# Regulation of Skeletal Muscle Tension Redevelopment by Troponin C Constructs with Different $Ca^{2+}$ Affinities

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ABSTRACT In maximally activated skinned fibers, the rate of tension redevelopment ($k_{tr}$) following a rapid release and restretch is determined by the maximal rate of cross-bridge cycling. During submaximal $Ca^{2+}$ activations, however, $k_{tr}$ regulation varies with thin filament dynamics. Thus, decreasing the rate of $Ca^{2+}$ dissociation from TnC produces a higher $k_{tr}$ value at a given tension level (P), especially in the [$Ca^{2+}$] range that yields less than 50% of maximal tension ($P_o$). In this study, native rabbit TnC was replaced with chicken recombinant TnC, either wild-type (rTnC) or mutant (NHdel), with decreased $Ca^{2+}$ affinity and an increased $Ca^{2+}$ dissociation rate ($k_{off}$). Despite marked differences in $Ca^{2+}$ sensitivity (>0.5 $\Delta pCa_{50}$), fibers reconstituted with either of the recombinant proteins exhibited similar $k_{tr}$ versus tension profiles, with $k_{tr}$ low (1–2 $s^{-1}$) and constant up to ~50% $P_o$, then rising sharply to a maximum (16 ± 0.8 $s^{-1}$) in fully activated fibers. This behavior is predicted by a four-state model based on coupling between cross-bridge cycling and thin filament regulation, where $Ca^{2+}$ directly affects only individual thin filament regulatory units. These data and model simulations confirm that the range of $k_{tr}$ values obtained with varying $Ca^{2+}$ can be regulated by a rate-limiting thin filament process.

## INTRODUCTION

Tension development in muscle results from the cyclic interactions between myosin cross-bridges and actin. This interaction is regulated by the troponin-tropomyosin complex on the thin filament and the initial step in tension generation is $Ca^{2+}$ binding to the troponin C (TnC) subunit of troponin (Chalovich, 1992; Farah and Reinach, 1995; Grabarek et al., 1992; Tobacman, 1996). In chemically skinned muscle fibers, where the level of activating $Ca^{2+}$ can be controlled, both steady-state tension (P) and the rate at which tension redevelops ($k_{tr}$) following a sudden release and restretch of fibers are $Ca^{2+}$-dependent (Brenner, 1988; Sweeney and Stull, 1990). Whereas the number of cycling cross-bridges determines P, $k_{tr}$ is thought to report the rate of cross-bridge transitions from detached or weakly attached states to tension-generating states (Brenner and Eisenberg, 1986). The relationship between P and $k_{tr}$ with varying [$Ca^{2+}$] is highly curvilinear in rabbit psoas fibers at 10–15°C, such that $k_{tr}$ is slow (1–2 $s^{-1}$) and unchanging at $P < 50\%$ of maximally activated tension ($P_o$) and increases 10- to 15-fold as P increases to $P_o$ at high levels of $Ca^{2+}$ activation (Brenner, 1988; Sweeney and Stull, 1990; Metzger et al., 1989; Metzger and Moss, 1992; Chase et al., 1994; Regnier et al., 1996, 1998b). This relationship indicates that $k_{tr}$ is inhibited at low levels of activation and that $Ca^{2+}$ controls the rate-limiting process in tension development (Brenner, 1988; Sweeney and Stull, 1990; Regnier et al., 1996, 1998b).

The mechanism by which $Ca^{2+}$ controls the rate of tension development in fibers is unclear. In maximally $Ca^{2+}$-activated fibers there is good evidence that the rate of cross-bridge cycling determines $k_{tr}$. Metzger and Moss (1990a) showed that maximal $k_{tr}$ correlates with the myosin isoform present in fast and slow mammalian skeletal muscle. Maximal $k_{tr}$ also correlates with changes in the cross-bridge cycling rate induced by varying temperature (Brenner and Eisenberg, 1986) or altering substrate conditions (Regnier and Homsher, 1998; Regnier et al., 1998b). However, an increasing amount of evidence suggests that $k_{tr}$ is regulated by the dynamics of thin filament activation during submaximal $Ca^{2+}$ activations in fast skeletal fibers. When the $Ca^{2+}$ binding kinetics of TnC are altered, the relationship between P and $k_{tr}$ with varying [$Ca^{2+}$] can be greatly affected. In the presence of calmidazolium, a compound that specifically reduces the rate of $Ca^{2+}$ dissociation from TnC (Johnson et al., 1994) and increases the $Ca^{2+}$ sensitivity of steady-state tension (El-Saleh and Solaro, 1987), $k_{tr}$ is increased at submaximal (but not maximal) levels of $Ca^{2+}$-activated tension (Regnier et al., 1996). Additionally, the $Ca^{2+}$ dependence of $k_{tr}$ is reduced by replacement of native TnC with cardiac forms of TnC and is completely abolished by an activated form of TnC (aTnC) (Chase et al., 1994). Furthermore, increasing the rate of cross-bridge cycling by substitution of ATP with 2-deoxy-ATP (Regnier and Homsher, 1998; Regnier et al., 1998b) has little or no effect on $k_{tr}$ at submaximal levels of $Ca^{2+}$ activation. Paradoxically, decreasing the rate of cross-bridge cycling by lowering the [ATP] of activation solutions (Regnier et al., 1998b) actually increases $k_{tr}$ slightly at submaximal levels of $Ca^{2+}$

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activation. These findings suggest that at low levels of $Ca^{2+}$, $k_{tr}$ can be greatly influenced by the kinetics of $Ca^{2+}$ binding to TnC and thin filament activation.

In the present work we have further studied the influence of the $Ca^{2+}$ binding kinetics of TnC on the rate of tension development in skeletal muscle. Because slowing the rate of $Ca^{2+}$ dissociation from TnC results in an elevation of $k_{tr}$ at low levels of $Ca^{2+}$ activation (Regnier et al., 1996), we tested whether a faster rate of $Ca^{2+}$ dissociation from TnC would have the opposite effect, i.e., decrease $k_{tr}$ at low levels of $Ca^{2+}$ activation. This was done by extracting the native TnC from skinned rabbit psoas fibers and reconstituting them with a mutant TnC (NHdel) that has a lower $Ca^{2+}$ affinity due to an increased $Ca^{2+}$ dissociation rate (Rennie et al., 1997). Possible structural correlates relating the N-helix deletion to changes in the $Ca^{2+}$ binding loops have been discussed previously (Chandra et al., 1994; Strynadka et al., 1997). NHdel confers a much lower apparent affinity for $Ca^{2+}$ when it replaces native TnC in skinned fibers, resulting in a large decrease in the $Ca^{2+}$ sensitivity of steady-state tension (Chandra et al., 1994; Rennie et al., 1997). We now show that, despite this large change in $Ca^{2+}$ sensitivity of tension, $k_{tr}$ with NHdel on the thin filament is no slower than in fibers containing the recombinant control, rTnC, at similar levels of tension. This lack of a further inhibition of $k_{tr}$ at low levels of $Ca^{2+}$ is predicted by our model (see Discussion) and suggests that, in fast skeletal muscle, the thin filament activation process may be maximally tuned to provide the greatest $Ca^{2+}$ control over the rate of tension development. A preliminary report has appeared (Regnier et al., 1998a).

## METHODS

### Fiber preparation, mechanical apparatus, and data acquisition

Segments of glycerinated, skinned rabbit psoas fibers were prepared according to the method of Chase and Kushmerick (1988). Fiber end compliance was minimized by regional application of 1% gluteraldehyde in $H_2O$ to chemically fix fiber end segments (Chase and Kushmerick, 1988). The fixed ends were then wrapped in aluminum foil T-clips for attachment to small wire hooks on the mechanical apparatus. A drop of silicone sealant was placed on each T-clip to further stabilize the attachment to the hook. Fiber diameter and total fiber length (length of fiber segments between T-clips, including both unfixed and fixed fiber regions) were measured from digital images at the beginning of the experiment and periodically throughout the experiment as previously described (Regnier et al., 1996). At the conclusion of each experiment the fiber was fully activated and allowed to maximally shorten for determination of the unfixed portion of fiber segments ($L_o$) from digital images (Chase and Kushmerick, 1988). In relaxed fibers (pCa 9.2) at a sarcomere length ($L_s$) of 2.6 $\mu$m, fiber diameter was 55 $\pm$ 2 $\mu$m (mean $\pm$ SE, $n = 21$), and $L_o$ was 1.3 $\pm$ 0.1 mm.

For mechanical measurements, the tension transducer, servo motor, and helium-neon laser diffraction systems used to measure $L_s$ during experiments were as previously described (Chase et al., 1993, 1994). Fiber properties were maintained for long periods of continuous activation using a protocol first described by Brenner (1983). Steady-state tension (P) was obtained from the initial portion of digitized records and normalized to cross-sectional area, assuming circular geometry. In maximum activation conditions (pCa 4.5), the control tension ($P_o$) was 252 $\pm$ 12 mN/mm$^2$ (mean $\pm$ SE, $n = 21$) and relaxed tension (pCa 9.2) was ~1% of maximally activated tension. To measure $k_{tr}$, a fiber was rapidly shortened by ~20% $L_o$ with a 4 $L_o \cdot s^{-1}$ ramp, which reduced tension to zero, followed by a rapid (300 $\mu$s), under-damped restretch to the initial fiber length. The subsequent tension redevelopment kinetics were characterized as previously described (Chase et al., 1994). Briefly, an apparent rate constant was obtained by a linear transformation of the half-time estimate, extrapolating from 50% to 63.2% ($k_{tr} = \tau^{-1} = -\ln 0.5 \cdot (t_{0.5})^{-1}$), and is expressed as reciprocal seconds ($s^{-1}$). Alternatively, exponential fits were performed using the simplex method for nonlinear least-squares regression (Caceci and Cacheris, 1984). Experiments were performed without $L_s$ feedback control to minimize the stress placed on fibers during the long and demanding experimental protocols. This is justified because previous experiments have shown that $k_{tr}$ measurements carried out (using fibers with fixed ends) with and without $L_s$ control are linearly correlated, with the latter underestimating $k_{tr}$ by 20–30% at all levels of $Ca^{2+}$ activation (Chase et al., 1994). Thus, all values reported are from $t_{1/2}$ estimates of $k_{tr}$.

### Fiber solutions

Relaxing and activating solutions were calculated according to Martyn et al. (1994). Solutions were maintained at 0.2 M ionic strength, pH 7.0, and contained 5 mM MgATP, 15 mM PCr, 12–15 mM EGTA, at least 30 mM MOPS, 1 mM free $Mg^{2+}$, 135 mM $Na^+ + K^+$, 1 mM dithiothreitol (DTT), and 250 units ml$^{-1}$ creatine kinase (CK, Sigma, St. Louis, MO). The $Ca^{2+}$ levels (expressed as pCa = $-\log [Ca^{2+}]$) were established by varying the amount of $Ca(propionate)_2$. Dextran T-500 (4% w/v) (Pharmacia, Piscataway, NJ) was included in all solutions to minimize covariation of myofilament lattice spacing (and fiber diameter) with tension (Martyn and Gordon, 1988; Matsubara et al., 1985; Brenner and Yu, 1985). All mechanical measurements were made at 15°-17°C.

### TnC extraction and reconstitution

After measurement of steady-state tension and $k_{tr}$ with a pCa series (pCa 6.4–4.5), the native TnC was extracted, followed by reconstitution with one of the recombinant proteins, and the pCa series was repeated with the range extended to pCa 3.5 in the case of NHdel. Native TnC was extracted at 15–17°C by repeated exposure to a solution containing trifluoperazine dihydrochloride (TFP) (Metzger et al., 1989). In preliminary experiments this solution contained 5 mM EDTA, 20 mM Tris-HCl (pH 7.2), and 0.5 mM TFP (Hannon et al., 1993). In later experiments, fibers were exposed repeatedly (15 s) to a solution of 150 mM K propionate, 20 mM MOPS (pH 7.0), 5 mM EDTA, and 0.5 mM TFP, alternating with 15 s in relaxing solution (pCa 9.2). After a total of ~5 min in the TFP-containing solution, fibers were washed for 5 min in pCa 9.2 and extraction was evaluated by testing maximal activation tension ($P_o$). This test was repeated after a second wash in pCa 9.2 (5 min) to ensure that TFP had been thoroughly washed out (Chandra et al., 1994). Average residual tension following extraction of native TnC was <5% of control $P_o$. In preliminary experiments, fibers were reconstituted with different TnC constructs by exposure for 15 min to relaxing solution (see above) containing 1 mM DTT and 0.5–1 mg/ml recombinant TnC protein, without Dextran or creatine kinase. In later experiments, we found that 1–2 min incubation at 1 mg/ml TnC was adequate to provide maximal reconstitution. The level of reconstitution is reported as $P/P_o$ and is based on maximal activation with each TnC isoform (Table 1).

The effects of replacing native rabbit TnC with chicken TnC were studied by comparing native TnC and the control rTnC with a TnC (I130T) that repairs a spontaneous mutation ($T_{130} \rightarrow I$) occurring in the chicken cDNA clone (Golosinska et al., 1991). This control is presented at the end of the Results section. Each TnC construct used for comparisons was studied in a separate group of fibers.

**TABLE 1 Maximal tension ($P_o$) and $k_{tr}$ following reconstitution with recombinant TnC isoforms**

| TnC isoform | Reconstituted force | Reconstituted $k_{tr}$ |
|---|---|---|
| Native | 1 | 1 |
| rTnC WT | 0.89 ± 0.03 | 0.82 ± 0.04 |
| NHdel | 0.81 ± 0.02 | 0.76 ± 0.04 |
| I130T | 0.93 ± 0.04 | 0.7 ± 0.11 |

Normalized to the maximal values obtained at pCa 4.5 prior to extraction of native TnC. Before extraction, the values of $P_o$ (252 ± 12 mN/mm$^2$) and maximal $k_{tr}$ (16 ± 0.8 s$^{-1}$) did not vary significantly between groups of fibers.

## Proteins

All of the chicken recombinant proteins used in this study were expressed in *Escherichia coli* strain QY13 as fusion proteins followed by cleavage with factor Xa, as described by Reinach and Karlsson (1988). These proteins have an unblocked Met preceding Ala, where Ala is the first residue in the sequence of the native muscle protein. In the native chicken TnC, the Met is absent and the Ala residue is blocked by an acetyl group. Residue 1 (Thr) of the native rabbit skeletal muscle TnC is also blocked; it corresponds to residue 4 in the chicken protein. Thereafter, the sequence of the entire N-terminal half of the chicken protein is 95% identical to that of the rabbit protein (see Fig. 1).

The N-helix deleted recombinant protein NHdel, lacking residues 1–11, was produced by site-directed mutagenesis (Chandra et al., 1994), followed by expression and purification according to Golosinska et al. (1991). Amino-terminal sequences of the control rTnC and NHdel were confirmed by automated Edman degradation methods. The mutant I130T was constructed as described (Golosinska et al., 1991) and the entire cDNA of the coding region of the M13 vector was sequenced to confirm the structure (Fig. 1).

## Modeling of results

The results of steady-state and kinetic tension measurements were modeled using a program developed by Dr. Neil Millar (Millar and Homsher, 1990). This program numerically solves a series of simultaneous differential equations descriptive of the model scheme using the Gear method. The initial conditions before a perturbation allow calculation of steady-state tension from model state populations and, following a perturbation, the rate of tension redevelopment is determined from $t_{0.5}$, as in fibers. Methods for simulating $k_{tr}$ were previously presented in detail (Regnier et al., 1995; Hancock et al., 1997).

# RESULTS

## Steady-state tension

In previous reports, the lower $Ca^{2+}$ affinity of the regulatory N-domain sites of the NHdel TnC construct in solution has been attributed to an approximately twofold faster rate of $Ca^{2+}$ dissociation (Chandra et al., 1994; Rennie et al., 1997). In muscle fibers this decrease in $Ca^{2+}$ affinity results in a greatly reduced $Ca^{2+}$ sensitivity of steady-state tension. The chart records in Fig. 2 compare the $Ca^{2+}$ dependence of tension for a fiber reconstituted with NHdel versus a fiber reconstituted with the control rTnC. Each fiber was exposed to progressively increasing concentrations of $Ca^{2+}$ (decreasing pCa values), both before extraction of native TnC and after reconstitution with one of the recombinant TnCs. It is clear that after reconstitution with NHdel (Fig. 2 *b*) the fiber required more $Ca^{2+}$ to generate a given level of tension. In comparison, reconstitution with rTnC had little effect on the $Ca^{2+}$ sensitivity of tension (Fig. 2 *a*). Before extraction, the fibers in Fig. 2 had essentially identical responses to $Ca^{2+}$, with $pCa_{50}$ ~6.1. These records also illustrate force transients resulting from ramp release/restretch cycles (every 5 s) that are used to maintain fiber properties during long periods of activation (see Methods) and for measurements of the rate of tension redevelopment ($k_{tr}$).

The $Ca^{2+}$ dependence of steady-state tension for all fibers reconstituted with rTnC or NHdel is summarized in Fig. 3, demonstrating a dramatic reduction in $Ca^{2+}$ sensitivity of tension with the NHdel TnC construct. The $pCa_{50}$ for fibers reconstituted with NHdel was shifted to the right by 0.56 pCa units compared with fibers reconstituted with rTnC. This is indicative of a 3.6-fold reduction in the $Ca^{2+}$ sensitivity of tension because $n$ is similar for both TnC isoforms. A similar shift was reported earlier (0.77 pCa units by Chandra et al. (1994); 0.32 pCa units by Rennie et al. (1997)) and is somewhat larger than the shift monitored by spectroscopic methods using isolated TnC of 0.2–0.4 pCa units (Chandra et al., 1994; Rennie et al., 1997). These data confirm that the lower $Ca^{2+}$ binding affinity measured for the NHdel construct in solution, which results from a faster rate of $Ca^{2+}$ dissociation, decreases $Ca^{2+}$ sensitivity of steady-state tension when reconstituted into fibers.

## The rate of tension redevelopment, $k_{tr}$

To determine whether the reduced $Ca^{2+}$ affinity of TnC affects the rate of tension development, we compared $k_{tr}$ with each recombinant TnC at varying levels of $Ca^{2+}$ activation. The maximal $k_{tr}$ (at $P_o$) following reconstitution with either rTnC or NHdel was ~80% of that obtained with

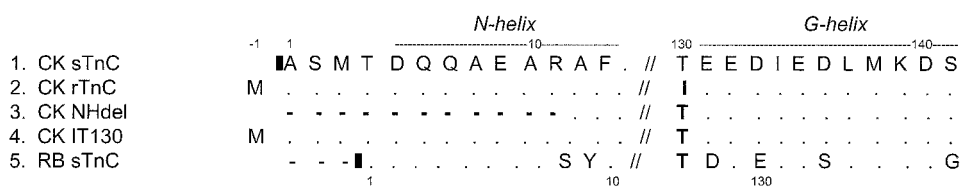



FIGURE 1 Comparison of partial primary sequences among TnC isoforms from chicken (1) and rabbit (5) skeletal muscle and the chicken recombinants (2–4) used in this study. Chicken and rabbit proteins differ in 9 additional positions (not shown), of which 7 are in the C domain. Dots and hyphens indicate repetition and absence, respectively, of the residues shown on line 1. Numbers above line 1 are for the chicken proteins and numbers below line 5 are for the rabbit protein. Sequence data are from Golosinska et al. (1991) (1, 2, and 4), Chandra et al. (1994) (3), and Collins et al. (1973) (5), with helices as determined by Herzberg and James (1985) and Sundaralingam et al. (1985).

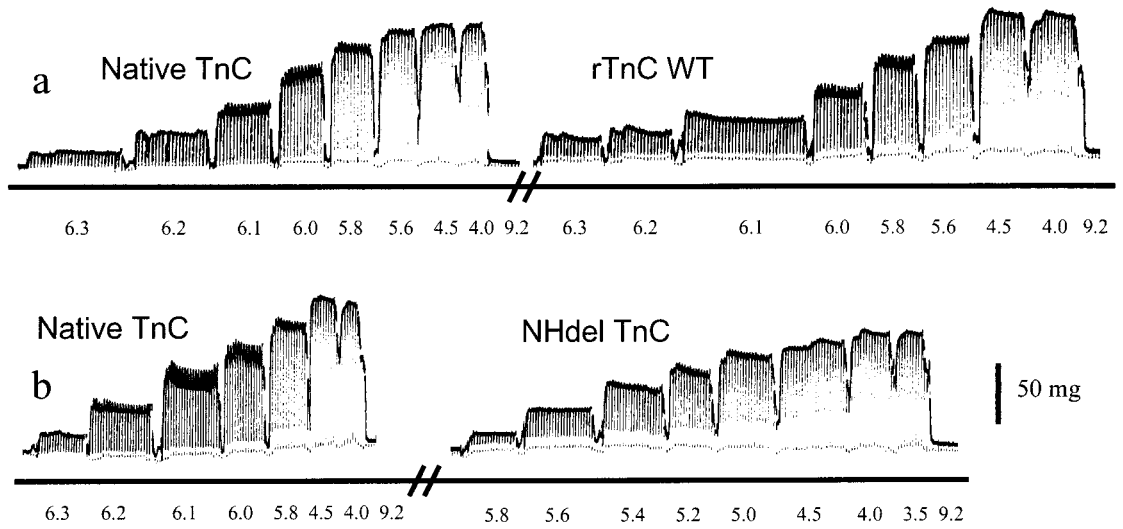



FIGURE 2 Chart records showing a $Ca^{2+}$ activation series for two fibers comparing contractions before TnC extraction and following extraction and reconstitution with either the chicken recombinant rTnC (*a*) or the NHdel construct (*b*). For each contraction series the $Ca^{2+}$ concentrations (pCa) are indicated below the force record. Force transients occur every 5 s because of ramp release/restretch cycles used to maintain the sarcomere integrity during prolonged activations (see Methods). Reconstitution with rTnC in (*a*) did not change the threshold [$Ca^{2+}$] and the [$Ca^{2+}$] needed to activate the fiber to half-maximal tension ($pCa_{50}$) was similar to when the native TnC was present, before extraction. In contrast, following reconstitution with NHdel in (*b*), the [$Ca^{2+}$] required for both threshold and half-maximal activation increased dramatically.

native TnC before extraction (Table 1). However, at pCa values greater than ~5.5, both steady state tension and $k_{tr}$ are decreased in fibers reconstituted with NHdel versus those with rTnC. Fig. 4 *a* shows example $k_{tr}$ traces obtained during activation at pCa 5.0. Both P (measured before the release step) and $k_{tr}$ are lower in the fiber reconstituted with NHdel than in the control fiber with rTnC. This reduction in both P and $k_{tr}$ was observed in all fibers reconstituted with NHdel. The $Ca^{2+}$ dependence of $k_{tr}$ for all fibers is summarized in Fig. 4 *b*. For ease of comparison of fibers reconstituted with NHdel and those reconstituted with rTnC, values for $k_{tr}$ were normalized to maximal $k_{tr}$ for each fiber before the extraction of native TnC. Fig. 4 *b* shows that $k_{tr}$ is $Ca^{2+}$-dependent with both NHdel and rTnC and changes by a similar magnitude when the [$Ca^{2+}$] is varied for both TnC constructs. This behavior is also comparable to the well-documented $Ca^{2+}$ dependence of $k_{tr}$ before extraction of the native TnC (Fig. 6). What differs between fibers reconstituted with NHdel versus rTnC is the range of activating $Ca^{2+}$ over which $k_{tr}$ increased to a maximum. For NHdel-reconstituted fibers a higher concentration of $Ca^{2+}$ is required before $k_{tr}$ increases above the slowest rate of 1–2 $s^{-1}$.

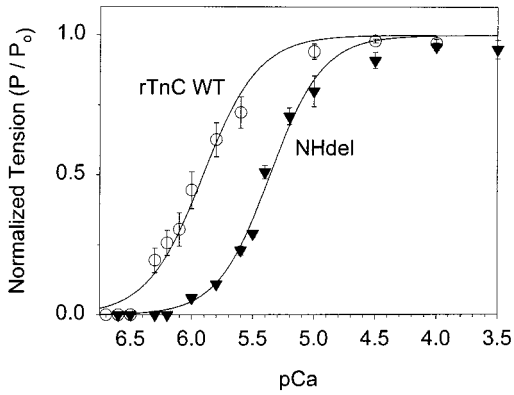



FIGURE 3 $Ca^{2+}$ sensitivity of steady-state tension in fibers reconstituted with rTnC (○) and NHdel (▼). Tension values are the means ± SE ($n$ = 7 fibers for each group) normalized to maximal reconstitution tension for each fiber before fitting with the Hill equation ($P = P_o/(1 + 10^{n(pCa50-pCa)})$) (*solid lines*) to determine $pCa_{50}$ and $n$. Fitted values for rTnC were $pCa_{50}$ = 5.91 ± 0.02 and $n$ = 1.9 ± 0.2. Fitted values for NHdel were $pCa_{50}$ = 5.35 ± 0.02 ($p < 0.001$) and $n$ = 2.0 ± 0.2. The lower $pCa_{50}$ for NHdel indicates that a 3.6-fold greater [$Ca^{2+}$] is needed to activate fibers to half-maximal tension.

The rightward shift observed for both the tension versus pCa (Fig. 3) and $k_{tr}$ versus pCa (Fig. 4 *b*) relationships suggests that both of these shifts may result solely from reduced numbers of tension-generating cross-bridges at submaximal levels of $Ca^{2+}$ activation in NHdel-reconstituted fibers. If this is indeed the case, $Ca^{2+}$ activation to similar levels of P should yield similar values of $k_{tr}$. An example of this type of comparison is shown in Fig. 5 *a*, demonstrating that at both low and nearly maximal levels of activation, $k_{tr}$ is correlated with P, not pCa. To compare $k_{tr}$ for NHdel and rTnC over the entire range of $Ca^{2+}$ activation, the data in Fig. 4 *b* are replotted in Fig. 5 *b* as $k_{tr}$ versus P (binned in 0.1 $P_o$ tension level increments). The values for both $k_{tr}$ and P were normalized to the maximal values obtained with native TnC (pCa 4.5) before extraction and reconstitution with either NHdel or rTnC to compare the two groups of fibers. Both NHdel and rTnC provide a $k_{tr}$-versus-P relationship with the same curvilinear profile seen with native TnC (see Fig. 7). As Fig. 5 *b* shows, at equivalent levels of P, $k_{tr}$ is similar for fibers with either NHdel or rTnC. Of

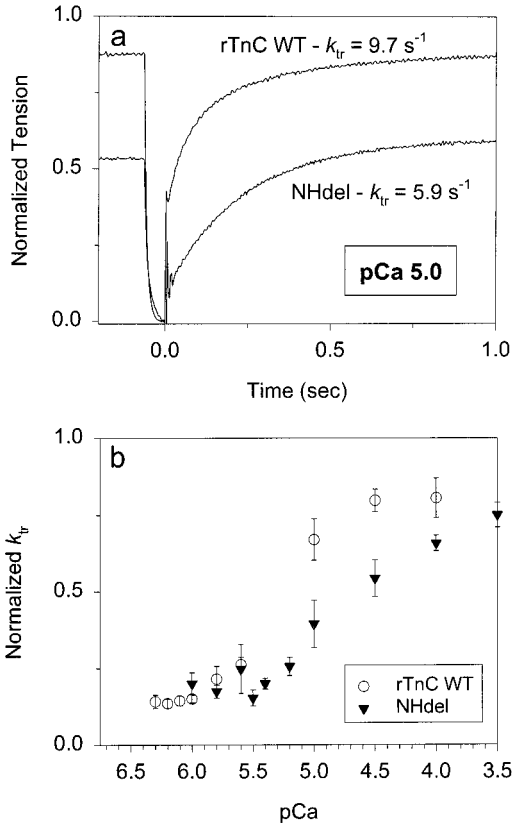



FIGURE 4 The rate of tension redevelopment ($k_{tr}$) following a release/restretch cycle. (*a*) Example tension traces (normalized to the maximum value for each fiber) comparing a fiber reconstituted with rTnC versus a fiber reconstituted with NHdel at pCa 5.0. Steady-state tension before release was less for the NHdel fiber and $k_{tr}$ was slower. (*b*) Summary of the $Ca^{2+}$ sensitivity of $k_{tr}$ for fibers with NHdel or rTnC. Values are normalized to maximal $k_{tr}$ with native TnC present, showing that with either recombinant TnC, maximal reconstituted $k_{tr}$ is similar (see Table 1). The inhibition of $k_{tr}$ at low levels of $Ca^{2+}$ activation is of similar magnitude for both TnC isoforms and the rate (1–2 $s^{-1}$) is comparable to the values obtained before extraction of native TnC (see Fig. 6b). For the rTnC-reconstituted fibers this inhibition is relieved at higher pCa than for NHdel-reconstituted fibers.

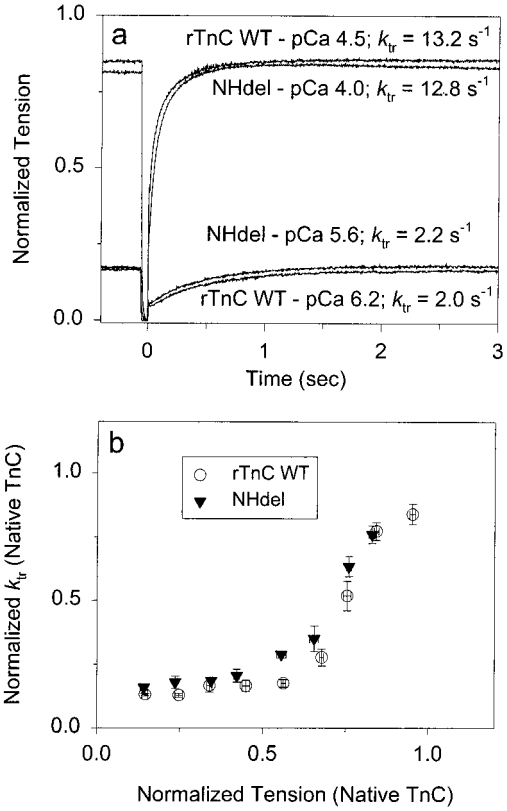



FIGURE 5 Comparison of $k_{tr}$ at similar levels of $Ca^{2+}$-activated tension. (*a*) Example tension-matched $k_{tr}$ traces at ~0.2 $P_o$ and near $P_o$. The pCa used to activate fibers to these tension levels and the measured $k_{tr}$ are indicated next to each trace. These examples show that more $Ca^{2+}$ is required to activate fibers in the presence of NHdel TnC, but $k_{tr}$ is similar for both recombinant proteins when $Ca^{2+}$ activation levels are matched. (*b*) The $k_{tr}$-versus-tension relationship for all fibers reconstituted with rTnC (○, $n$ = 7 fibers) or NHdel (▼, $n$ = 7 fibers). Values for the means ± SE for $k_{tr}$ replotted from Fig. 4 *b* after binning the data in 0.1 $P_o$ bins. As in Fig. 4 *b*, the data for each fiber are normalized to $P_o$ and maximal $k_{tr}$ (pCa 4.5) before extraction of native TnC. Throughout the entire range of $Ca^{2+}$-activated tensions, $k_{tr}$ differs significantly between the two sets of fibers only in one tension bin (0.5–0.6 $P_o$).

particular note is that there is no difference in $k_{tr}$ at low levels of activation (i.e., $P < 0.5\ P_o$). Thus the slower $k_{tr}$ values seen with NHdel at intermediate concentrations of $Ca^{2+}$ (Fig. 4 *b*) most likely result solely from a decrease in the number of cycling cross-bridges, with no additional influence of the *increased* rate of $Ca^{2+}$ dissociation from TnC on $k_{tr}$. This contrasts with the increase in $k_{tr}$ at similar low levels of P when the rate of $Ca^{2+}$ dissociation from TnC is *decreased* (Regnier et al., 1998b; see Discussion). Taken together, these results suggest the $Ca^{2+}$ dissociation rate of TnC influences both thin filament activation kinetics and cross-bridge recruitment and, at low levels of $Ca^{2+}$, modulates the rate of tension development in fast skeletal muscle.

## Controls

Because different sets of fibers were used to test the two recombinant proteins, it is essential to determine that the mechanical behavior of each set of fibers is similar before extraction of the native TnC. The P versus pCa (Fig. 6 *a*) and $k_{tr}$ versus P (Fig. 6 *b*) relationships for each group of fibers were identical, within the range of experimental error, before extraction of native TnC and reconstitution with rTnC or NHdel. Although reconstitution with the control recombinant TnC (rTnC) results in the characteristic curvilinear $k_{tr}$-versus-tension relationship seen with native TnC, Fig. 7 *a* shows that $k_{tr}$ consistently begins to increase at lower levels of $Ca^{2+}$-activated tension with rTnC (Fig. 7 *a*). This difference between native TnC and rTnC is probably not due to the reconstitution procedure, because we have previously shown that reconstitution with purified rabbit skeletal TnC results in a $k_{tr}$-versus-tension relationship identical to that produced before extraction of native TnC (Chase et al., 1994). The $Ca^{2+}$ dissociation rate for chicken recombinant TnC has been measured in solution using a protein in which Phe 29 was replaced with Trp to provide a

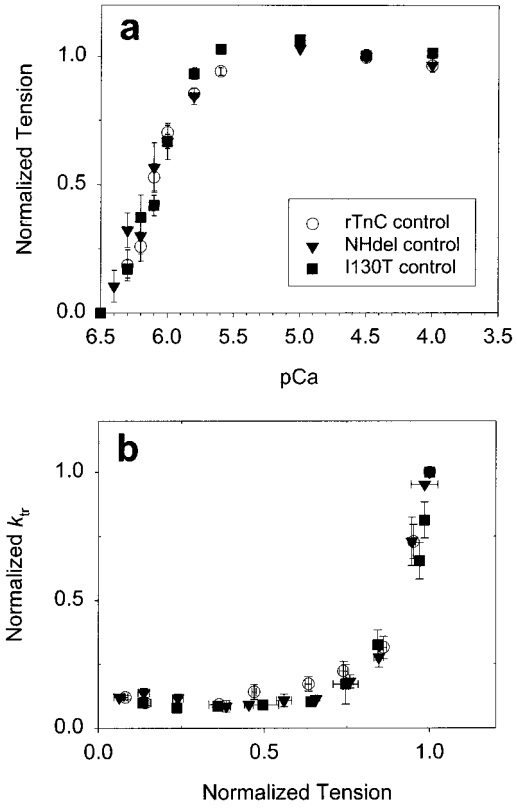



FIGURE 6 Comparison of fibers used for rTnC, NHdel, and I130T reconstitutions, measured before extraction of the native TnC. Data are normalized to values obtained at pCa 4.5. (*a*) P-versus-pCa relationship; data were fitted with the Hill equation (lines not shown) and the $pCa_{50}$ values for native TnC (indicated by reconstitution group) were 6.11 ± 0.01 (rTnC), 6.11 ± 0.02 (NHdel), and 6.08 ± 0.01 (I130T). Hill *n* values were 3.5 ± 0.3 (rTnC), 2.9 ± 0.3 (NHdel), and 3.1 ± 0.3 (I130T). (*b*) $k_{tr}$-versus-P relationship for fibers shown in (*a*), grouped in bins of 0.1 $P_o$. Maximal $k_{tr}$ ranged from 14–21 $s^{-1}$ for all the fibers and there were no significant differences from the mean maximal rate (16 ± 0.8 $s^{-1}$). The $k_{tr}$-versus-P relationship is essentially identical for all three groups of fibers used for reconstitution experiments. In some fibers steady-state tension was slightly greater at pCa 5.0, but $k_{tr}$ was always maximal at pCa 4.5–4.0.

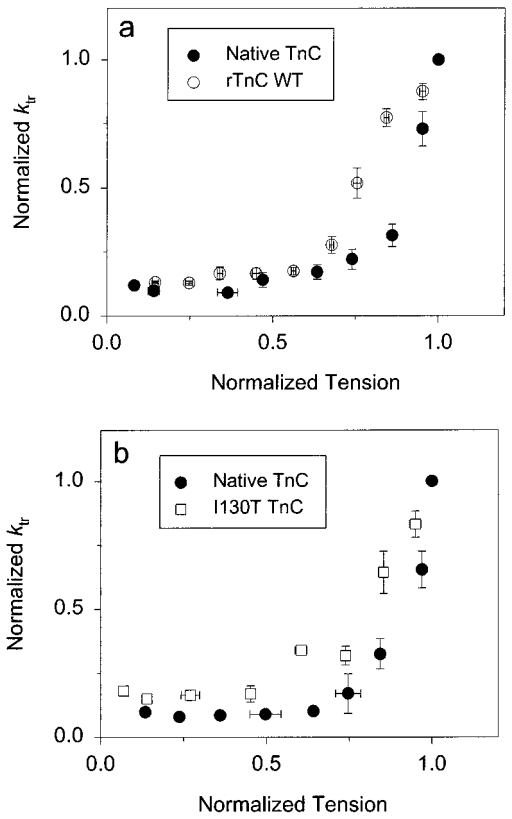



FIGURE 7 Effect of I130 conversion to the original Thr residue of chicken TnC on the $k_{tr}$ versus P relationship. (*a*) Following extraction of native (rabbit) TnC and reconstitution with the recombinant rTnC, $k_{tr}$ begins to increase toward its maximum at slightly lower levels of P. (*b*) A similar shift occurs in fibers reconstituted with I130T, suggesting that the spontaneous mutation at this position is not responsible for the differences between chicken and rabbit TnC isoforms in the $k_{tr}$-versus-P relationship.

fluorescent reporter of $Ca^{2+}$ binding to the N-terminal sites (Johnson et al., 1994). This protein, F29W, has the same $Ca^{2+}$ affinity as rTnC (Golosinska et al., 1991; Chandra et al., 1994) and presumably a similar $Ca^{2+}$ dissociation rate, which is similar to that for rabbit skeletal muscle TnC (Johnson et al., 1994). Thus some other factor must be involved in the small differences seen in Fig. 7 *a*.

The primary sequence of rTnC (residues 4–162) is 90% identical to that of the rabbit protein (residues 1–159), and the homology increases to 96% when conservative substitutions are taken into account. Among the 16 residues that differ between the two proteins, 12 lie in the C domain. A spontaneous mutation in the cDNA that was cloned to produce the recombinant proteins originally led to replacement of Thr 130 (corresponding to Thr 127 in the rabbit protein) with Ile. In a previous report, this was shown to reduce the $Ca^{2+}$ affinity of the C-terminal sites by about 0.4 log units (Golosinska et al., 1991). For this reason, we tested the recombinant chicken protein I130T, which was constructed to restore the original Thr residue at position 130, and has normal $Ca^{2+}$ affinity (Golosinska et al., 1991). The results (Fig. 7 *b*) show that the $k_{tr}$-versus-P relationship for I130T is essentially the same as with rTnC (compare with Fig. 7 *a*), which contains the residue Ile 130. We therefore conclude that the difference between rabbit TnC and the chicken recombinant TnC proteins cannot be attributed to this residue alone. Possibly one or more of the other differences in sequence produces a relatively minor change in the $Ca^{2+}$ binding kinetics or a mismatch in TnC-TnI interactions in the reconstituted hybrids.

## DISCUSSION

The main finding of the present experiments is that a large decrease in the $Ca^{2+}$ affinity of TnC due to an *increased* rate of $Ca^{2+}$ dissociation from TnC decreases the $Ca^{2+}$ sensitivity of both steady-state tension (P) and the rate of tension redevelopment ($k_{tr}$), but has little or no effect on $k_{tr}$ at similar levels of $Ca^{2+}$-activated P (Fig. 5). Previously, we have shown that *decreasing* the rate of $Ca^{2+}$ dissociation

from TnC increases the $Ca^{2+}$ sensitivity of P and $k_{tr}$ and elevates $k_{tr}$ at similar low levels of $Ca^{2+}$-activated P (Regnier et al., 1996; Chase et al., 1994). Taken together, these results indicate that at low [$Ca^{2+}$], the kinetics of $Ca^{2+}$ binding to TnC, followed by thin filament activation, is sufficiently slow to limit or inhibit the rate of tension development in fast skeletal muscle, and that reducing the rate of $Ca^{2+}$ dissociation from TnC relieves the inhibition of $k_{tr}$, whereas increasing the rate of $Ca^{2+}$ dissociation does not inhibit $k_{tr}$ further.

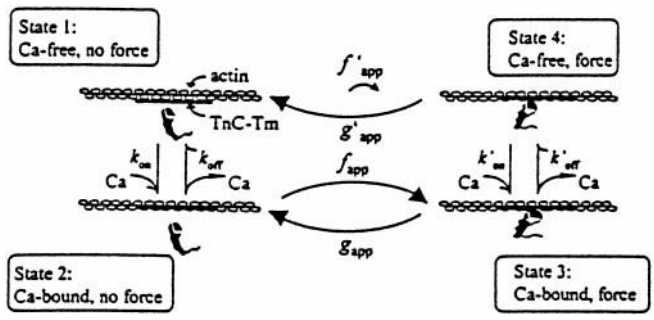
This study provides further evidence that the $Ca^{2+}$ regulation of $k_{tr}$ occurs via the thin filament activation process, without major influence from a second regulatory site such as a cross-bridge transition rate (Brenner, 1988; Metzger and Moss, 1990b) or the regulatory light chain of myosin (Metzger and Moss, 1992; Diffee et al., 1996; Patel et al., 1996). If either of these mechanisms were predominant, we would expect $k_{tr}$ to increase at similar levels of $Ca^{2+}$ regardless of whether NHdel or rTnC was present. However, this was not the case. As Fig. 4 shows, $k_{tr}$ remained slow (1–2 $s^{-1}$) in fibers with NHdel at much higher levels of $Ca^{2+}$. Instead, the relationship between $k_{tr}$ and $Ca^{2+}$-activated tension was maintained with NHdel (Fig. 5 *b*), and we therefore conclude that differences in $Ca^{2+}$ sensitivity of steady-state tension and $k_{tr}$ in fibers resulted from the different properties of the recombinant TnCs.

The finding that increasing the $Ca^{2+}$ dissociation rate of TnC does not slow $k_{tr}$ at low levels of $Ca^{2+}$ activation (this study), but decreasing this rate can dramatically increase submaximal $k_{tr}$ (Chase et al., 1994; Regnier et al., 1996), suggests the $Ca^{2+}$ binding kinetics of TnC in fast skeletal muscle may be tuned to provide for maximal $Ca^{2+}$ control of the rate of tension development. This idea of TnC tuning of the regulatory system is supported by experiments in which the native TnC of fast fibers was replaced with cardiac TnC. In these fiber preparations $k_{tr}$ was faster at low levels of $Ca^{2+}$ activation and increased less during maximal activations (compared to control), resulting in a much reduced $Ca^{2+}$ dependence of $k_{tr}$ (Chase et al., 1994). Indeed, the $k_{tr}$-versus-P relationship in such hybrid preparations resembles that of cardiac muscle (Hancock et al., 1993, 1996; Wolff et al., 1995; Araujo and Walker, 1996), indicating that the TnC isoform controls the extent to which changes in [$Ca^{2+}$] can affect the rate of tension development.

What is the rate-limiting process within thin filament activation regulates $k_{tr}$ during submaximal $Ca^{2+}$ activations? Because the rate of $Ca^{2+}$ binding to N-terminal sites of both skeletal and cardiac muscle TnC appears to be diffusion-limited (or very nearly so) (Johnson et al., 1979, 1980, 1994; Rosenfeld and Taylor, 1985), another step in the thin filament activation process is probably rate-limiting. This could be the rate of $Ca^{2+}$ dissociation from TnC, a $Ca^{2+}$-dependent conformational transition within TnC, or a $Ca^{2+}$-dependent interaction between TnC and another thin filament regulatory protein (TnI, TnT). Recent experimental evidence from solution studies suggests that conformational changes in TnC induced by $Ca^{2+}$ binding and dissociation occur more slowly than $Ca^{2+}$ binding and dissociation per se (Dong et al., 1997; Hazard et al., 1998). These conformational changes appear to be independent of [$Ca^{2+}$], however, suggesting this is not how $Ca^{2+}$ regulates $k_{tr}$ (Dong et al., 1997). Conversely, the rate at which $Ca^{2+}$ dissociates from TnC could confer $Ca^{2+}$ regulation of $k_{tr}$ by directly affecting the time thin filament regulatory units spend in the "on" state, thereby controlling strong cross-bridge attachment and tension generation. Evidence from regulatory protein complexes (in solution) suggests $Ca^{2+}$ dissociation from TnC may be slow enough (1–2 $s^{-1}$ at 4°C and 15–20 $s^{-1}$ at 20°C) to confer a $Ca^{2+}$ dependence of $k_{tr}$ (Rosenfeld and Taylor, 1985). However, the rate of $Ca^{2+}$ dissociation from TnC has not been measured in fibers.

To evaluate the relative importance of $k_{on}$ and $k_{off}$ for thin filament activation in the regulation of $k_{tr}$ we have modeled our experimental results using a four-state reaction mechanism (Scheme 1). This reaction mechanism, originally proposed by Landesberg and Sideman (1994) and modified by Hancock et al. (1997), is based on the coupling of thin filament activation and cross-bridge cycling kinetics. Scheme 1 depicts an individual thin filament regulatory unit of 7 actin monomers, 1 tropomyosin (Tm), and 1 troponin (Tn), and the associated cross-bridges. Its features have been described in detail elsewhere (Hancock et al., 1997; Regnier et al., 1998b) and therefore will not be presented here.

To simulate experimental steady-state tension and $k_{tr}$ at 15°C the apparent rate constants regulating cross-bridge cycling ($f_{app}$, $g_{app}$, and $g'_{app}$) were set to generate a maximal $k_{tr}$ of 16–17 $s^{-1}$ (controlled by $f_{app}$ + $g_{app}$) and a minimal $k_{tr}$ of 1.5 $s^{-1}$ (controlled by $g_{app}$) (see Fig. 8 legend). To simulate varying [$Ca^{2+}$], we assumed that $Ca^{2+}$ affects only thin filament activation kinetics and values for $k_{on}$ and $k_{off}$ were selected in the following manner. A wide range of $k_{off}$ values simulated the control $k_{tr}$-versus-P relationship, as $k_{on}$ was varied to simulate changing [$Ca^{2+}$]. However, larger values of $k_{off}$ (> 100 $s^{-1}$) did not allow $k_{tr}$ to increase at low [$Ca^{2+}$], as when $k_{off}$ was reduced two- to threefold experimentally (Regnier et al., 1996), so we arbitrarily set $k_{off}$ at 10 $s^{-1}$ to simulate control conditions. An apparent second-order binding constant of $Ca^{2+}$ was calculated (~$10^7$ $M^{-1}$ $s^{-1}$) that is within the range reported by Rosenfeld and Taylor (1985) for the thin filament in solution, and this allowed us to simulate a P-versus-pCa relationship (not shown) and a $k_{tr}$-versus-P relationship for rTnC (closed circles in Fig. 8).




Scheme 1

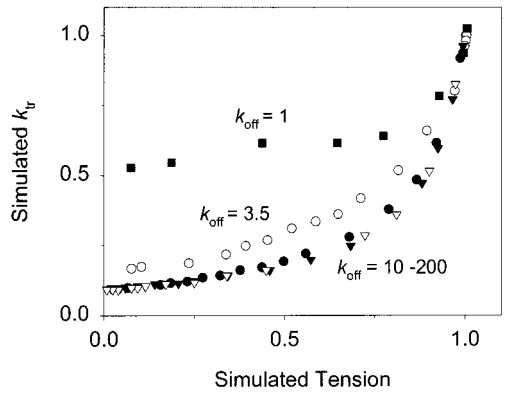



FIGURE 8 Simulations of P and $k_{tr}$ using Scheme 1. For simulations, the apparent rate constants (in $s^{-1}$) for cross-bridges were $f_{app} = 17$, $f'_{app} = 0.01$, $g_{app} = g'_{app} = 1.5$. Values for $k_{off}$, $k'_{off}$ were (in $s^{-1}$) 1 (■), 3.5 (○), 10 (●), 70 (▽), or 200 (▼). $k_{on}$ and $k'_{on}$ were varied from 0–144 $s^{-1}$ to simulate the pCa range used for experiments (described in the Discussion). These simulations predict no effect on the $k_{tr}$-versus-P relationship when $k_{off}$ is increased above the control value (10 $s^{-1}$), in accordance with experimental data for NHdel (Fig. 5 *b*). In contrast, decreasing $k_{off}$ below 10 $s^{-1}$ can dramatically reduce the $Ca^{2+}$ dependence of $k_{tr}$, as we have previously shown (Regnier et al., 1996, 1998b; Chase et al., 1994).

To simulate the results with NHdel, $k_{off}$ was increased sevenfold (to 70 $s^{-1}$), such that $pCa_{50}$ was reduced to 5.35, as in the reconstituted fiber experiments (see Fig. 3). Interestingly, this increase in $k_{off}$ is much larger than the two- to threefold increase measured for NHdel compared with F29W in solution (Rennie et al., 1997). However, this and even larger increases in $k_{off}$ (up to 100-fold), had no effect on the $k_{tr}$-versus-P relationship, except to reduce $P_o$ at maximal activation (Fig. 8). Thus, in accordance with the experimental data (Fig. 5 *b*), higher values for $k_{off}$ do not reduce $k_{tr}$ at low levels of activation. Scheme 1 also suggests that the rate of cross-bridge dissociation, i.e., $g_{app}$, sets the lower limit for $k_{tr}$ (at low levels of fiber activation) and that reducing $k_{off}$ (Fig. 8, *upper curves*) can markedly increase $k_{tr}$ at low levels of activation, as shown earlier (Regnier et al., 1996, 1998b).

As mentioned above, Scheme 1 describes a simple coupled relationship between activation of the thin filament and cross-bridge cycling. We have not incorporated any cooperative mechanisms of $Ca^{2+}$ or cross-bridge binding on the thin filament activation mechanism. Because of this we cannot model the cooperativity that occurs in the P versus pCa relationship, but simulations are able to predict the $k_{tr}$ versus P relationship as $[Ca^{2+}]$ is varied. This suggests the $Ca^{2+}$ dependence of $k_{tr}$ can be described by the kinetics of individual thin filament regulatory units without cooperative mechanisms, unlike steady-state tension. This idea is supported by the experiments of Metzger and Moss (1991) showing that partial extraction of TnC does not alter the $Ca^{2+}$ dependence of $k_{tr}$. Additionally, we have recently investigated cooperativity between individual regulatory units along the thin filament by extracting endogenous TnC and reconstituting with mixtures of purified native TnC and a non-$Ca^{2+}$-binding skeletal TnC mutant. In these preparations, even when the percentage of thin filament units containing native TnC was 10–20%, the activation dependence of $k_{tr}$ was unaffected, even though $pCa_{50}$ was decreased by 0.3–0.5 units and the slope of the pCa-tension relationship was reduced (Regnier et al., 1999).

In summary, an increasing amount of evidence suggests the $Ca^{2+}$ binding kinetics of TnC can regulate $k_{tr}$ during submaximal activations in fast skeletal muscle fibers. Whereas maximal $k_{tr}$ is determined by the myosin isoform present and the rate of cross-bridge cycling (Metzger and Moss, 1990a; Regnier et al., 1998b; Regnier and Homsher, 1998), our studies indicate the rate of $Ca^{2+}$ dissociation from TnC may determine $k_{tr}$ at low levels of $Ca^{2+}$ activation (Regnier et al., 1996; Chase et al., 1994). The present study suggests the major point of $Ca^{2+}$ regulation for $k_{tr}$ is the thin filament, not the regulatory light chain of myosin or a kinetic step in the cross-bridge cycle. Additionally, simulating the regulation of $k_{tr}$ by $Ca^{2+}$ can be done without modeling cooperativity between regulatory units along the thin filament or cooperative activation of the thin filament by strong cross-bridge binding, even though these mechanisms are necessary to explain the steep $Ca^{2+}$ dependence of steady-state tension.

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