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Effects of Two Hypertrophic Cardiomyopathy Mutations in α-Tropomyosin, Asp175Asn and Glu180Gly, on Ca^{2+} Regulation of Thin Filament Motility

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The functional properties of wild type α-tropomyosin expressed in E. coli with an alanine-serine N-terminal leader (AS-α-Tm) were compared with those of AS-α-Tm with either of two missense mutations (Asp175Asn and Glu180Gly) shown to cause familial hypertrophic cardiomyopathy (FHC). Wild type AS-α-Tm and AS-α-Tm(Asp175Asn) binding to actin was indistinguishable from rabbit skeletal muscle ab-tropomyosin whilst the affinity of AS-α-Tm (Glu180Gly) was about threefold weaker. *In vitro* motility assays were performed with AS-α-tropomyosin incorporated into skeletal muscle actin-rhodamine phalloidin filaments moving over skeletal muscle heavy meromyosin. Under relaxing conditions (pCa9), troponin added to actin filaments containing AS-α-tropomyosin or mutant tropomyosins resulted in normal switch-off, with a decrease in the fraction filaments moving from $>$80% to $<$20%. Under activating conditions (pCa5), troponin had a minor effect upon actin-AS-α- tropomyosin filament velocity (increased by 5$\pm$1%, n=10), whereas the velocity increased by 18$\pm$3%(n=7) with actin filaments containing AS-α-tropomyosin(Asp175Asn) and by 21$\pm$2%(n=8) with filaments containing AS-α-tropomyosin(Glu180Gly) ($p<0.05$ compared with AS-α- tropomyosin). Thus FHC mutations in α-tropomyosin produce detectable changes in the Ca^{2+}- regulation of thin filaments, presumably via altered interaction with troponin. © 1997 Academic Press

Familial hypertrophic cardiomyopathy (FHC) has been shown to be caused by dominant mutations in several genes coding for cardiac sarcomeric proteins, including b myosin heavy chain, myosin light chains, myosin binding protein C, troponinT and α-tropomyosin (1-6).

Troponin T and tropomyosin are involved in the Ca^{2+} dependent regulation of muscle contractility through the troponin-tropomyosin complex which is associated with actin in the thin filaments of striated muscle (7,8). Investigations of a missense mutation of troponin T (9) and a mutation leading to truncation of the C terminus (10) have suggested that mutant troponin T is incorporated into the sarcomere and that it alters thin filament regulation, apparently leading to an increase in unloaded shortening velocity and reduced force output.

The missense mutations in α-tropomyosin detected in FHC patients, Asp175Asn and Glu180Gly, introduce changes in surface charge in the region of the molecule that has been implicated in troponin T binding and have been shown to cause partial unwinding of the tropomyosin coiled-coil in this region (11). In order to determine the functional effects of the mutations we have expressed chicken α-tropomyosin in *E. coli* incorporating the Asp175Asn and Glu180Gly mutations (1). The expressed α-tropomyosin also includes an N-terminal Alanine-Serine extension which confers normal end-to-end interactions on tropomyosin in the absence of postranscriptional acetylation (12).

In this study we have used binding measurements to document that the mutant tropomyosin does bind to actin, and then the in vitro motility assay (13,14) to determine how the missense mutations in α-tropomyosin alter the movement of actin-tropomyosin-troponin filaments over immobilised skeletal muscle HMM. We have found that the missense mutations specifically alter the interaction of tropomyosin with troponin leading to an increase in filament velocity in the presence of Ca^{2+}.

MATERIALS AND METHODS

Protein preparations. Rabbit skeletal muscle actin and heavy meromyosin (HMM) were prepared by standard procedures (15).

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HMM was centrifuged in the presence of actin and ATP to remove rigor heads as previously described (13). Tropomyosin and troponin were extracted from rabbit skeletal muscle according to the method described by Potter (16). Tropomyosin was purified by repeated isoelectric precipition followed by chromatography on hydroxyapatite using a phosphate gradient (10-200mM) (17). Smooth muscle tropomyosin was prepared from sheep aorta by previously reported methods (18).

The AS- α-tropomyosin expressing plasmid (pET-MASTmy) was a gift from Professor Reinach, Sao Paulo (12). Constructs encoding AS-α-tropomyosin with either the Asp175Asn or the Glu180Gly mutation were made by a standard single-stranded oligo-directed mutagenesis method (19). A HindIII-XbaI fragment from pET-MASTmy was subcloned into pBluescript KS- and this construct used to transform the E. coli strain CJ236. A single-stranded DNA template was prepared and the oligonucleotides D175N (5′-CCCGCTCCAGGTTACCCTCAATG-3′) and E180G (5′-CAGCACGCTCCCCAGCCCGCTCC-3′) used to introduce the mutations. The pBluescript constructs were verified by dideoxy sequencing and HindIII-XbaI fragments ligated back into HindIII-XbaI cut pET-MASTmy. Wild type and mutant constructs were used to transform the E. coli strain BL21(DE3)pLysS. Cultures were grown, induced for 3 hours and harvested and the bacteria lysed according to standard protocols (20). Both wild type and mutant AS α-tropomyosin were expressed at a high level and were purified by a modification of the method described by Monteiro et al., 1994 (12). The proteins were dialysed against our standard motility assay buffer (50mM KCL, 25mM Imidazole-HCL, 4mM MgCl2, 1mM EDTA, 5mM dithiothreitol, pH 7.4, Buffer A) for 2-4hours and aliquoted and stored at −20°C. Protein concentration were determined by the method of Lowry.

In vitro motility assays. *In vitro* motility assays were performed as described by Fraser and Marston (13) using 100 μg/ml skeletal muscle HMM on siliconized cover glasses. Rabbit skeletal muscle actin was labelled with rhodamine phalloidin(ϕ) as described by Kron et al (21). Actin-ϕ, recombinant tropomyosin and skeletal troponin were pre mixed as follows :100nM of actin-ϕ, 200nM tropomyosin and skeletal troponin (200nM -3200nM) were mixed and diluted tenfold to the working concentration of 10nM actin-ϕ in buffer A immediately prior to infusion into the motility cell. Filaments were observed with a fluorescence microscope (Zeiss Axiolab,X46/1.4 NA Planapochromat lens, DAGE-SIT68 Camera). Filament movement was initiated by infusing 1 mM MgATP, 0.5% methyl cellulose at 25°C as described by Fraser and Marston (13), and recorded on videotape. Recordings were digitised and the movement was analysed to determine the fraction of filaments moving and the velocity of motile filaments using the automatic tracking programme described by Marston et al. (22)

Binding assays. Rhodamine-phallodin labelled actin (100nM) was incubated for 30 minutes on ice with 0-800nM tropomyosin in buffer A. Following incubation the actin-bound tropomyosin was sedimented by centrifugation at 200,000g for 20 minutes. The resultant pellet was dissolved in SDS, separated by electrophresis in polyacrylamide gels (8-18%/0.1%SDS) and stained with PAGE blue83. Quantitative densitometry was carried out using a "Scanmaster3" flat-bed scanner with "Quantity One" software(pdI Inc.). The quantity of tropomyosin was calculated as a proportion of the corresponding actin band.

RESULTS

Tropomyosin Binding to Actin

We measured tropomyosin binding to actin in cosedimentation experiments. The affinity of alanine-serine-α-tropomyosin (AS-α-tropomyosin) for actin was the same as skeletal muscle ab-tropomyosin. The mutant

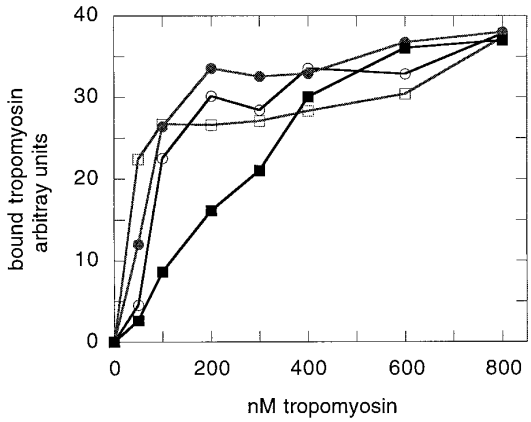

FIG. 1. Binding of tropomyosin to actin. Conditions: 50 mM KCL, 25 mM Imidazole-HCL, 4 mM MgCL2, 1 mM EDTA, 5 mM DTT, pH 7.4 (Buffer A), 100nM Actin, 25 °C. Binding was assayed by sedimenting the actin-tropomyosin complex and analysing the pellets by SDS gel electrophorsis. Tropomyosin bund as percentage of the actin bound area is plotted. □, skeletal muscle tropomyosin. ○, AS-α-tropomyosin. ●, AS-α-tropomyosin(Asp175Asn). ■, AS-α-tropomyosin (Glu180Gly)

AS-α-tropomyosin (Asp175Asn) was indistinguishable from AS-α-tropomyosin whilst the mutant AS-α-tropomyosin (Glu180Gly) bound slightly weaker (Figure 1).

The Effects of Tropomyosin on Actin-ϕ Filament Movement over a Skeletal HMM Surface in the in Vitro Motility Assay

Smooth muscle tropomyosin has been shown to potentiate actin activation of the myosin ATPase under most conditions in vitro (23). In the *in vitro* motility assay the activating property of smooth muscle tropomyosin is manifested as an increase in velocity of actin tropomyosin filaments relative to actin alone (24, 25). In contrast skeletal muscle $\alpha\beta$-tropomyosin often inhibits ATPase and has no effect on velocity of filaments under our conditions (13). We found that the AS-α-tropomyosin wild type functioned similarly to smooth tropomyosin in vitro motility assay. The velocity increased by 25% with AS-α-tropomyosin wild type compared with 28% with 4nM-48nM smooth muscle tropomyosin, whilst skeletal tropomyosin did not affect the velocity of actin filament (Table 1, Figure 2). None of the tropomyosins affected the fraction of filaments that were motile.

The AS-α-tropomyosin mutants Asp175Asn and Glu180Gly also increased the speed of actin filament by 23% and 22% respectively with no change of fraction of filaments motile observed (Table 1). Thus in this assay the mutants were not significantly different from the AS-α-tropomyosin.

Effects of Skeletal Troponin on Actin-ϕ-AS-α-Tropomyosin Filament Movement

We titrated skeletal troponin with actin- tropomyosin filaments made up of 10nM actin-ϕ plus 10-20nM

TABLE 1

The Effect of Tropomyosin on Actin-ϕ Filament Velocity

	Effect of tropomyosin on actin-ϕ filament velocity			Effect of troponin at pCa5 on actin-ϕ-tropomyosin filament velocity		
	Velocity increase	Standard error	n	Velocity increase	Standard error	n
AS-α-tropomyosin	25%	±3.5%	9	5%	±1%	10
AS-α-tropomyosin (Asp175Asn)	23%	±4%	12	18%*	±3%	7
AS-α-tropomyosin (Glu180Gly)	22%	±4%	4	21%*	±2%	8
Skeletal muscle tropomyosin	0%*	±1%	3			
Smooth muscle tropomyosin	28%	±2%	3			

Note. The movement of actin-ϕ-tropomyosin filaments over immobilised HMM was analyzed. There were two populations of filaments, motile (velocity 2.5-4 μm/sec) and stationary. Fraction of filaments in the motile population and the mean velocity of motile filaments were determined from 200-600 individual filament vectors. Mean and standard error of relative velocity from n measurements with 2-4 separate protein preparations is presented. *$p<0.05$ compared with AS-α-tropomyosin.

of AS-α-tropomyosin, 20nM AS-α-tropomyosin (Asp175Asn) or 40nM of AS-α-tropomyosin(Glu180Gly). In activating conditions (pCa5) we found that troponin had minor effects upon wild type AS-α-tropomyosin filament velocity (increased by 5±1%, n=10), whereas the velocities increased by 18±3%(n=7) with filaments containing AS-α-tropomyosin(Asp175Asn) and by 21±2%(n=8) with filaments containing AS-α-tropomyosin (Glu180Gly) (Figure 3, Table 1). The tropomyosins had no effect on the fraction of filaments motile.

In relaxing conditions(pCa9), troponin added to actinf filaments containing AS-α-tropomyosin, or the mutants, Asp175Asn and Glu180Gly resulted in normal switch-off, with a decrease in the fraction of filaments moving from >80% to <20% and slightly decreased filament velocity (Figure 4). There were no significant differences in this decrease in velocity between the three tropomyosins when several experiments were analysed.

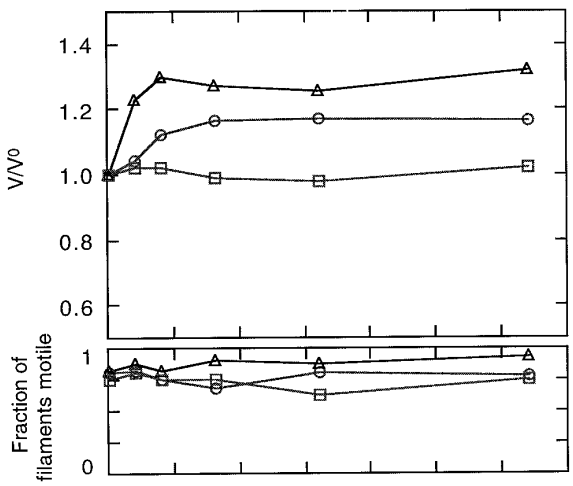

FIG. 2. Comparison of the effects of tropomyosin on actin-ϕ filament movement over a skeletal HMM surface in the *in vitro* motility assay. Conditions: 100ug/ml skeletal muscle HMM, buffer A at 25°C. ○, AS-α-tropomyosin. △, smooth muscle tropomyosin. □, skeletal muscle tropomyosin. Top: Effect of the tropomyosin on filament velocity relative to initial velocity (V/V0). Initial velocities were 2.4μm/sec, 2.2μm/sec and 2.35μm/sec respectively. Bottom: Effect of the tropomyosin on fraction of filaments that were motile. Each point on these graphs is derived from 300-600 filament vector measurements. Vectors fell into two populations; stationary and moving. Moving filaments showed a Gaussian distribution of velocities with a standard deviation equal to about 0.3× median velocity. Single determinations of mean velocity of the filaments that were motile and the fraction of filaments that were motile are plotted from a typical experiment. Averaged results from 2-4 experiments are analysed in the table.

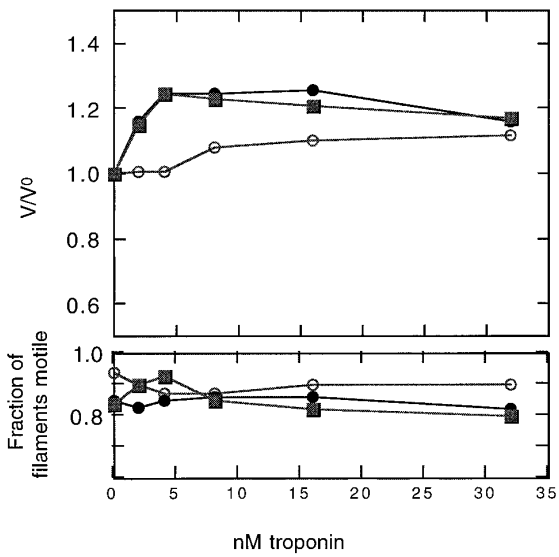

FIG. 3. Effects of skeletal muscle troponin on actin-ϕ-AS-α-tropomyosin filament movement at pCa5. Conditions: As for figure 2. ○, 20nM AS-α-tropomyosin wild type. ●, 20nM AS-α-tropomyosin(Asp175Asn). ■, 40nM AS-α-tropomyosin (Glu180Gly). Top: Effects of the skeletal troponin on filament velocity. Initial velocities were 2.7μm/sec, 3.7μm/sec and 2.6 μm/sec respectively. Bottom: Effects of the skeletal troponin on fraction of filaments that were motile.

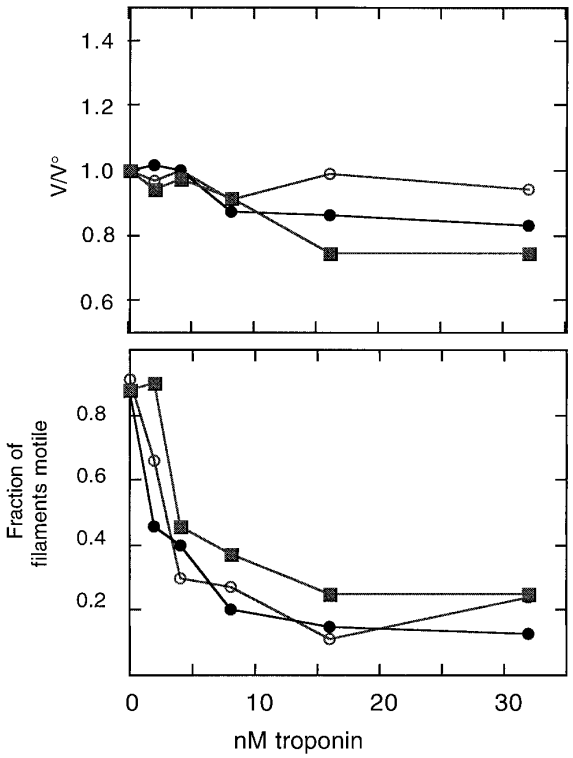

FIG. 4. Effects of skeletal muscle troponin on actin-ϕ-AS-α-tropomyosin filament movement at pCa9. Conditions: As for figure 3. Top: Effect of the skeletal troponin on filament velocity. Initial velocities were 3.2μm/sec, 3.5μm/sec and 3.5μm/sec respectively. Bottom: Effect of the skeletal troponin on fraction of filaments that were motile.

DISCUSSION

In this paper we have studied the effects of two missense mutations in α-tropomyosin which have previously been shown to cause familial hypertrophic cardiomyopathy (1). Chicken α-tropomyosin cDNA was expressed in *E. coli* with an N terminal alanine-serine extension since this has been reported to mimic the functional effects of N-terminal acetylation which occurs *in vivo* (12). We have been able to confirm that the AS-α-tropomyosin does indeed bind to actin with a similar affinity to natural skeletal muscle ab-tropomyosin and that the expressed tropomyosin causes an increase in actin-ϕ filament velocity over skeletal muscle HMM in the *in vitro* motility assay which is similar to the increasein velocity caused by smooth muscle tropomyosin (Table 1)(13,18,24). Thus this is a suitable material for studying the effects of mutations in tropomyosin. A recent report of unmodified recombinant α-tropomyosin binding to actin differed somewhat from these results. Overall affinity for actin was almost two orders of magnitude less and the α-tropomyosin(Asp175Asn) bound twofold weaker than wild type α-tropomyosin and α-tropomyosin(Glu180Gly) (11)

Using the *in vitro* motility assay to compare wild type AS-α-tropomyosin and AS-α-tropomyosin with the two missense mutations Asp175Asn and Glu180Gly we have observed little or no difference in their interaction with actin or their mediation of the inhibitory effect of troponin at pCa9. On the other hand there was a significant change in the interaction with troponin at pCa5. The addition of 5-10nM troponin resulted in a 18% increase in velocity of actin-ϕ filaments containing the mutant AS-α-tropomyosin(Asp175Asn) and 21% with AS-α-tropomyosin (Glu180Gly), compared with only 5% with the wild type AS-α-tropomyosin.

Our data indicate that the increased velocity is likely to be due to an altered interaction between tropomyosin and troponinT, as the interactions of the mutant tropomyosins with actin alone in the motility assay were not affected. TroponinT is the only component of the troponin complex which binds strongly to tropomyosin and the mutations Asp175Asn and Glu180Gly introduce changes in surface charge of tropomyosin in the region of the molecule that has been implicated in troponin T binding. The nearby amino acids Val170 and Ileu171 form a hydrophobic patch which has been predicted to be part of a troponinT binding site (26). The cysteine190 can be labelled with fluorophores which are sensitive to troponin binding (27,28); studies with these fluorophores has suggested that the mutations cause an altered conformational response when myosin heads switch the thin filament from the off to the on state (11). This region is further implicated in troponinT binding since the smooth muscle isoform of α-tropomyosin, which has charge change amino acid substitutions in the 189-213 region is unable to bind to the T1 fragment of troponinT (29,30). Finally it has been demonstrated that a missense mutation in rat cardiac TnT (equivalent to the human FHC mutation TnT-(Ile79Asn)) leads to an increase in filament velocity at high pCa which is similar in magnitude to the velocity increase seen with the α-tropomyosin mutations (9). This mutation is in a pututive tropomyosin-binding region of troponin T (8,29).

It is well documented that troponinT causes a Ca^{2+}-dependent 'switching on' of partially activated thin filaments which may be detected both in ATPase assays and in the *in vitro* motility assay (31,32) and the mutations in α-tropomyosin appear to enhance or mimic this effect. The apparent increase in unloaded velocity that occurs with the FHC α-tropomyosin and troponinT mutations could be due to a shift in the equilibrium favouring the ON (or open) state of actin-tropomyosin or due to a change in the parameters of the ON state itself (33). In the case of the troponinT mutation ($Int^{15G>A}$) there also appears to be a decrease in force production (10).

We conclude that the point mutations in tropomyosin which cause FHC in humans affect the tropomyosin-troponinT interaction at pCa5 enough to produce a

measurable change in thin filament regulatory function, but the effect is not so severe as to render the muscle non-viable. Increased velocity of unloaded shortening may be associated with less force and hence reduced power output in the intact heart, thus leading to compensatory hypertrophy.

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