Calmidazolium Alters Ca^{2+} Regulation of Tension Redevelopment Rate in Skinned Skeletal Muscle

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ABSTRACT To examine if the Ca^{2+}-binding kinetics of troponin C (TnC) can influence the rate of cross-bridge force production, we studied the effects of calmidazolium (CDZ) on steady-state force and the rate of force redevelopment (k_{tr}) in skinned rabbit psoas muscle fibers. CDZ increased the Ca^{2+}-sensitivity of steady-state force and k_{tr} at submaximal levels of activation, but increased k_{tr} to a greater extent than can be explained by increased force alone. This occurred in the absence of any significant effects of CDZ on solution ATPase or in vitro motility of fluorescently labeled F-actin, suggesting that CDZ did not directly influence cross-bridge cycling. CDZ was strongly bound to TnC in aqueous solutions, and its effects on force production could be reversed by extraction of CDZ-exposed native TnC and replacement with purified (unexposed) rabbit skeletal TnC. These experiments suggest that the method of CDZ action in fibers is to bind to TnC and increase its Ca^{2+}-binding affinity, which results in an increased rate of force production at submaximal [Ca^{2+}]. The results also demonstrate that the Ca^{2+}-binding kinetics of TnC influence the kinetics of k_{tr}.

INTRODUCTION

The initial step in regulation of force generation in skeletal muscle fibers is Ca^{2+} binding to the thin filament regulatory protein troponin C (Chalovich, 1992; Farah and Reinach, 1995; Gergeley et al., 1993; Tobacman, 1996). The mechanism of Ca^{2+} action could be either regulation of the rate of a specific step in the cross-bridge cycle or regulation of cross-bridge recruitment. In skeletal muscle fibers, evidence supporting the former hypothesis includes the observation that the rate of isometric tension redevelopment (k_{tr}) increases nonlinearly as [Ca^{2+}] increases (Brenner, 1988; Metzger et al., 1989; Metzger and Moss, 1990; Metzger and Moss, 1991; Millar and Homsher, 1990; Regnier et al., 1995; Swartz and Moss, 1992; Sweeney and Stull, 1990; Walker et al., 1992). Because k_{tr} is thought to report the rate of transition from detached or weakly bound cross-bridge states to force-generating states, the strong Ca^{2+} dependence of k_{tr} implies that Ca^{2+} regulates the rate-limiting step in force generation. Using a two-state model, it has been suggested that the forward rate constant of force generation is the Ca^{2+}-dependent process (Brenner, 1988; Sweeney and Stull, 1990). Such models predict a unique relationship between force and k_{tr} as Ca^{2+} is varied. Furthermore, this relationship should not be altered by changes in thin filament Ca^{2+}-binding properties; this prediction is not supported by experimental evidence (Chase et al., 1994).

The relationship between steady-state force and k_{tr} can be altered by manipulation of troponin C (TnC). For example, replacing native skeletal TnC (sTnC) in skinned rabbit psoas fibers with either purified rabbit cardiac TnC (cTnC), a modified form of cTnC (aTnC), or recombinant TnC mutants can alter the force-k_{tr} relationship (Chase et al., 1994; Pan et al., 1994). In addition, Metzger and Moss (1991) showed that partial extraction of TnC decreased isometric force at a given [Ca^{2+}] without affecting k_{tr}. Taken together, these studies indicate the Ca^{2+}-binding properties of TnC are important modulators of k_{tr}, specifically at submaximal Ca^{2+} activations. Thus, the kinetics of activation-dependent thin filament processes could alter the kinetics of acto-myosin interactions, and this alteration correlates with solution measurements of the Ca^{2+} dissociation rate from TnC (Chase et al., 1994). To more directly examine this correlation we measured the effect of an agent that changes the rate of Ca^{2+} dissociation from endogenous TnC on the k_{tr}-force relationship.

Calmidazolium (CDZ, Fig. 1) is a Ca^{2+}-sensitizing compound, which stabilizes the interaction between TnC and Ca^{2+} by specifically reducing the Ca^{2+} dissociation rate from TnC (El-Saleh and Solaro, 1987; Johnson et al., 1994; Wahr et al., 1993). We hypothesized that CDZ should increase the Ca^{2+} sensitivity of force and k_{tr}, and also alter the force-k_{tr} relationship obtained by varying the steady-state activation level (Landesberg and Sideman, 1994). To test these hypotheses, the Ca^{2+} dependencies of force and k_{tr} were measured both in the absence and presence of CDZ using single glycerinated psoas fibers from rabbit. We also examined the specificity of CDZ action: i.e., whether the effects of CDZ were mediated by binding to TnC, rather than by affecting cross-bridge interactions directly. The results show that CDZ 1) increases the Ca^{2+} sensitivity of force and k_{tr} (at 10°C), 2) alters the relation between force and k_{tr} by increasing k_{tr} at a given submaximal force level, and 3) appears to act specifically via its interaction with TnC. Thus, via its influence on TnC, CDZ modifies the kinetics of the thin filament activation process of submaxi-

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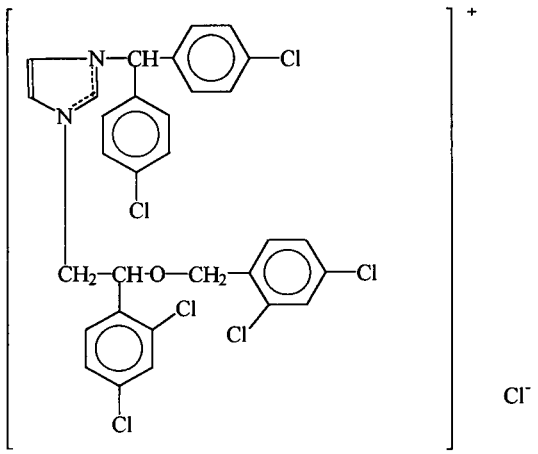

FIGURE 1 Structure of CDZ. Adapted from van Belle (1981); drawn using ChemWindows (SoftShell, Grand Junction, CO).

mal activations, resulting in an increase in the rate of cross-bridge transition from nonforce-bearing to force-bearing states. A preliminary report of these data has appeared in abstract form (Regnier et al., 1996).

METHODS

Fiber preparation

Glycerinated segments of individual, fast fibers from rabbit psoas muscle were prepared according to Chase and Kushmerick (1988). Isolated fiber segments were treated for 10 min with the nonionic detergent Trition X-100 (1%) in a 50% glycerol/relaxing (v:v) solution to remove residual membrane fragments. Fiber end compliance was minimized by regional microapplication of 1% gluteraldehyde (in H_2O) to chemically fix fiber segment ends (Chase and Kushmerick, 1988). After fixation of the ends, fiber segments were wrapped in aluminum foil T-clips for attachment to small wire hooks on the mechanical apparatus. For most experiments a drop of silicone sealant was placed on the T-clip to further stabilize the attachment to the hook. Digital images of relaxed fibers and fibers during steady-state contractions were obtained with 0.32 μm/pixel (32 × objective) or 2.56 μm/pixel (4 × objective) using an XR-77 CCD camera (Sony, Japan), DT3155 frame grabber and Global Lab Image software (Data Translation, Marlboro, MA). Fiber diameter and total fiber length (length of fiber segment between T-clips, including both unfixed and fixed fiber regions) were measured at the beginning of each experiment. The unfixed portion of fiber length (L_o) was determined at the conclusion of each experiment as previously described (Chase and Kushmerick, 1988). In relaxed fibers (pCa 9.2) at a sarcomere length (L_s) of 2.56 ± 0.07 (mean ± SD, N = 23), diameter was 52 ± 1.7 μm and L_o was 1.18 ± 0.25 mm (N = 18 fibers; determinations not made on five fibers).

Mechanical apparatus and data acquisition

For mechanical measurements, the force transducer was either a Model 400A with a 2.2-kHz resonant frequency (Cambridge Technology, Watertown, MA) or a Model AE801 with a ≥5-kHz resonant frequency (SensoNor, Horton, Norway). Fiber length was controlled using a model 300 servo motor (Cambridge Technology, Watertown, MA) tuned for a 300-μs step response. Helium-neon laser diffraction was used to measure L_s during experiments (Chase et al., 1993). All signals were recorded digitally with 12-bit resolution at a rate of 0.3–2.0 kHz per channel (Chase et al., 1994). To avoid high frequency aliasing, all signals were low pass filtered ($f_c \leq$ 40% of the sampling rate) using a CyberAmp 380 (Axon Instruments, Foster City, CA) before digitization. Fiber properties were maintained during continuous activation by using a protocol first described by Brenner (1983). An example of an activation series can be seen in Fig. 2.

Measurements of k_{tr} (Brenner and Eisenberg, 1986) shown in Figs. 5, 6, and 7 were made according to the following procedure. First, steady-state isometric force was obtained from the initial portion of each record and was normalized to a cross-sectional area using the measured diameter and assuming circular geometry. In maximum activating solutions (pCa 4.0) the control force was 266 ± 71 mN/mm^2 (mean ± SD, n = 23) and relaxed force (pCa 9.2) was 1.6 ± 0.7% of the maximally activated force. To measure k_{tr}, the fiber was then shortened by ~20% L_o with a 4 $L_o \cdot s^{-1}$ ramp, which reduced force to zero, followed by a rapid (300 μs), underdamped restretch to the initial fiber length. Subsequent force 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 ($k_{tr} = \tau^{-1} = -\ln 0.5 \cdot (t_{1/2})^{-1}$), and is expressed as reciprocal seconds (s^{-1}). Exponential curve fits were performed using the simplex method for nonlinear least-squares regression (Caceci and Cacheris, 1984). For most experiments, k_{tr} was measured without L_s feedback control to minimize the stress placed on fibers during long experimental protocols; this is justified because previous experiments

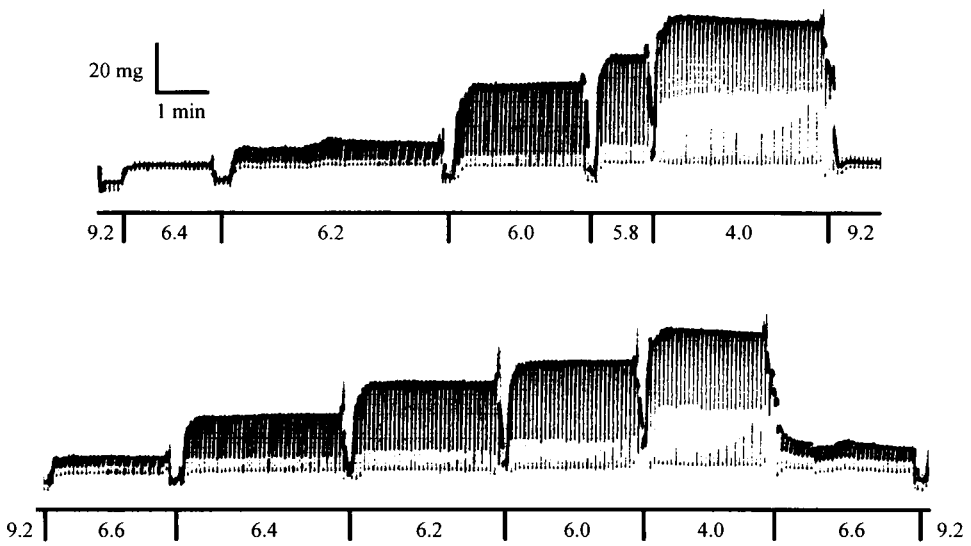

FIGURE 2 Record of an activation series for a single glycerinated rabbit psoas fiber in the absence (*A*) and presence (*B*) of CDZ (30 μM). Ca^{2+} concentrations (pCa) and solution changes are indicated below the force record. Force transients occur every 5 s due to ramp release/restretch cycles (see Methods). After the control activation series (*A*) the fiber was incubated in a relaxation solution (pCa 9.2) containing 30 μM CDZ for 15 min, followed by maximal activation (pCa 4.0) in the presence of 30 μM CDZ to optimize CDZ binding to TnC (not shown). The activation series was then repeated to compare steady-state force and k_{tr} with control activations (*B*). Note that both force and k_{tr} were consistently increased with CDZ at all submaximal Ca^{2+} concentrations.

have shown that k_{tr} measured with and without L_s control are linearly correlated, yielding qualitatively similar results (Chase et al., 1994).

Fiber solutions

Relaxing and activating solutions were calculated according to Martyn et al. (1994). Solutions were maintained at 0.17 M ionic strength, pH 7.0 and contained (in mM): 5 MgATP, 15 PCr, 1 Pi, 15 EGTA, at least 40 MOPS, 1 free Mg^{2+}, 135 Na^+ + K^+, 1 dithiothreitol (DTT), 250 units ml^{-1} creatine kinase (Sigma, St. Louis, MO), and Dextran T-500 (4% w/v; Pharmacia, Piscataway, NJ). The Ca^{2+} levels (expressed as pCa = $-\log [Ca^{2+}]$) were established by varying the amount of $Ca(proprionate)_2$. Dextran T-500 was included in all solutions to minimize covariation of myofilament lattice spacing (and fiber diameter) with force (Martyn and Gordon, 1988; Matsubara et al., 1985). All mechanical measurements were made at 10°-12°C. CDZ (1-[bis-(4-Chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[2,4-dichlorophenyl)methoxy]-ethyl]-^{1}H-imidazolium chloride) was obtained from Sigma (St. Louis, MO) and was solubilized in EtOH to a concentration of 10 mM. For experiments, CDZ was diluted into relaxing and activation solutions to the desired concentration. Control experiments confirmed that the small amount of added EtOH (≤1%) had no effect on either force or k_{tr} (data not shown).

TnC was extracted from fibers by incubation in a solution containing (in mM): 5 EDTA, 20 Tris, pH 7.2, with 0.5 trifluoperazine dihydrochloride (TFP; Aldrich Chemical Co., Milwaukee, WI) (Hannon et al., 1993; Metzger et al., 1989). Fibers were exposed to extracting solution until Ca^{2+}-activated force was <5% of control maximal force. Extraction of TnC from fibers before CDZ exposure was complete in 10 min at 10°C. After extraction, force was restored by reconstitution with purified rabbit skeletal TnC for 30 min (1 mg/ml in relaxing solution without creatine kinase or Dextran T-500) (Chase et al., 1994).

Protein preparation

Myosin was prepared from rabbit back muscle according to Margossian and Lowey (1982) and was used immediately or stored at −20°C in 50% glycerol for up to 4 weeks. Heavy meromyosin (HMM) was prepared by chymotryptic digestion of myosin as described by Kron et al., (1991) and was stored on ice for up to 5 days. F-actin was prepared from rabbit back and leg muscle ether powder (Potter, 1982) using the procedure of Pardee and Spudich (1982) and was stored for up to 2 weeks on ice. For visualization by fluorescence microscopy (in vitro motility assay), F-actin was labeled with rhodamine-phalloidin (Rh-Ph; Molecular Probes, Eugene, OR) according to Kron et al. (Kron et al., 1991) and maintained in a low light environment until used. Tropomyosin (Tm) was made from rabbit muscle ether powder as described by Smillie (1982). After purification, Tm was aliquoted and stored at −20°C. The method of Potter (1982) was used to make troponin (Tn) from rabbit muscle ether powder. Tn was stored on ice for up to 1 week. Regulated actin filaments were made by incubating Rh-Ph labeled F-actin (400 nM) with Tm (100 nM) and Tn (80 nM) on ice for at least 12 h before use. The molecular weight and extinction coefficients for proteins were: myosin − 520 kDa and 0.53 cm^{-1} at 280 nm; HMM − 350 kDa and 0.6 cm^{-1} at 280 nm, F-actin − 42 kDa and 0.62 cm^{-1} at 290 nm; Tm − 68 kDa and 0.29 cm^{-1} at 276 nm; and Tn − 70 kDa and 0.45 cm^{-1} at 276 nm, respectively.

Solution ATPase

ATPase activities (22°C) were measured in triplicate using a colorimetric method (White, 1982). Measurements were made over the linear range of the assay, which included the first 20 min for all reactions except myosin and HMM Mg^{2+}-ATPases, which were followed to 150 min. Conditions for Ca^{2+}-and K-EDTA-ATPase activities (pH 7.9) were as described by Margossian and Lowey (1982); reaction solutions for Mg^{2+}-ATPase activity (pH 7.0) contained (in mM): 10 imidazole, 2 $MgCl_2$, 0.1 K-EGTA, 1 DTT, and 2.5 Mg^{2+}ATP. Myosin and HMM concentrations for reactions were (in μM): 0.2 for Ca^{2+}-ATPase assay, 0.1 for K-EDTA-ATPase assay, and 1.5 for Mg^{2+}-ATPase assay. Protein concentrations for actin-activated Mg^{2+}-ATPase were 0.1 μM HMM and 20 μM F-actin. For each condition, the ATP hydrolysis rate was determined from the regression slope of phosphate generation versus incubation time. ATPase rates are reported as P_i production per S1 head per second (P_i/S1/s).

In vitro motility assay

In vitro motility assays were carried out at 30°C in flow cells made with nitrocellulose-coated coverslips as described by others (Homsher et al., 1996; Howard et al., 1993; Kron et al., 1991; Sellers et al., 1993). ATP-insensitive heads were removed from an aliquot of HMM by ultracentrifugation with F-actin and MgATP as described by Kron et al. (1991). Solutions were added to the flow cell in the order described by Homsher et al. (1996). Before infusion into motility flow cells, Rh-Ph F-actin or Rh-Ph F-actin-TnTm was diluted 1:100 in AB buffer (Krom et al., 1991); 80 nM Tn and 100 nM Tm were added to the diluted Rh-Ph F-actin-TnTm to retain complete Ca^{2+} regulation in the motility assay (Homsher et al., 1996). Motility assays were carried out either with RhPh F-actin in AB solution (Kron et al., 1991) or with RhPh F-actin-TnTm at 0.085 M ionic strength (Γ/2). In all motility assay buffers, 3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase (Boeringher-Mannheim), and 40 mM DTT (BioRad) were added to minimize photo-oxidation and photobleaching (Kron et al., 1991). In 0.085 M γ/2 motility buffer, the conditions were (in mM): 2 MgATP, 10 EGTA, and 1 Mg^{2+}, with MOPS and K-proprionate (KPr) added to adjust ionic strength (28–70 mM and 65 mM, respectively), pH 7.0 (30°C), and $CaPr_2$ was either omitted (pCa 9.2) or added to attain pCa 5.0; 0.4% methylcellulose (with 0.3 mM NaN_3) was added to prevent F-actin diffusion away from the assay surface at elevated γ/2 (Homsher et al., 1996; Kron et al., 1991; Uyeda et al., 1990). Filament speeds were obtained from video tape by analysis of centroids using hardware and software from Motion Analysis Systems (Santa Rosa, CA) (Homsher et al., 1996; Sellers et al., 1993).

RESULTS

Effects of CDZ on the Ca^{2+}-sensitivity of force and k_{tr}

CDZ increased the Ca^{2+} sensitivity of force in skinned skeletal muscle fibers. Fig. 3 compares the force-pCa relationships in the absence or presence of either 10 μM or 30 μM CDZ. The data are scaled relative to maximally activated (pCa 4.0) force (P_o) in the absence of CDZ and fitted to the Hill equation

$$P = P_o/(1 + 10^{n(pK-pCa)}) \quad (1)$$

where pK is the pCa that gives 0.5 P_o (midpoint) and n is the Hill coefficient (slope). pK was increased from 6.05 ± 0.02 (mean ± SE) for control measurements to 6.23 ± 0.01 with the addition of 10 μM CDZ with no significant changes in n or P_o, simply indicating an increase in the Ca^{2+} sensitivity of steady-state force. Increasing [CDZ] to 30 μM resulted in a further increase in pK to 6.32 ± 0.01, with small decreases in both n and P_o. Additional increases in [CDZ] caused a depression of P_o, as summarized in Fig. 4. Although the qualitative results were similar with 10 μM and 30 μM CDZ, the higher [CDZ] yielded quantitatively greater enhancement of activation at low Ca^{2+} levels (Fig. 3) and thus merited further study.

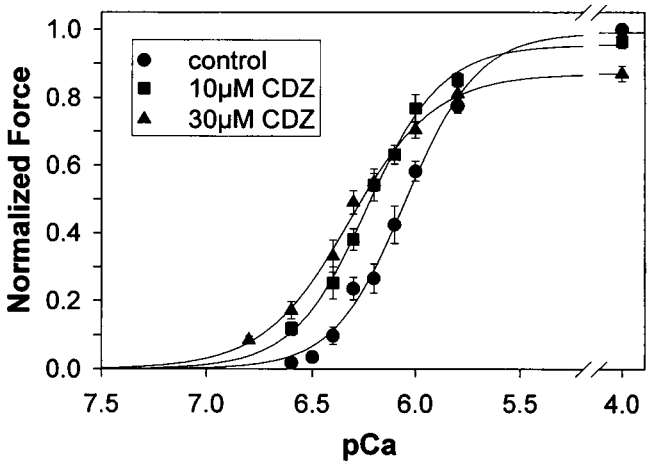

FIGURE 3 CDZ enhances the Ca^{2+} sensitivity of steady-state force in skinned fibers. Force was normalized to maximal force at *p*Ca 4.0 for control (●) activations (1.0). Maximum activation force was 0.95 ± 0.02 (mean ± SE) for 10 μM CDZ (■) and 0.87 ± 0.02 for 30 μM CDZ (▲). The number of fibers used for measurement of force at each *p*Ca varied from 7 to 24. Values in the plots are means ± SE for each *p*Ca. Data were fit to Eq. 1 and fits yielded midpoint *p*Ca (*K*) values (mean ± standard deviation): ● 6.05 ± 0.02, ■ 6.23 ± 0.01, and ▲ 6.32 ± 0.01; and cooperativity (*n*) values: ● 2.5 ± 0.2, ■ 2.4 ± 0.1, and ▲ 2.1 ± 0.1. Fits for the data were $r^2 > 0.997$.

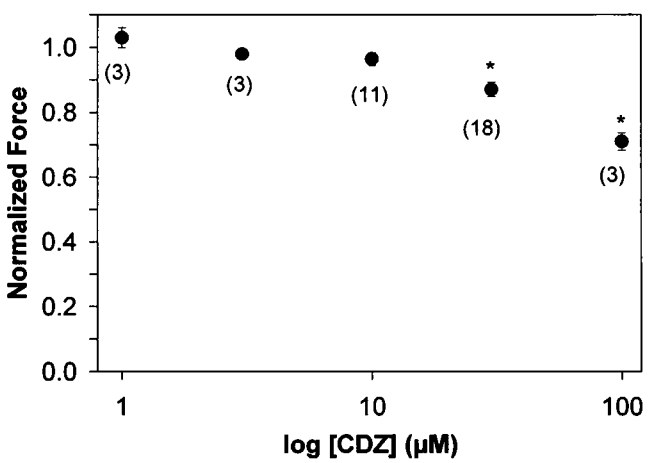

FIGURE 4 Effects of [CDZ] on steady-state force at *p*Ca 4.0. Values are expressed relative to maximum force (1.0) in the absence of CDZ and are reported as means ± SE. ⋆ – $p < 0.05$ level of significance.

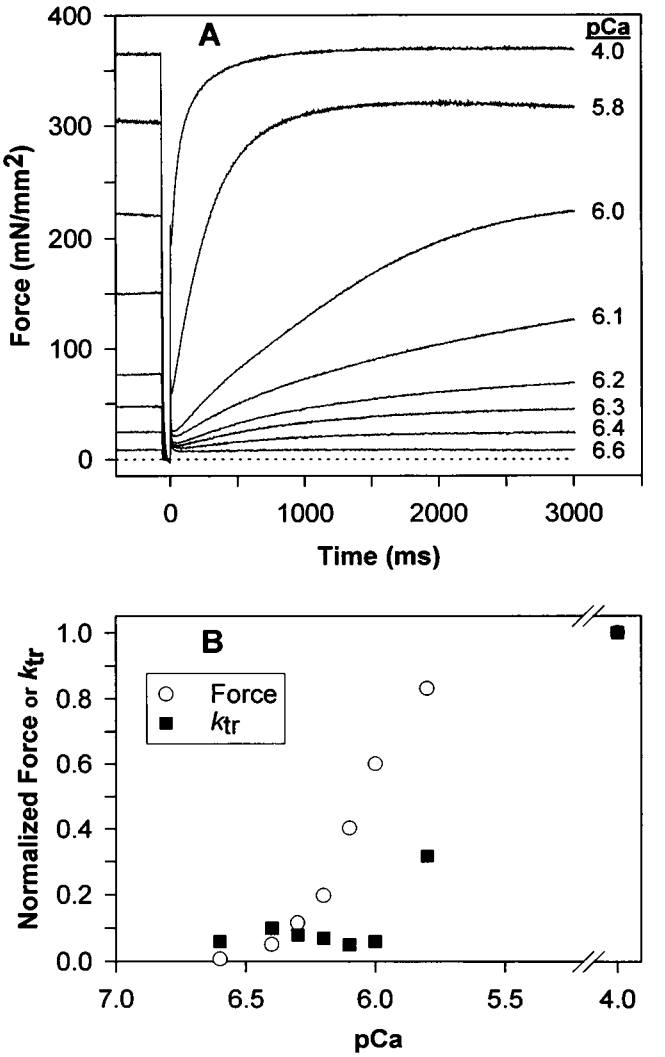

FIGURE 5 (*A*) Example force records of k_{tr} at various activation (*p*Ca) levels. All traces are from the same fiber. *p*Ca values are shown next to the respective records. (*B*) Values of steady-state force and ktr from *A* were normalized to values obtained for force (365 mN/mm²) or k_{tr} (13 s^{-1}) at *p*Ca 4.0 and plotted versus the *p*Ca of the activation solution to compare the relationship between steady-state force and the rate of force redevelopment with increasing Ca^{2+}.

To determine if CDZ also affected the Ca^{2+} sensitivity of force redevelopment kinetics, k_{tr} was measured at various Ca^{2+} concentrations with and without CDZ. Example force transients (from a single fiber) used to measure k_{tr} are shown in Fig. 5 *a*. Increasing [Ca^{2+}] caused both isometric force and k_{tr} to increase. In Fig. 5 *b*, the force and k_{tr} are normalized to maximal values (*p*Ca 4.0) and plotted versus *p*Ca. The data emphasize the different Ca^{2+} dependencies of force and k_{tr}. Whereas increasing [Ca^{2+}] resulted in elevated force, k_{tr} was not affected by changes in [Ca^{2+}] that produced <50% P_o. At Ca^{2+} concentrations that produced >50% P_o, k_{tr} exhibited a steep dependence on [Ca^{2+}]. This data is consistent with that described by others (Brenner, 1988; Chase et al., 1994; Metzger et al., 1989; Metzger and Moss, 1990; Metzger and Moss, 1991; Millar and Homsher, 1990; Swartz and Moss, 1992; Sweeney and Stull, 1990; Walker et al., 1992). As with steady-state force, the Ca^{2+} sensitivity of k_{tr} was enhanced by CDZ (Fig. 6). Fig. 6 *a* shows representative k_{tr} traces in a partially activated fiber (*p*Ca 6.2) in the absence and presence of 30 μM CDZ. CDZ increased both force and k_{tr} more than twofold. Force and k_{tr} were also enhanced by CDZ at other submaximal Ca^{2+} concentrations for this fiber, but maximal force and k_{tr} were reduced by ~15% and 30%, respectively (data not shown). The effects of CDZ on the relationship between *p*Ca and k_{tr} is summarized for all fibers in Fig. 6 *b*. k_{tr} was

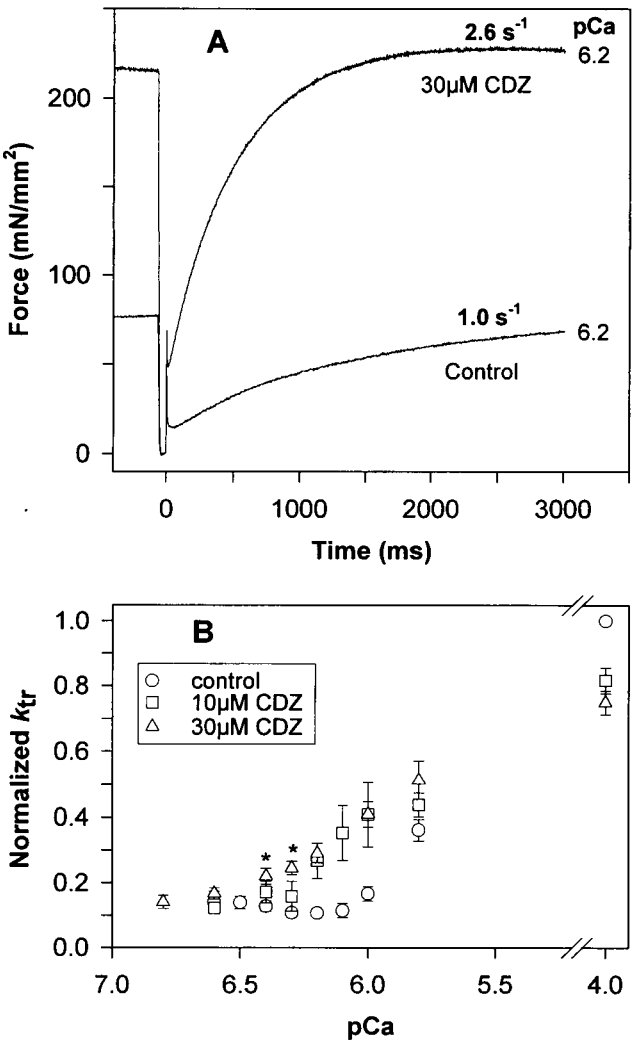

FIGURE 6 CDZ enhances the Ca^{2+}-sensitivity of k_{tr}. (*A*) Example records comparing force and k_{tr} at *p*Ca 6.2 in the same fiber in the absence (control, *lower record*) and presence of 30 μM CDZ (*upper record*). Traces are from the same fiber as in Fig. 5. (*B*) k_{tr} data for all fibers, normalized to maximal k_{tr} (*p*Ca 4.0, no CDZ). Values are means ± SE of 7 fibers for 10 μM CDZ and 12 fibers for 30 μM CDZ. At *p*Ca 4.0, k_{tr} was reduced to 0.82 ± 0.04 of control for 10 μM CDZ and to 0.75 ± 0.04 of control for 30 μM CDZ. k_{tr} was significantly faster ($p < 0.01$) at all submaximal *p*Ca < 6.3 for both 10 μM CDZ and 30 μM CDZ, and the symbol (*) indicates that values for 30 μM CDZ are significantly greater ($p < 0.01$) than control values at *p*Ca 6.3 and *p*Ca 6.4.

not influenced by CDZ at *p*Ca ≥ 6.5, even though isometric force was enhanced at these Ca^{2+} levels (see Fig. 3). CDZ (10 μM) caused a significant increase in k_{tr} at all submaximal *p*Ca < 6.3; and increasing the [CDZ] to 30 μM resulted in elevation of k_{tr} at even lower Ca^{2+} levels (i.e., *p*Ca 6.4 and *p*Ca 6.3, Fig. 6 *b*). Additionally, CDZ reduced k_{tr} at *p*Ca 4.0, which suggests that, at submaximal Ca^{2+} concentrations, the relative potentiation of k_{tr} by CDZ may actually be underestimated.

The CDZ-induced enhancement of k_{tr} was greatest in the range of Ca^{2+} levels that also had the greatest CDZ-induced enhancement of isometric force. This suggested the possibility that increases in k_{tr} resulted solely from an increase in the number of cross-bridges available to participate in the force-generating process. To examine this hypothesis, the data was replotted as steady-state force (measured just before the release/restretch cycle) versus k_{tr}. If the CDZ-induced enhancement in k_{tr} resulted from an increased population of force bearing cross-bridges, then CDZ would not affect the force versus k_{tr} relationship. However, this was not the case. As illustrated in Fig. 7 *a*, CDZ elevated k_{tr} at similar submaximal force levels. In Fig. 7 *b* the k_{tr} values from Fig. 6 *b* are replotted versus steady-state force. The data show that CDZ increased k_{tr} at all submaximal force levels >20% P_o. These results show that the CDZ-induced enhancement of k_{tr} (at submaximal Ca^{2+} levels) cannot be completely explained by an increased number of cross-bridges to form strongly bound, force-bearing states.

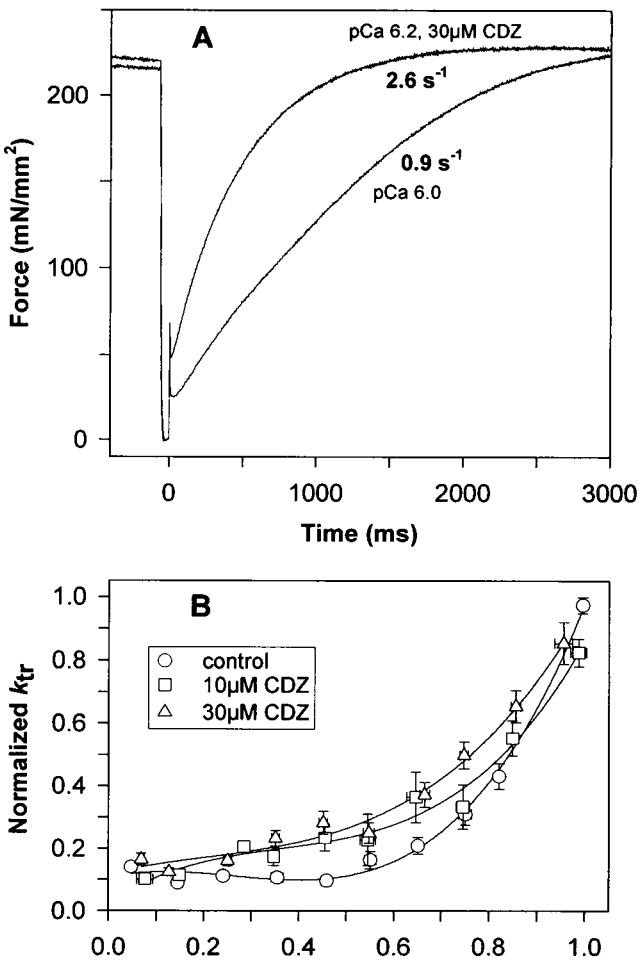

FIGURE 7 Effect of CDZ on the force versus k_{tr} relation. (*A*) Example force-matched traces from the same fiber for control and 30 μM CDZ. *p*Ca and k_{tr} values are indicated in the figure. (*B*) Force versus k_{tr} relationship for control, 10 μM and 30 μM CDZ k_{tr} data, replotted from Fig. 6 after binning data by force, rather than *p*Ca. Measurements were binned in 10% force level increments and values are normalized means ± SE. Data were empirically fit with a nonlinear regression (*solid lines*) and show that CDZ affects k_{tr} independent of the level of steady-state force.

The effects of CDZ on force and k_{tr} in fibers can either be reversed or maintained, in the absence of CDZ in the bath, by taking advantage of the solubility of CDZ. Fig. 8 shows that when a relaxation solution that contains 1% ethanol is used to wash CDZ out of the fiber (after Ca^{2+} sensitization), the CDZ-induced enhancement of steady-state force (*open bars*) and k_{tr} (*filled bars*) were readily reversible (*left panel*). This agrees with El-Saleh and Solaro (1987), who demonstrated reversibility of CDZ effects on isometric force in cardiac myofibrils. If, however, the washout relaxation solution contained no ethanol, the effects of CDZ on force and k_{tr} were maintained (*right panel*). These results are consistent with the idea that CDZ binds to a hydrophobic region on TnC (Reid et al., 1990) and forms a complex that is stabilized in an aqueous environment.

Mechanism of CDZ action in skinned muscle fibers

To determine if the CDZ induced enhancement of force and k_{tr} resulted solely from binding to TnC, we tested the effects of CDZ on HMM, myosin, and acto-HMM ATPase activity under a variety of conditions. The results, summarized in Table 1, show that CDZ (10 μM or 30 μM) had little or no effect on Mg^{2+}-ATPase, Ca^{2+}-ATPase, or K^{+}-ATPase activity of myosin or HMM in solution. F-actin activated Mg^{2+}-ATPase was similarly unaffected by 10 μM CDZ, but was inhibited slightly (10%) by 30 μM CDZ. The magnitude of acto-HMM ATPase inhibition is similar to the effect of CDZ on P_o (Fig. 4), in agreement with previous results in cardiac preparations (El-Saleh and Solaro, 1987). These results also support the hypothesis that the primary site of CDZ action is not cross-bridges. As a further test of the specificity of CDZ action, we used a more sensitive indicator of acto-myosin interactions, the in vitro motility assay. Results from >1500 Rh-Ph F-actin filament paths recorded in AB conditions (see Methods) showed little or no effects of 10 mM CDZ or the EtOH carrier on filament speed (Table 1) or fraction (>85%) of smoothly moving filaments. To distinguish between smoothly versus erratically and nonmoving filament paths, we used the criterion that the ratio of the standard deviation of speed to mean speed of each smoothly moving filament path was less than a cutoff of 0.5 (Homsher et al., 1996; Sellers et al., 1993). With Rh-Ph F-actin, we observed filament breakage at 30 mM CDZ; such observations are in concert with previous work demonstrating that examination of motility of individual filaments is considerably more sensitive to minor perturbations than is the solution ATPase assay. Therefore, we also examined the motility of filaments reconstituted with TnTm (Rh-Ph F-actin-TnTm), which stabilizes filament structure. Under the conditions used for regulated filament assays (see Methods), only 1% of the 273 filament paths met the criterion for smooth movement in the absence of Ca^{2+} (pCa 9.2), indicating that the filaments were well regulated. At pCa 5.0, the criterion for smooth movement was met by 59% to 82% of the filament paths examined (>1400). The speed attained at pCa 5.0 has previously been shown to be unaffected by further increases in $[Ca^{2+}]$ (Gordon et al., 1996; Homsher et al., 1996) and was not decreased by 10 mM CDZ or the EtOH carrier, but increased slightly by 30 mM, indicating that, in agreement with the ATPase measurements (Table 1), CDZ (at the concentrations examined) has little or no direct effect on cross-bridge cycling.

The observation that CDZ has little or no effect on cross-bridges, combined with enhanced Ca^{2+} activation of fibers (Figs. 3, 6–9) and Ca^{2+} affinity of TnC in solution (Johnson et al., 1994), strongly suggests that the effects of CDZ on skinned fibers results from binding to TnC. If this is the case, then extraction of TnC from fibers exposed to CDZ followed by reconstitution with purified rabbit skeletal TnC (sTnC) should result in a return of steady-state force and k_{tr} to control values. Fig. 9 demonstrates that this is the case. Extraction of native TnC (exposed to CDZ) and replacement with sTnC reversed the effects of CDZ on both submaximal force (Fig. 9 *a*) and k_{tr} (Fig. 9 *b*). Subsequent reintroduction of CDZ resensitized the fiber to Ca^{2+}, further suggesting that CDZ acts by binding to TnC. We also observed that extraction of TnC from CDZ-exposed fibers averaged 50 min (at room temperature), compared with 10 min (at 10°C) for fibers not exposed to CDZ. Similar results were obtained from two additional fibers and the data are summarized in Table 2. These results indicate that CDZ

TABLE 1 Effects of CDZ on myosin and heavy meromyosin (HMM) ATPase activity and on in vitro motility

	Ca^{2+}-ATPase* (Pi/S1/s)		K^{+}-ATPase* (Pi/S1/s)		Mg^{2+}-ATPase* (Pi/S1/s)			In vitro motility speed# ($\mu m\ s^{-1}$)	
	Myosin	HMM	Myosin	HMM	Myosin	HMM	Acto§-HMM	Rh-Ph F-actin	Rh-Ph F-actin-Tn-Tm
CNT	4.6 ± 0.1	3.0 ± 0.1	7.8 ± 0.3¶	10.8 ± 0.2	0.02 ± 0.001	0.03 ± 0.001	10.0 ± 0.1	5.9 ± 0.7	7.1 ± 1.2
CNT + 1% Et	4.5 ± 0.4	2.9 ± 0.1	8.4 ± 0.3	10.7 ± 0.2	0.02 ± 0.001	0.03 ± 0.001	9.7 ± 0.2	6.4 ± 0.8¶	7.3 ± 1.6
10 μM CDZ	4.7 ± 0.1	3.0 ± 0.1	8.5 ± 0.3	10.9 ± 0.4	0.02 ± 0.001	0.03 ± 0.002	9.7 ± 0.2	4.8 ± 0.6¶	7.1 ± 1.0
30 μM CDZ	4.4 ± 0.3	2.9 ± 0.1	8.6 ± 0.3	11.0 ± 0.5	0.02 ± 0.001	0.03 ± 0.001	9.0 ± 0.1¶	—	9.2 ± 0.9¶

Conditions for solution ATPase (22°C) and in vitro motility (30°C) measurements are given in Methods.
*Values are means ± S.E. for three runs.
#Values are means ± S.D.
§20 μM F-actin.
¶Significant difference ($P < 0.05$).

TABLE 2 Reversal of CDZ effects by extraction and replacement of TnC

Condition	P_0	pCa50	Slope	k_{tr}(pCa6.0)
Control	1	5.92 ± 0.02	3.8 ± 0.7	0.09 ± 0.01
+CDZ	0.97 ± 0.04	6.13 ± 0.04	2.4 ± 0.4	0.25 ± 0.05
+sTnC	0.79 ± 0.08	6.00 ± 0.01	2.2 ± 0.3	0.17 ± 0.04
+sTnC + CDZ	0.75 ± 0.07	6.21 ± 0.05	1.6 ± 0.2	0.35 ± 0.03

n = 3; values are means ± S.E. [CDZ] = 30 μM.
Values for k_{tr} are normalized to the maximal rate obtained (pCa 4.0) in the absence of CDZ.

binding to TnC is the primary mechanism by which CDZ increases the Ca^{2+} sensitivity of force and k_{tr} (at 10°C) in skeletal muscle fibers.

DISCUSSION

The present experiments were designed 1) to characterize the effects of CDZ on the Ca^{2+} dependence of contractile properties in skinned, skeletal muscle fibers, and 2) to study the role of Ca^{2+} dissociation kinetics from TnC as a modulator of isometric force redevelopment kinetics (k_{tr}). The results show that, at submaximal Ca^{2+} activation, CDZ reversibly increased steady-state force (Figs. 3, 6–9) and increased k_{tr} to an even greater extent than can be explained by increased force alone (Figs. 6–9). These observations support our previous conclusion that the activation dynamics of thin filament regulatory units (specifically in this study, the dissociation rate of Ca^{2+} from TnC) are major determinants of k_{tr}, as well as steady-state force, at submaximal Ca^{2+} concentrations (Chase et al., 1994).

Several lines of evidence demonstrate that the effects of CDZ are consequences of enhanced Ca^{2+}-binding affinity of TnC in skeletal muscle fibers. First, CDZ has been shown to enhance Ca^{2+} binding at the low affinity regulatory site of cTnC (El-Saleh and Solaro, 1987). This enhancement has also been shown to occur in sTnC and is due to an approximately threefold decrease in the Ca^{2+} off-rate from the

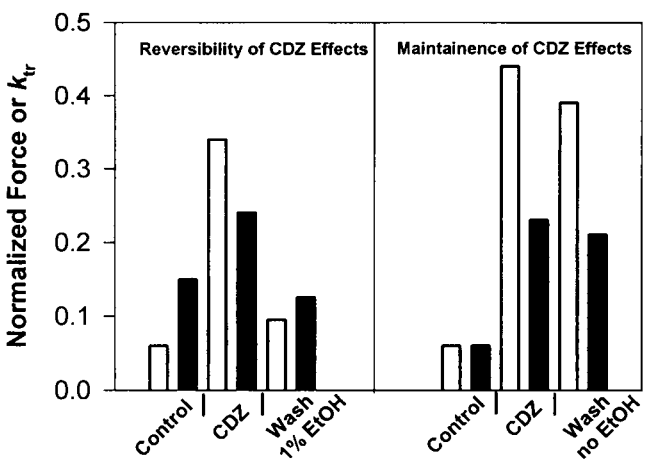

FIGURE 8 Reversibility of CDZ effects on submaximal force and k_{tr}. Fibers were activated at pCa 6.4, and were then exposed to 30 μM CDZ by the procedure described in Fig. 2. After a second activation at pCa 6.4 (with 30 μM CDZ) fibers were washed for 15 min in a CDZ-free relaxation solution containing 1% ethanol (*left panel*) or without ethanol (*right panel*). Fibers were then activated at pCa 6.4 to determine the reversibility of CDZ effects. Force (*open bars*) and k_{tr} (*filled bars*) measurements consistently showed reversal of CDZ effects when the fiber was washed in a 1% ethanol solution, and no reversal of CDZ effects when the wash contained no ethanol. Submaximal and maximal Ca^{2+}-activated force were not affected by ethanol concentration ≤ 1%.

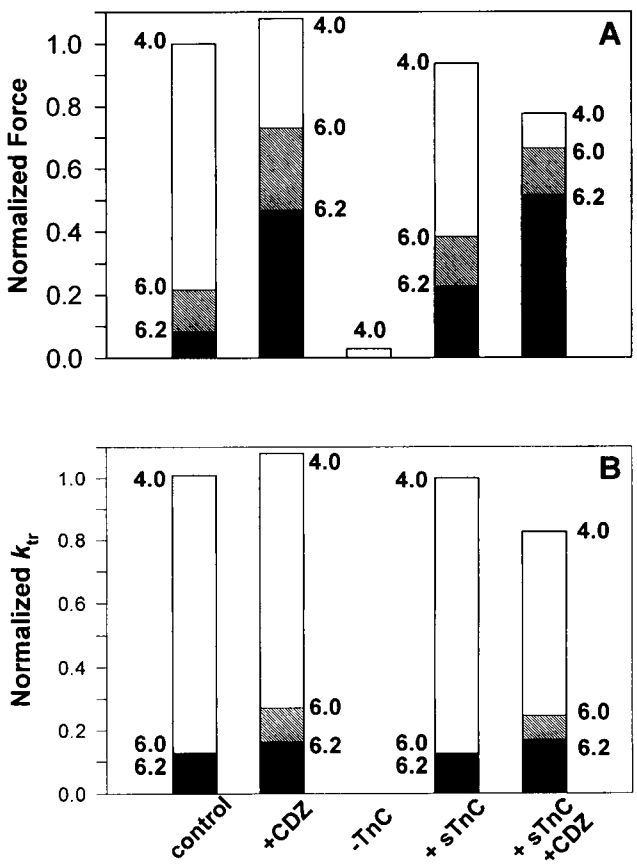

FIGURE 9 Native TnC extraction (−TnC) and replacement with purified rabbit skeletal TnC (+sTnC) reverses CDZ effects on force (*a*) and k_{tr} (*b*). After activations at pCa 6.2, 6.0, and 4.0 (control), fibers were exposed to 30 μM CDZ (as described in Fig. 2), and activations were repeated to show CDZ sensitization of force and k_{tr} (+CDZ). Fibers were then washed in CDZ-free solutions (no ethanol), as in Fig. 8, and the activation series was repeated a third time to verify that CDZ-induced enhancement of force and k_{tr} persisted (data not shown here). CDZ-TnC was then extracted from fibers (−TnC), as described in Methods, and the thin filament was reconstituted with sTnC. After reconstitution, the activation series was then repeated in the absence (+sTnC) and presence (+sTnC + CDZ) of 30 μM CDZ.

Ca^{2+} specific sites with no effect on the diffusion-limited Ca^{2+} on-rate (Johnson et al., 1994; Johnson et al., 1981). If the Ca^{2+}-binding properties of TnC in fibers are similarly affected by CDZ, the result should be an increase in steady-state force at low Ca^{2+} concentrations with minimal effects at maximal Ca^{2+} activation. Furthermore, models that include thin filament activation kinetics predict that k_{tr} (at submaximal activations) should also be affected by CDZ (Hancock et al., 1995; Landesberg and Sideman, 1994; Peterson et al., 1991; Zahalak and Ma, 1990). These predictions were confirmed by our results (Figs. 3, 4, 6–9) (Chase et al., 1994). Second, the CDZ enhancement of force and k_{tr} (at 10°C) was reversed by extraction of TnC from CDZ-exposed fibers followed by reconstitution with purified rabbit skeletal TnC. Additionally, CDZ enhanced steady-state force and k_{tr} of these sTnC-reconstituted fibers (Fig. 9, Table 2). Third, the slower extraction of TnC from fibers previously exposed to CDZ (see Results) is consistent with the idea that CDZ binds strongly to TnC. This inhibition could occur either by CDZ competing for the same site on TnC that trifluoperazine dihydrochloride (TFP) binds to, or by stabilizing TnC interactions with the thin filament, at least under extraction conditions (see Methods). The former possibility is supported by the observation that CDZ binds to a hydrophobic depression of the N-terminal domain of TnC that is exposed with Ca^{2+} binding (Reid et al., 1990), similar to the binding domain proposed for TFP (Ovaska and Taskinen, 1991); both compounds are thought to stabilize the Ca^{2+}-bound configuration of TnC. Fourth, solution ATPase measurements (at 22°C) and in vitro motility assays (at 30°C) were not affected by 10 μM CDZ (Table 1), and 30 μM CDZ similarly had no effect on K-EDTA- and Ca^{2+}-ATPases of myosin and HMM or in vitro motility of Rh-Ph F-actin-Tn-Tm (pCa 5), but decreased acto-HMM Mg^{2+}-ATPase activity by 10%. This decrease correlates with effects on maximum Ca^{2+}-activated steady-state force (Fig. 4), in vitro motility of Rh-Ph F-actin (Table 1), and k_{tr} (Fig. 6) in fibers. It has been suggested that high concentrations of CDZ may inhibit maximal force and ATPase by binding to myosin light chains (El-Saleh and Solaro, 1987). However, taken together, the above four lines of evidence suggest the primary site of CDZ action in fibers is TnC, via increased Ca^{2+} affinity resulting from a reduction of the "off-rate" for Ca^{2+} from TnC (Johnson et al., 1994; Wahr et al., 1993).

Thin filament influence on the rate of tension redevelopment

An increase in k_{tr} without a corresponding change in force (Fig. 7) is not consistent with a two-state model (Brenner, 1988). This is because, in the two-state model, a unique relationship should exist between steady-state force and k_{tr}, such that an increase in force results from a Ca^{2+}-dependent increase in the forward transition rate (f), which also results in an increase in k_{tr} (Brenner, 1988). New, more detailed models should include the kinetics of interactions occurring on the thin filament, as well as the kinetics of acto-myosin interactions. Recently, models of this type have been proposed that may account for the dissociation of steady-state force and k_{tr} without requiring a Ca^{2+}-sensitive kinetic transition in the actomyosin cycle (Hancock et al., 1995; Landesberg and Sideman, 1994; Peterson et al., 1991; Zahalak and Ma, 1990). Other models have been proposed that include Ca^{2+}-dependent activation of the thin filament as a step preceding an isomerization of the cross-bridge to a strongly bound state that can produce force (Geeves and Conibear, 1995; McKillop and Geeves, 1993; Regnier et al., 1995). There is growing evidence to validate consideration of these types of models.

The observed effect of CDZ on the force versus k_{tr} relationship, shown in Fig. 7, demonstrates that TnC strongly influences this relationship in skinned rabbit psoas fibers (Chase et al., 1994). This is in concert with previous reports that with a Ca^{2+}-insensitive, constitutively activating form of cTnC (reconstituted into skinned skeletal fibers from which the endogenous sTnC had been extracted) rendered k_{tr} independent of the level of force and similar to maximal k_{tr} obtained in control conditions (pCa 4.0 with native TnC) (Chase et al., 1994). Furthermore, when skinned skeletal fibers were reconstituted with cTnC, k_{tr} was elevated at submaximal Ca^{2+}-activated force levels (Chase et al., 1994). A common feature between cTnC-and CDZ-exposed sTnC is that both have a slower Ca^{2+} dissociation rate than native sTnC (Johnson et al., 1980; Johnson et al., 1994). Thus these data provide important new evidence that activation-dependent thin filament processes influence the kinetics of acto-myosin interactions in skeletal muscle.

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