = 5; determinations were not made on 7 fibers) and 70 ± 8.5% (mean ± SD; n = 7 determinations on 6 fibers; determinations were not made on 6 fibers), respectively; the elastic modulus was higher than force at submaximal [Ca^{2+}], as has been previously reported (Martyn and Gordon, 1992). For cTnC-reconstituted fibers, force was measured at pCa 6.6, 6.4, 6.2, 6.0, and 4.0 (Fig. 1 *B*) because of the lower sensitivity of force to [Ca^{2+}] in fibers containing cTnC (Gulati et al., 1990; Moss et al., 1986). At pCa 4.0, after reconstitution with cTnC, force was 59.0 ± 4.7% of control (mean ± SD; n = 3). Gulati et al. (1990) contend that the lower maximum Ca^{2+}-activated force in cTnC-reconstituted psoas fibers does not result from incomplete occupancy of TnC binding sites. However, others have indicated that reconstitution with cTnC may be incomplete after extraction of endogenous TnC from skinned skeletal fibers (Moss et al., 1991). Maximum force obtained after cTnC reconstitution of fully extracted fibers was lower than that for fibers that were partially TnC extracted before cTnC reconstitution (Gulati et al., 1990; Moss et al., 1986), but was equivalent to or higher than that reported by others for a similar degree of TnC extraction at 0.2 M ionic strength (Gulati et al., 1990). Although fibers reconstituted with cTnC generated less force than control at maximum Ca^{2+} activation, normalization of all variables to the initial, maximum Ca^{2+}-activated control allowed comparison at comparable levels of submaximal force.

Examples of superimposed force records obtained during slack tests are shown for one fiber before extraction of TnC (Fig. 2 *A*). Individual records in Fig. 2 were obtained at different pCas using a constant amplitude release. The slack time for these records is indicated by the symbols below the force baseline and was obtained as indicated in the inset to Fig. 2 *A*. For a constant amplitude release, the slack time became shorter as the level of activation increased for control fibers (Fig. 2 *A*). The relationship between slack time and ΔL_F was clearly nonlinear at pCa 6.6, although the curvature decreased considerably at pCa 4.0 (Fig. 2 *B*), in accord with observations by others (Brenner, 1980, 1986; Farrow et al., 1988; Gulati and Babu, 1985; Julian et al., 1986; Metzger and Moss, 1988; Moss, 1986). We attempted to fit the data to an exponential function (Brenner, 1986), but found that although data obtained at pCa 6.6 could be well fit to a monoexponential, data obtained at higher [Ca^{2+}] were not. The data were more accurately described (typically for V_E, R^2 > 0.96) by two linear segments: an early fast phase (V_E) and a

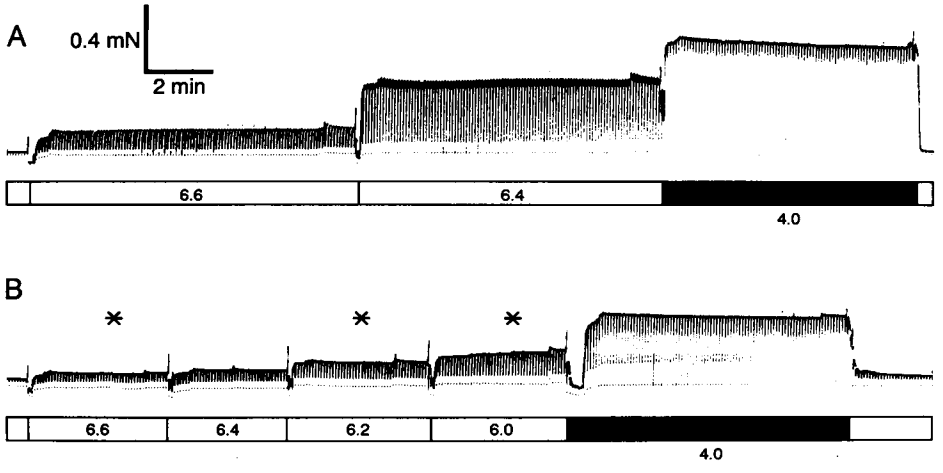

FIGURE 1 Force obtained from a glycerinated rabbit psoas fiber at the pCa indicated below the traces (pCa 9.2 where no value is given) before (*A*) and then after (*B*) complete extraction of endogenous sTnC and reconstitution with cTnC. The record was broken at the points indicated by (*). The force transients apparent throughout the steady contractures resulted from periodic (0.2 s^{-1}) releases (Materials and Methods). After reconstitution with cTnC, the force at pCa 4.0 (*B*) was 61% of that with endogenous sTnC (*A*). L_F was 1.62 mm, and diameter was 64.7 μm. Calibrations: force, 0.4 mN; time, 2 min.

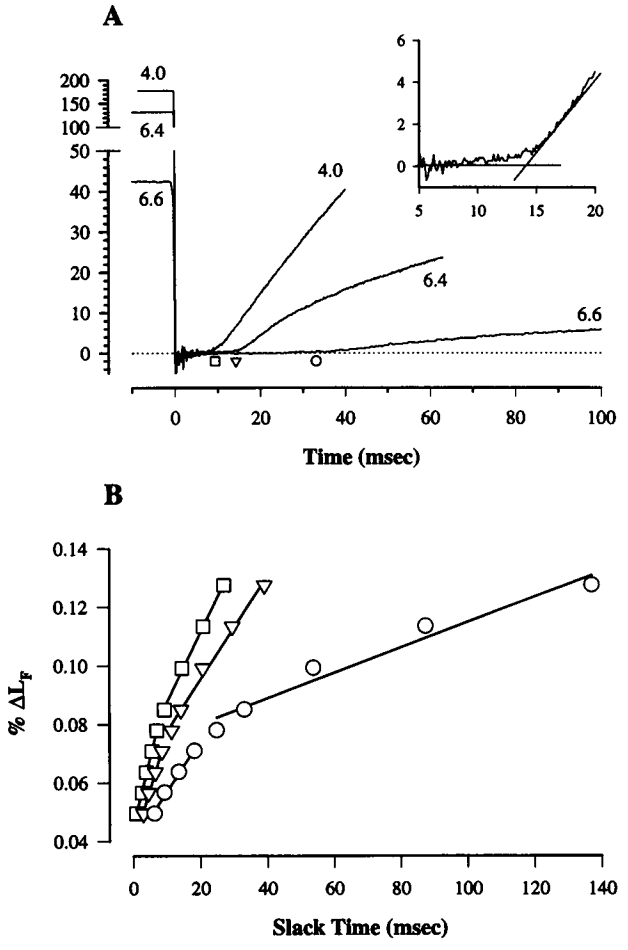

FIGURE 2 Determination of shortening velocity at various pCas in a single fiber with endogenous sTnC (*A*, *B*). (*A*) Force traces obtained during slack releases of 7.9% L_F at different pCas, as indicated at the beginning and end of each trace. The method used to determine slack time (Materials and Methods) is illustrated in the inset. The times thus obtained are indicated below the force baseline (·····) for the traces obtained at pCa 4.0 (□), 6.4 (▽), and 6.6 (○). In *B* the slack times obtained at each pCa are plotted against the corresponding ΔL_F (4.7–12.0% ΔL_F). Symbols correspond to the same pCas as in *A*. At each pCa, the plots were nonlinear and velocity was characterized by two slopes corresponding to early (V_E) and later phases (V_L) (Materials and Methods). L_F was 1.62 mm.

later slow phase (V_L), as previously described (Metzger and Moss, 1988; Moss, 1986). Control determinations (mean ± SD; n = 22) at pCa 4.0 for V_E and V_L were 3.6 ± 0.8 L_F·s^{-1} and 2.4 ± 0.3 L_F·s^{-1}, respectively, and the length intercept was 3.8 ± 0.9% L_F.

To compare the effect of activation level upon V_E and V_L, isometric force and velocities during both phases were obtained at submaximal [Ca^{2+}] and normalized to the corresponding values obtained at pCa 4.0 in the same fiber before extraction of sTnC. As is evident in Figs. 2 and 3, both V_E (Fig. 3 *A*) and V_L (Fig. 3 *B*) decreased at lower levels of activation in nonextracted control fibers. The apparent activation dependence of V_E shown in Fig. 3 *A* is similar to that previously described for skinned psoas fibers using slack tests (Hofmann et al., 1990; Metzger and Moss, 1988; Moss, 1986). Least-squares regression of the data in Fig. 3 *A*, fit to a linear function which passes through unity,

$$y = \beta_1(x - 1) + 1$$

indicated that V_E was reduced at submaximal activation (β_1 = 0.553 ± 0.046, SE) and that the data deviated significantly from that predicted if there were no activation dependence (β_1 = 0; $p < 0.01$). Similar measurements were made in three fibers that had been extracted and reconstituted with cTnC (Fig. 3, *C* and *D*; ▼). As in Fig. 3, *A* and *B*, V_L, and to a lesser extent V_E, exhibited an activation dependence in cTnC-reconstituted fibers. Although our data suggest that shortening depends upon activation at the earliest times examined, we were not able to determine whether instantaneous shortening velocity immediately after the slack release varied with activation. This is because of the discrete nature of the slack measurements such that small variations (on the order of 0.5–1.0 ms) associated with the determination of slack time, at the highest velocities and earliest times, have a disproportionately large influence on the calculated velocity.

To determine whether the extraction/reconstitution protocol affected V_{US}, V_E and V_L were measured after TnC extraction and subsequent reconstitution with sTnC (Fig. 3, *C* and *D*; ◇). In Table 1 the values of force, V_E, and V_L obtained from five fibers at pCa 6.6, 6.4, and 4.0 before and after extraction/reconstitution with sTnC are expressed as a fraction of that found at pCa 4.0 before extraction. At either pCa 6.6 or 6.4, the extraction/reconstitution protocol did not significantly affect isometric force, V_E or V_L ($p > 0.05$). Furthermore, comparing data obtained after extraction/reconstitution with either sTnC (Fig. 3, *C* and *D*; ◇) or cTnC (Fig. 3, *C* and *D*; ▼) indicates that alteration of TnC isoform has little or no effect on V_{US}.

Because extraction of LC_2 has been shown to affect V_{US} (Hofmann et al., 1990), we tested for changes in myosin light chain content resulting from the TnC-extraction/reconstitution protocol using Ag-stained SDS-PAGE to compare treated with nonextracted control fiber segments. Densitometric analysis of the gels indicated that no significant changes in protein content had occurred. The ratio of LC_2:(LC_1 + LC_3) (mean ± SD; n = 13) was 0.69 ± 0.12 in fibers from which sTnC had been fully extracted once (and reconstituted with TnC) compared with 0.66 ± 0.15 for control segments; the corresponding ratios for another group of fibers that had undergone the extraction/reconstitution protocol twice (see Fig. 4) were 0.74 ± 0.15 compared with 0.85 ± 0.14 for control (mean ± SD; n = 8). Thus, there was no significant evidence for selective extraction of any myosin light chain isoform ($p > 0.05$). Furthermore, to determine whether a uniform extraction of all LC isoforms had occurred, the fiber content of LC_1 was compared with the tropomyosin (Tm) content. The ratio of LC_1:Tm was 0.89 ± 0.13 in fibers that had undergone the extraction/reconstitution protocol once compared with 0.87 ± 0.14 for control (mean ± SD; n = 13); the corresponding values for the second group of fibers that had undergone the extraction/reconstitution protocol twice were 1.07 ± 0.24 vs. 0.91 ± 0.21 for control (mean ± SD; n = 8). Thus, no

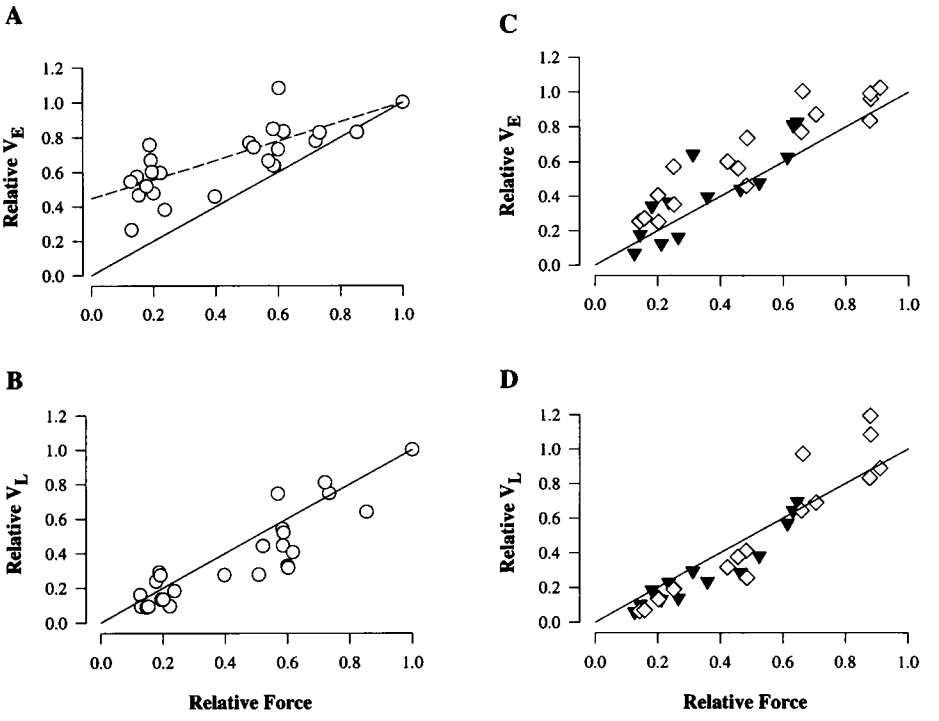

FIGURE 3 Relationship between V_E (*A*, *C*) or V_L (*B*, *D*) and steady-state isometric force obtained before endogenous sTnC extraction (*A*, *B*) or after TnC extraction and reconstitution with either sTnC or cTnC (*C*, *D*). Force, V_E, and V_L were normalized to those obtained at pCa 4.0 before TnC extraction. In *A* and *B*, data were obtained from 12 fibers using slack tests at pCa 6.6, pCa 6.4, or pCa 4.0, as illustrated in Fig. 2. V_E (*C*) and V_L (*D*) are shown for fibers after full extraction of sTnC and reconstitution with either cTnC (▼; 3 fibers) or sTnC (◇; 5 fibers). Control force (100%) was 283 ± 76 mN·mm^{-2} (mean ± SD; n = 12); the corresponding values for V_E and V_L were 3.7 ± 0.9 L_F·s^{-1} and 2.4 ± 0.3 L_F·s^{-1}, respectively. In all panels, the solid lines were drawn according to $y = x$. In panel *A*, the dashed line was drawn according to the least-squares regression line $V_E = 0.553(x - 1) + 1$.

TABLE 1 Force and unloaded velocities of rabbit psoas fibers measured before and after sTnC extraction/reconstitution

TnC	pCa	Relative force	Relative V_E	Relative V_L
Endogenous sTnC	6.6	0.17 ± 0.03	0.50 ± 0.15	0.14 ± 0.08
Reconstituted sTnC	6.6	0.20 ± 0.05	0.35 ± 0.12	0.13 ± 0.06
Endogenous sTnC	6.4	0.55 ± 0.12	0.68 ± 0.13	0.41 ± 0.13
Reconstituted sTnC	6.4	0.50 ± 0.09	0.63 ± 0.13	0.40 ± 0.15
Endogenous sTnC	4.0	1	1	1
Reconstituted sTnC	4.0	0.89 ± 0.02	0.95 ± 0.08	1.00 ± 0.17

Force, V_E, and V_L are expressed as a fraction of the value obtained at pCa 4.0 before TnC extraction. All values are the means ± SD for data obtained from five fibers (for relative V_E and V_L, n = 4 at pCa 4.0 for fibers reconstituted with sTnC).

significant extraction of myosin light chains resulted from the extraction/reconstitution protocols ($p > 0.05$). The lack of detectable changes in protein composition correlates well with the minimal effects of the TnC-extraction/reconstitution protocol on the activation dependence of V_{US}.

Taken together, these observations validate comparisons made between control fibers and fibers reconstituted with various TnCs. The results in Fig. 3 suggest that V_E and V_L both decline with decreasing level of thin filament activation by Ca^{2+}, thus confirming previous reports (Farrow et al., 1988; Metzger and Moss, 1988; Moss, 1986). In addition, this activation dependence of shortening velocity was similar whether sTnC or cTnC was bound to the thin filament.

Fibers reconstituted with aTnC

The activation dependence of isometric force, elastic modulus, and shortening velocity were also investigated in fibers from which endogenous sTnC was fully extracted and which were then reconstituted with a mixture of aTnC and cTnC. The protocol used to achieve partial activation with aTnC is illustrated in Fig. 4. After extraction of sTnC and reconstitution with the aTnC:cTnC mixture, force generation consisted of Ca^{2+}-insensitive and Ca^{2+}-sensitive components (Fig. 4 *A*). For the mixture of aTnC:cTnC used (1:8.5), the Ca^{2+}-insensitive component of force, which was caused by activation by aTnC at pCa 9.2, was 16.2 ± 4.1% of control (mean ± SD; n = 16 determinations on 8 fibers), whereas the corresponding value of the elastic modulus was 24.6 ± 4.5%. At pCa 4.0, force increased to 54.9 ± 4.6% (mean ± SD; n = 8), whereas the elastic modulus increased to 64.1 ± 3.7% of control.

The protocol illustrated in Fig. 4 was designed to achieve submaximal activation by a homogeneous distribution of aTnC within the fiber (longitudinally along individual thin filaments or radially across the fiber). Several observations indicate that this is true. First, aTnC appears to have approximately the same binding affinity for thin filament sites as cTnC because the relative amount of Ca^{2+}-independent force after reconstitution with the mixture was similar to the proportion of aTnC used; if aTnC had a much higher affinity to actin-binding sites, the level of steady force at pCa 9.2 would have been much higher than the relative proportion of aTnC in the reconstitution mixture. Second, it is unlikely that differences in the diffusion of aTnC and cTnC (18 kDa molecular mass) into the interior of the fiber could create significant gradients because the rise of Ca^{2+}-insensitive force during reconstitution was on the order of minutes and was comparable with the time necessary to attain complete reconstitution of fibers with sTnC or cTnC. To detect more directly any gradients in aTnC distribution, we reconstituted

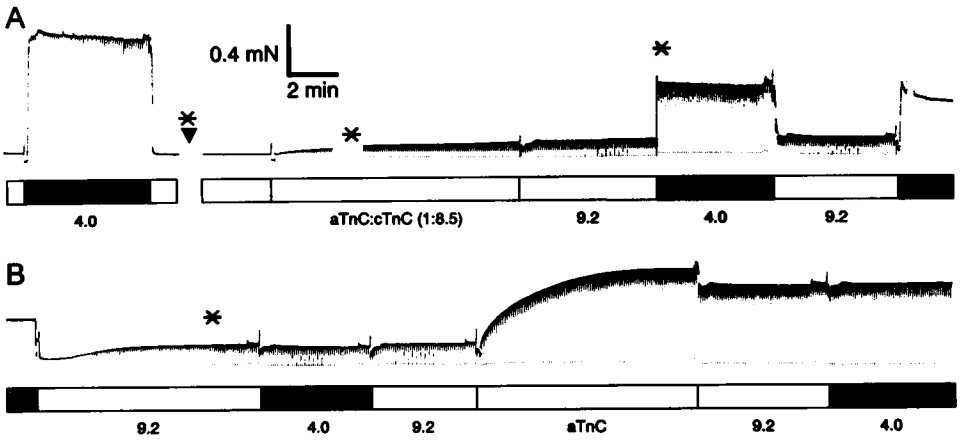

FIGURE 4 Force traces illustrating the protocol used to achieve partial activation of skinned fibers with aTnC. As in Fig. 1, the bars below the force traces in *A* and *B* indicate the solution conditions. The record was broken at the points indicated by (*). (*A*) After determination of the control values for isometric force, V_{US} and elastic modulus at pCa 4.0, endogenous sTnC was completely extracted (Materials and Methods). The fiber was then exposed to a mixture of aTnC and cTnC (1:8.5; 0.22 mg·ml^{-1} in pCa 9.2 solution) and allowed to reach a steady-state force (10–20 min) before being placed in pCa 9.2 solution. Subsequently, the force at pCa 4.0 was 57.6% of control. At the end of record *A*, the fiber was again exposed to TnC extraction solution (*hatched bar*). (*B*) The second exposure to TnC extraction solution (*hatched bar*) resulted in a loss of the Ca^{2+}-dependent component of force. After mechanical measurements at pCa 9.2 and 4.0, the fiber was reconstituted with aTnC (0.22 mg·ml^{-1} in pCa 9.2 solution) until steady isometric force was attained; the slight elevation of force observed during loading with aTnC is probably caused by a lack of CK in the loading solution. The level of force achieved with full aTnC reconstitution was 67.8% of control and was unaffected by [Ca^{2+}].

three fibers with the aTnC/cTnC mixture, then extracted the cTnC and finally reconstituted the fiber with Rhod-sTnC (Materials and Methods; similar to Fig. 4). Force was measured at pCa 4.0 to verify that Rhod-sTnC was incorporated into thin filaments and was functional. Fluorescent striations were observed, indicating binding specificity of Rhod-sTnC within sarcomeres and presumably on thin filaments. In comparison with Fig. 4, a higher proportion of aTnC-activated force was chosen for the imaging experiment shown in Fig. 5 to enhance detection of TnC gradients that might be present (at pCa 9.2, force was approximately 50% of maximum at pCa 4.0). To prevent shortening of the aTnC-activated fiber during confocal microscopy, it was first washed in pCa 9.2 solution devoid of protein and Dextran T-500, then fixed with 10 mM glutaraldehyde for 30 min at pCa 9.2. If aTnC was not evenly distributed across the fiber, then areas with greater occupancy by aTnC would be revealed by reduced fluorescence compared with areas less densely occupied by aTnC, and thus having greater occupancy by Rhod-sTnC. The distribution of fluorescence in the aTnC/Rhod-sTnC-reconstituted fibers was measured by laser scanning fluorescence confocal microscopy, and the results are shown in Fig. 5. Rhodamine fluorescence was excited with 568-nm light, and emission was collected at 600–630 nm. No significant difference in the distribution of fluorescence intensity across the fiber was detected (Fig. 5). These observations indicate that the extraction/reconstitution protocol used in this study resulted in a homogeneous distribution of TnC radially throughout fibers.

Shortening velocity was measured under the various conditions described in Fig. 4 *A*. For fibers that were submaximally activated by reconstitution with a mixture of aTnC and cTnC (1:8.5), both V_E and V_L obtained at pCa 9.2 were comparable with values obtained at submaximal [Ca^{2+}] in fibers that contained only cTnC (Figs. 2 *D* and 3 *C*). At pCa 4.0, the velocity during both phases increased in proportion to the isometric force because of the presence of Ca^{2+}-sensitive TnC. Force at pCa 9.2 was 16.2 ± 4.1% (mean ± SD; n = 8), V_E was 27.4 ± 7.4%, and V_L was 26.5 ± 7.8% normalized to maximum Ca^{2+}-activated values obtained before TnC extraction; because of the presence of cTnC, the corresponding values of force, V_E, and V_L at pCa 4.0 increased to 54.9 ± 4.6%, 60.3 ± 12.1%, and 49.0 ± 9.1%, respectively.

The Ca^{2+}-sensitive component of force was removed by a second TnC extraction of fibers that contained a mixture of aTnC and cTnC, the remaining submaximal force being because of aTnC alone (Fig. 4 *B*). This Ca^{2+}-insensitive force was 23.4 ± 6.8% of control (mean ± SD; n = 16 determinations on 8 fibers) at pCa 9.2 and 24.1 ± 7.9% (mean ± SD; n = 8) at pCa 4.0; corresponding elastic modulus values were also unaffected by [Ca^{2+}], being 36.2 ± 7.8% (mean ± SD; n = 16 determinations on 8 fibers) and 36.1 ± 8.5% of control (mean ± SD; n = 8), respectively. At submaximal activation levels attained with aTnC, the elastic modulus was greater relative to force, as was found for control fibers at submaximal levels of Ca^{2+}-activation. Finally, when fibers were fully reconstituted with aTnC, relative force remained insensitive to [Ca^{2+}] and was 69.6 ± 4.7% (pCa 9.2, mean ± SD; n = 15 determinations on 8 fibers) and 71.7 ± 4.5% of control (pCa 4.0, mean ± SD; n = 8); corresponding elastic modulus values were 68.3 ± 6.6% (mean ± SD; n = 15 determinations on 8 fibers) and 70.6 ± 6.1% (mean ± SD; n = 8), respectively.

V_{US} was measured at pCa 9.2 and 4.0 in fibers that were submaximally activated by partial reconstitution with aTnC

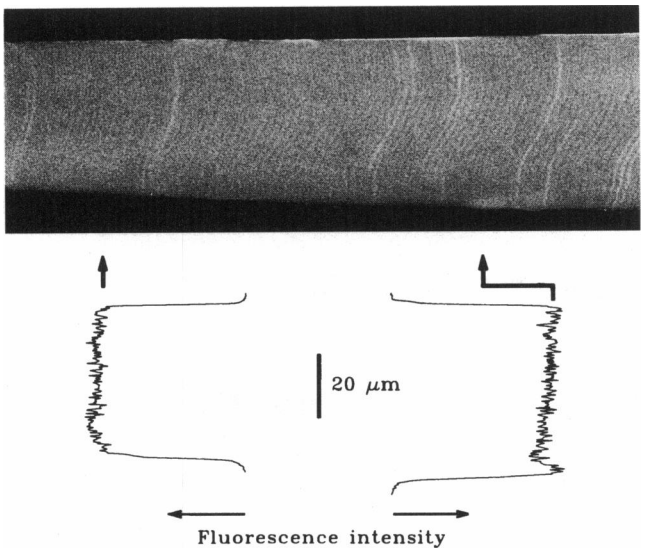

FIGURE 5 (*A*) Laser scanning fluorescence confocal microscopic image (average of 6 sequential scans) of a rabbit psoas fiber reconstituted with aTnC and Rhod-sTnC (*top*). Originally, endogenous sTnC was completely extracted and the fiber was reconstituted with an aTnC/cTnC mixture; next, cTnC was extracted, similar to the procedure illustrated in Fig. 4 *B*. Subsequently, the fiber was reconstituted with Rhod-sTnC, resulting in approximately equal proportions of Ca^{2+}-sensitive and -insensitive force. Images were obtained as described in Materials and Methods. 25-pixel-wide averages of image intensity perpendicular to the fiber axis (*bottom*) were obtained across the regions indicated by arrows. Intensity units are arbitrary. Length calibration bar is 20 μm and applies to both top and bottom portions of the figure.

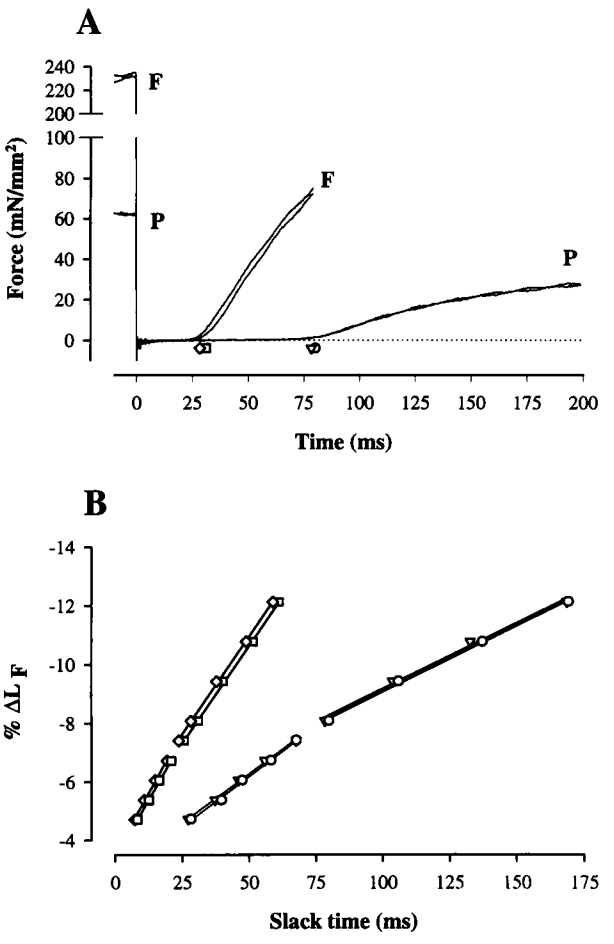

FIGURE 6 (*A*) Force records obtained during slack tests on one fiber which was first partially activated with aTnC only (beginning and end of traces marked P), and then fully reconstituted with aTnC (beginning and end of traces marked F). For both, force traces obtained at pCa 4.0 and 9.2 are superimposed and are nearly identical. ΔL_F was 7.3% L_F. The slack times obtained with each trace are indicated below the force baseline (·····) for full (□) and partial (○) aTnC reconstitution at pCa 9.2 and for full (▽) and partial (◇) aTnC reconstitution at pCa 4.0. (*B*) ΔL_F is plotted against slack time for full aTnC reconstitution at pCa 4.0 (◇) and pCa 9.2 (□) and with partial aTnC reconstitution at pCa 4.0 (▽) and 9.2 (○) As in Fig. 2, the shortening was nonlinear and was characterized by two slopes.

alone. The fibers were then fully reconstituted with aTnC as illustrated in Fig. 4, and V_{US} was again measured with and without Ca^{2+}. For comparison with Fig. 2 *A*, representative force traces from slack tests of the same ΔL_F are shown in Fig. 6 *A*; the data were obtained from a fiber that contained only aTnC, with either partial activation by aTnC (subsequent to the second extraction, traces labeled P) or after full reconstitution of the same fiber with aTnC (traces labeled F). Slack times and the time course of force recovery after the length step were unaffected by $[Ca^{2+}]$. At both partial and full activation by aTnC alone, fiber shortening versus slack time could be described by two linear phases (Fig. 6 *B*), as found in control (Fig. 2) and cTnC-reconstituted fibers. This supports the proposal by Moss (1986) that the transition from fast to slow shortening was not caused by dissociation of Ca^{2+} from thin filaments, which might have resulted from altered Ca^{2+} affinity of TnC caused by cross-bridge detachment during shortening (Gordon and Ridgway, 1987). At both partial and full activation by aTnC alone, neither phase of velocity was affected by $[Ca^{2+}]$.

In Fig. 7, *A* and *B*, individual determinations of V_E and V_L obtained during partial and full activation by aTnC are plotted against the corresponding values of relative force. With aTnC alone, both phases of velocity varied with the level of activation. In Fig. 8, values (mean ± SD) of force, V_E, and V_L relative to pCa 4.0 controls are compared for fibers that contained either a mixture of aTnC and native cTnC or were partially or fully reconstituted with aTnC alone (as illustrated in Fig. 4). Whereas V_{US} varied with isometric force during activation by Ca^{2+} in control fibers and fibers reconstituted with either sTnC or cTnC (Figs. 2 and 3), the results summarized in Figs. 7 and 8 indicate that V_{US} correlates with force and not $[Ca^{2+}]$.

DISCUSSION

Variation of V_{US} with activation level

In single, skinned fibers from rabbit psoas muscle, unloaded shortening velocity measured by the slack method decreased as shortening progressed, with this phenomenon being more pronounced at low levels of activation. This apparent activation dependence of V_{US} we observed in fibers activated

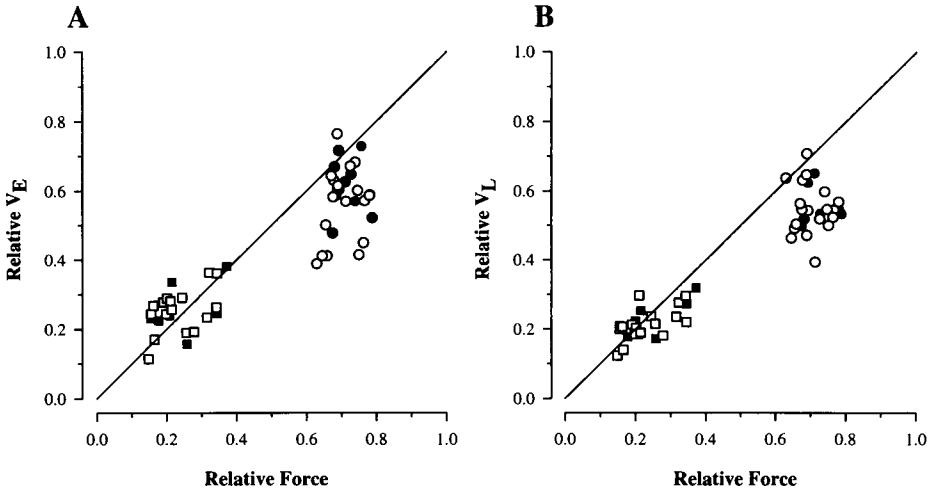

FIGURE 7 Relation between V_E (*A*) or V_L (*B*) and steady-state isometric force in fibers containing only aTnC; such fibers were obtained as illustrated in Fig. 4 *B*. Velocity measurements at partial aTnC-activation were made at pCa 9.2 (□) and pCa 4.0 (■). Similar measurements were also obtained after full reconstitution with aTnC at pCa 9.2 (○) and pCa 4.0 (●). Control force (100%) was 302 ± 100 $mN \cdot mm^{-2}$ (mean ± SD; $n = 8$); the corresponding values for V_E and V_L were 3.5 ± 0.8 $L_F \cdot s^{-1}$ and 2.4 ± 0.2 $L_F \cdot s^{-1}$, respectively. In both *A* and *B*, the solid lines were drawn according to $y = x$.

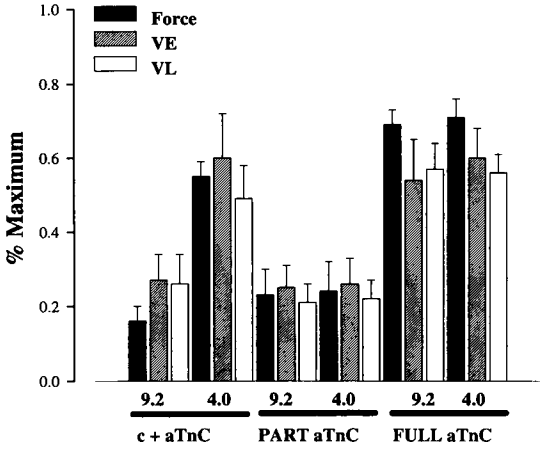

FIGURE 8 Variations of force (*filled columns*), V_E (*hatched columns*), and V_L (*open columns*) with TnC composition and $[Ca^{2+}]$ for eight fibers treated by the TnC swapping protocol illustrated in Fig. 4. Values are expressed as the fraction (mean ± SD) of the control value obtained at pCa 4.0 in the same fiber. pCas are indicated below the columns. Data were obtained after reconstitution with a mixture of cTnC and aTnC (c + aTnC), subsequent to extraction of cTnC (PART aTnC), or after full reconstitution with aTnC (FULL aTnC).

with aTnC (Figs. 5 and 7) or in Ca^{2+}-activated fibers containing sTnC or cTnC (Figs. 2 and 3), are in general agreement with several other reports (Farrow et al., 1988; Julian, 1971; Julian and Moss, 1981; Julian et al., 1986; Metzger and Moss, 1988, 1986; Wise et al., 1971). The absence of an effect of Ca^{2+} on V_{US} in fibers activated with aTnC alone (Figs. 6–8) suggests that Ca^{2+} binding to sites other than TnC does not modulate V_{US} (Hofmann et al., 1990; Wagner, 1984). The similarity of the relationship of V_{US} and force observed in Ca^{2+}-activated fibers compared with aTnC-activated fibers indicates that, over the extent of shortening measured, V_{US} is affected more by the level of thin filament activation than by the properties of TnC.

The early phase of unloaded shortening was less dependent on Ca^{2+} activation than V_L in control fibers containing endogenous sTnC (Fig. 3, *A* and *B*) as previously reported by Moss (Metzger and Moss, 1988, 1986). Both V_E and V_L varied in a similar manner with force in TnC-extracted fibers reconstituted with either sTnC or cTnC (Fig. 3, *C* and *D*; Table 1). For comparison, in skinned soleus fibers from rat, which probably contain the cTnC isoform (Parmacek and Leiden, 1989; Wilkinson, 1980), V_{US} was Ca^{2+}- and force-dependent (Metzger and Moss, 1988). Thus, it is clear that with either TnC isoform, V_{US} varied with the level of thin filament activation, and this dependence was not significantly affected by the extraction/reconstitution protocol (Table 1).

Comparison with other studies

Others have concluded that V_{US} does not vary with activation level (Brenner, 1980, 1986; Gulati and Babu, 1985; Podolin and Ford, 1983; Podolsky and Teichholz, 1970). Possible reasons for the differing results from these earlier studies have been discussed in detail (Julian et al., 1986; Moss, 1986, 1992; Podolin and Ford, 1986). Certainly, the presence of a small internal load, whether resulting from cross-bridge attachment (Moss, 1986) or passive load-bearing structures that impede shortening (Brenner, 1986), would cause an apparent activation dependence of unloaded shortening. For example, no activation dependence was found for velocity extrapolated from the linear relationship between sarcomere length and velocity during load clamps to zero force, after 3–4% of sarcomere shortening (Brenner, 1986). However, shortening velocity during zero load clamps was activation-dependent over the range of steady shortening (Brenner, 1986). Our slack test data do not allow us to determine whether the instantaneous velocity immediately after a length step is activation-dependent (Fig. 2 *B*; see also Results) because we cannot extrapolate with the required precision to the earliest times. Our results do indicate that over the range of shortening studied, unloaded velocity was affected by the level of activation, as observed by others using similar approaches (Hofmann et al., 1990; Metzger and Moss, 1988; Moss, 1986).

In our study, 4% Dextran T-500 was used to compress osmotically the myofilament lattice (Matsubara et al., 1985) and to minimize potential effects of force-dependent changes in myofilament lattice spacing (Brenner and Yu, 1985) on V_{US}. For example, at maximum activation, V_{US} has been shown to decrease with myofilament lattice compression (Metzger and Moss, 1987). In the absence of Dextran, myofilament lattice spacing is greater at low force than at maximum activation (Brenner and Yu, 1985). Therefore, one could speculate that at a given submaximal force, V_{US} would be higher in the absence of Dextran and, thus, the apparent activation dependence would appear to be less than that found in the presence of Dextran. The observation that the early phase of shortening of skinned soleus fibers is more affected by activation in the presence than in the absence of Dextran (Metzger and Moss, 1988) is consistent with this possibility.

V_{US} of intact fibers at 0–5°C was apparently independent of force in twitches or tetanii, and when force was varied pharmacologically by dantrolene or by altered filament overlap (Claflin et al., 1989; Edman, 1979; Gordon et al., 1966; Gulati and Babu, 1985). However, stretched intact fibers almost certainly had fully activated thin filaments and, thus, according to the skinned fiber data, one would not expect a change in V_{US}. One could speculate that inhibition of force in intact fibers by dantrolene did not result in a uniform decrease in thin filament activation throughout the fiber radius, perhaps because of spatial gradients of $[Ca^{2+}]$ such as was found during fatigue in frog fibers (Westerblad et al., 1990), and in contrast with skinned fibers in which well buffered solutions of submaximal $[Ca^{2+}]$ are more likely to achieve spatially homogeneous activation. Thus, in pharmacologically inhibited intact fibers at submaximal force, it is possible that the outer annulus of myofilaments could be fully activated while the center was not activated. If V_{US} of intact fibers does vary with thin filament activation as in skinned fibers, no reduction in V_{US} would be expected in the intact fiber experiments because the measurements may have been dominated by a maximally activated subpopulation of myofilaments.

Implications for the mechanism of regulation of V_{US}

To explain changes in V_{US} with activation, it has been suggested by others that the decrease of V_{US} observed at reduced activation levels may be caused either by a passive internal load (Brenner, 1980, 1986; Brenner and Yu, 1985; Gulati and Babu, 1985; Wise et al., 1971) or by a cross-bridge-based internal load resulting from a reduced rate of detachment during shortening (Metzger and Moss, 1988; Moss, 1986; Wise et al., 1971). Although plausible, a passive internal load in skinned fibers may not be sufficient to explain our observed relation between V_{US} and force because such a mechanism would imply that any perturbation that inhibits force but does not directly influence velocity would ultimately decrease V_{US} also. For example, the observation that V_{US} is unaffected by elevated Pi even though the maximum Ca^{2+}-activated force was depressed by at least 30% is inconsistent with a passive internal load (Chase and Kushmerick, 1988; Chase et al., 1993; Cooke and Pate, 1985; Pate and Cooke, 1989).

Alternatively, it is possible that the kinetics of cross-bridge detachment during shortening are altered by the average level of thin filament activation. In support of this, V_{US} has been shown both theoretically and empirically to be sensitive to detachment rate (Cooke and Bialek, 1979; Cooke and Pate, 1985; Ferenczi et al., 1984; Huxley, 1957). Also, V_{US} has been shown to decline when the level of thin filament activation is lowered by extraction of TnC or whole troponin in skinned skeletal fibers (Moss, 1986). If the rate of cross-bridge detachment decreased in regions between activated regulatory units, as has been proposed (Moss, 1986), then negatively strained cross-bridges in such regions would themselves present an internal load to shortening. The fraction of attached cross-bridges in such a slowly detaching state would decrease as activation level increased, thus providing a plausible explanation for our observed increase of V_{US} with average thin filament activation level. Our results indicate that it made little or no difference whether thin filaments were activated by dynamic equilibrium binding of Ca^{2+} to TnC or were activated continuously with aTnC binding even in the absence of Ca^{2+} (Hannon et al., 1993; Putkey et al., 1993). Thus, it appears that the average level of thin filament activation affects unloaded shortening rather than the activation dynamics of individual regulatory units, in sharp contrast to the regulation of isometric tension redevelopment kinetics (Chase et al., 1994).

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