Inhibition of rabbit skeletal muscle acto-S1 ATPase by troponin T

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1. INTRODUCTION

For full calcium-sensitive control of the actomyosin ATPase of both vertebrate skeletal and cardiac muscle, the concerted action of both troponin (Tn) and tropomyosin (TM) are required [1,2]. In contrast, the inhibition of the actomyosin ATPase, but not the calcium-triggered release of inhibition, can be mediated by TnI and TM alone [3–5]. Though TnI, the inhibitory subunit of the troponin complex, possesses the ability to inhibit the ATPase activity of actomyosin itself, this inhibition, at ionic strengths where TM by itself neither binds nor inhibits, is greatly enhanced by the addition of TM [3,5–8]. At ionic strengths where binding and inhibition by TM is significant, TnI enhances the inhibition [3,5].

The mechanism of regulation proposes that, in the absence of Ca^{2+}, corresponding to the relaxed state, the position of TM and TnI are such as to prevent the actin–myosin interaction. In the presence of Ca^{2+}, corresponding to contraction, Ca^{2+} binds to TnC causing conformational changes that are transmitted to TnI, TnT and TM, resulting in movement of both TnI and TM on the actin filament such that the actomyosin ATPase is activated (reviews [9–11]).

Though the inhibitory effects of TnI [3–8] and the TnI–TnT complex, which produces a Ca^{2+}-independent inhibition of the actomyosin ATPase similar to TnI alone, are well documented [4,12,13], no demonstration of an inhibitory role for TnT (pure preparations of TnT devoid of TnI contamination) has been made. This communication is the first report of a substantial inhibition of the actomyosin ATPase by rabbit skeletal TnT in the presence of TM. This inhibition is compared to that of TnI and native troponin.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

Rabbit skeletal troponin and troponin subunits were prepared as in [14]. α-Tropomyosin was prepared from rabbit hearts and purified as in [15]. Actin was purified from rabbit skeletal muscle acetone powder as in [16]. Myosin S1 was made as in [17] except for the elution conditions on the DEAE-cellulose column [18]. The two peaks of S1 eluting from the column corresponding to myosin S1 containing the A1 or A2 light chains were pooled together and the mixture used in all experiments. Protein concentrations were determined either by amino acid analysis or absorbance

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Abbreviations: Tn, troponin; TM, tropomyosin; TnI, troponin I; TnT, troponin T; TnC, troponin C; acto-S1, actin and myosin subfragment 1; SDS, sodium dodecyl sulphate; EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol

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measurements at 280 nm with the extinction coefficients summarized in [18]. SDS–urea–polyacrylamide gel electrophoresis was done as in [19] except that the gel was 15% polyacrylamide, 0.1% SDS and 6 M urea in 0.375 M Tris–HCl (pH 8.8).

2.2. *ATPase assays*

ATPase assays (2 ml) were performed on a pH stat apparatus as in [20]. The actin-activated ATPase was measured, unless otherwise stated, in 30 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$, 2 mM disodium ATP and 2 mM Tris at pH 7.8. Assays to investigate the effect of α-tropomyosin on the actin-activated ATPase of S1 (fig.1) were carried out as follows: The ATP hydrolysis rate in the absence of regulatory proteins was taken as 100. TM was added to the assay, and its effect on the hydrolysis rate recorded (a separate assay was carried out for each of the TM concentrations shown). After addition of TM, Tn was added to the assay vial. The Tn concentration was kept constant at ~1:7 with respect to actin. Finally, 0.2 mM calcium was added to the assay (giving 0.1 mM 'free' calcium) to provide an estimate of calcium-sensitive release of inhibition.

In fig.2,3, the acto-S1–TM ATPase activity was taken as 100. Inhibitor was added to the assay and its effect on the hydrolysis rate recorded (a separate assay was carried out for each inhibitor

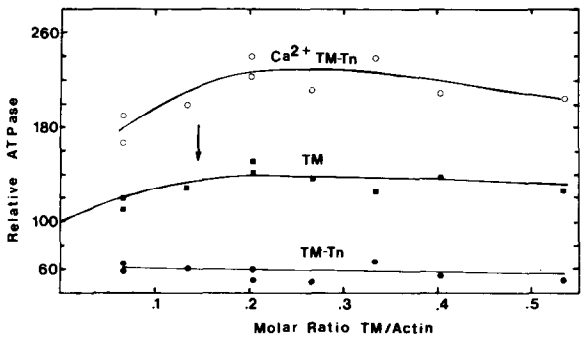

Fig.1. Effect of α-tropomyosin on the acto-S1-ATPase. The assay contained 4 μM S1, 2 μM actin and 0.25 μM troponin. α-TM was varied from 0–1.15 μM: (■—■) effect of TM alone on the ATPase activity in the absence of calcium; (●—● and ○—○) the ATPase activity of TM-Tn in the absence and presence of Ca^{2+}, respectively. The ATPase activity of acto-S1 was taken as 100. The arrow indicates a molar ratio of TM/actin of 1/7.

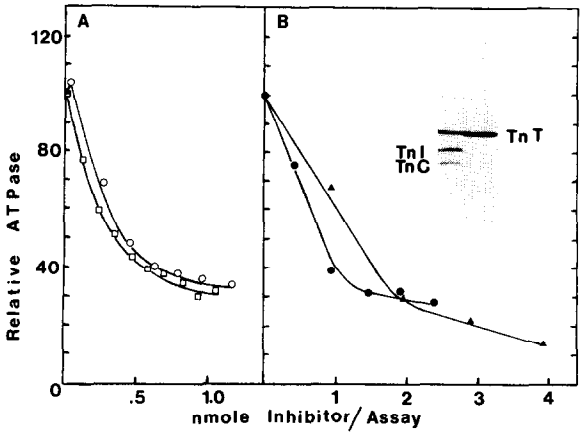

Fig.2. Inhibition of the acto-S1–TM-ATPase by native Tn, TnI and TnT. The acto-S1–TM-ATPase activity was taken as 100. All assays contained a molar ratio of S1:actin:TM of 14:7:1. The open and closed symbols represent two different preparations of actin and S1: (□) TnI; (○,●) native Tn; (▲) TnT. Inset: SDS–urea Laemmli gel electrophoresis of TnT and native Tn.

concentration shown). The only exception to this procedure was in fig.3C, where increasing concentrations of TnC were added to one assay vial in the presence of calcium and one assay vial in the absence of calcium. Similar results were obtained by the above method. The ATPase activities of acto-S1–TM varied from 322–351 nmol $PO_4 . min^{-1} . mg\ S1^{-1}$ depending on the preparation used. In all experiments, the concentrations of S1 were either 3 μM or 4 μM. S1:actin was always 2:1.

TnT and TnI are well known for their solubility problems. In these experiments, both were dissolved (~3 mg/ml) in 8 M urea 1 M KCl–2 mM DTT–10 mM Tris–HCl buffer (pH 7.8) dialyzed firstly against 1 M KCl–2 mM DTT–10 mM Tris–HCl buffer (pH 7.8) and finally against 0.5 M KCl–2 mM DTT–10 mM Tris–HCl buffer (pH 7.8). Control experiments indicated that the small increase in KCl concentration from the titration of TnI or TnT in 0.5 M KCl did not affect the ATPase assay.

3. RESULTS AND DISCUSSION

Here, we have chosen S1:actin ratios in the ATPase assay to examine the inhibition of TnI, Tn

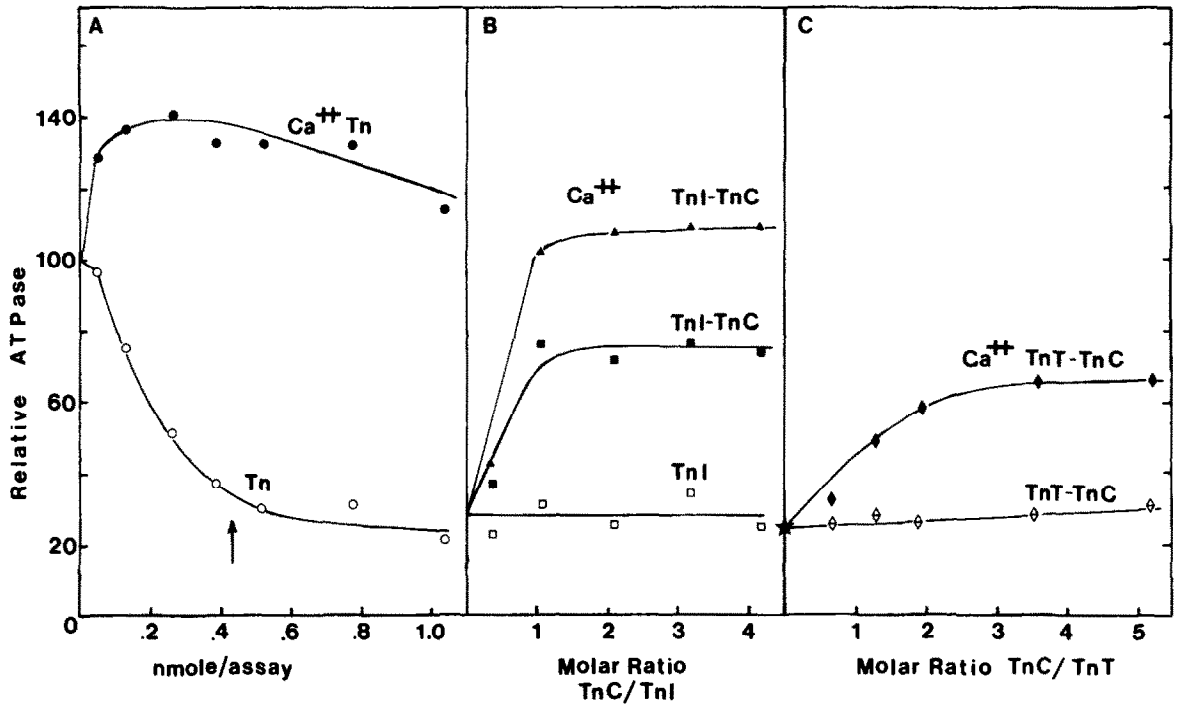

Fig.3. Inhibition and release of inhibition of acto-S1–TM-ATPase. The acto-S1–TM-ATPase activity was taken as 100. All assays contained a molar ratio of S1:actin:TM of 14:7:1. (A) (⟶) Molar ratio of Tn:TM of 1:1; (○—○ and ●—●) ATPase activity of Tn in the absence and presence of Ca^{2+}, respectively. (B) (□—□) Inhibition by TnI; (■—■ and ▲—▲) release of inhibition of TnC in the absence and presence of Ca^{2+}, respectively. (C) (*) Inhibition by TnT alone; (◇—◇ and ◆—◆) effect of TnC on the inhibition of TnT in the absence and presence of Ca^{2+}, respectively.

and TnT such that TM does not inhibit but slightly potentiates the actin-activated ATPase (fig.1). Potentiation by tropomyosin of the acto-S1–ATPase has been observed in [13,21–25]. Under the conditions used in this assay (30 mM KCl, 5 mM $MgCl_2$ and 2 mM ATP) tropomyosin has been shown to bind to F-actin [3]. However, with S1:actin at 2:1, tropomyosin alone no longer inhibits the acto-S1–ATPase, but potentiates [24,25]. Addition of troponin resulted in an inhibition of the ATPase and the calcium-sensitive release of inhibition approached 220% higher than the original acto-S1 activity.

The effectiveness of TnI, Tn and TnT to inhibit the acto-S1–TM-ATPase was examined in fig.2. TnI and native Tn were equally effective in inhibiting the ATPase (A) whereas TnT was ~2-fold less effective than TnI or native Tn (B). All 3 inhibitors were capable of reducing the ATPase activity to 30%. This is the first report of a significant inhibition of the acto-S1–TM-ATPase (70%) by TnT. Previous workers have shown a small inhibition by TnT using different ATPase assay conditions. However, it was concluded that the inhibition was probably due to small contamination of TnT by TnI [12,13]. In this study, we have prepared TnT devoid of any contamination by other troponin components as shown by the SDS gel electrophoresis patterns in fig.2.

Fig.3 compares the release of inhibition of native Tn by calcium and the release of inhibition of TnI and TnT by calcium and TnC. Almost complete inhibition of the acto-S1–TM-ATPase was achieved at a 1:1 molar ratio of Tn to TM (A). Calcium results in release of the inhibition and potentiation of the ATPase. When the acto-S1–TM-ATPase is inhibited by TnI, addition of TnC results in a partial release of inhibition in the

absence of calcium (fig.3B). Complete release of inhibition is only obtained with TnC in the presence of calcium (fig.3B). The partial (25%) Ca^{2+} sensitivity which is observed is the result of a difference in binding of the IC complex to TM–actin in the presence and absence of calcium. That is, the IC complex binds to TM–actin only in the absence of calcium and not in the presence of calcium where TnC can dissociate TnI from the TM–actin filament [26–28]. TnI and TnC form a 1:1 complex in the absence or presence of Ca^{2+} [29–31]. Maximum release of TnI inhibition by TnC in the presence or absence of Ca^{2+} occurs at a 1:1 molar ratio (fig.3B). The difference in inhibiting power between TnI and IC complex (minus Ca^{2+}) can be explained by the structural change induced in TnI by TnC [32,33].

When the acto-S1–TM-ATPase is inhibited by TnT, addition of TnC does not release the inhibition in the absence of Ca^{2+} but does show a partial release in the presence of Ca^{2+} (fig.3C). The partial (35%) Ca^{2+} sensitivity can be explained by both structural change induced in TnT by TnC and the stronger interaction in the presence of Ca^{2+} [34–37]. The maximum release of inhibition of TnT by TnC in the presence of Ca^{2+} occurs at a TnC/TnT molar ratio of 3:1 (fig.3C) which reflects a much weaker binding between TC complex compared to the IC complex. The IC complex in the presence of Ca^{2+} is stable in 6 M urea [29,31].

The inhibitory role of TnT demonstrated here is a reasonable observation, since TnT binds to all components (TnI, TnC and TM) of the regulatory complex and the Ca^{2+}-induced conformational changes in TnC are transmitted through TnT to TM–actin. TnT interacts extensively with TM (essentially the COOH-terminal half of TM, including the head-to-tail overlap region) [38,39]. TM can either inhibit or activate the acto-S1–ATPase and has been shown to occupy at least 3 positions on actin [25]. Since TnT binds to TM, and weakly if at all to actin, it must be altering the position of TM on actin from the non-inhibitory to the inhibitory state under the conditions reported here. The ability of TnT to affect the inhibitory role of TnI in the absence of TM has also been demonstrated [4], where the IT complex was able to inhibit in a similar fashion to TnI–TM. Obviously, the concerted effects of TM, TnI, TnT and TnC are required to obtain complete regulation (inhibition, release of inhibition, potentiation).

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