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Effects of Troponin T Mutations in Familial Hypertrophic Cardiomyopathy on Regulatory Functions of Other Troponin Subunits[1]

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We have previously shown that mutations in troponin T (TnT), which is associated with familial hypertrophic cardiomyopathy (HCM), cause an increase in the Ca^{2+} sensitivity and a potentiation of cardiac muscle contraction. To gain further insight into the pathophysiological role of these mutations, four mutations (Arg92Gln, Phe110Ile, Glu244Asp, Arg278Cys) were introduced into recombinant human cardiac TnT, and the mutants were exchanged into isolated porcine cardiac myofibrils. The effects of mutations were tested on maximal ATPase activity, the inhibitory function of troponin I (TnI) in the absence of troponin C (TnC), and the neutralizing function of TnC. Arg92Gln, Phe110Ile, and Glu244Asp markedly impaired the inhibitory function of TnI. Arg278Cys also impaired the inhibitory function of TnI, but the effect was much smaller. Phe110Ile and Glu244Asp markedly enhanced the neutralizing function of TnC and potentiated the maximum ATPase activity. Arg92Gln and Arg278Cys only slightly enhanced the neutralizing function of TnC, and they conferred no potentiation on the maximum ATPase activity. These results indicate that mutations in TnT impair multiple processes of Ca^{2+} regulation by troponin, and there are marked differences in the degree of impairment from mutation to mutation.

Key words: ATPase, calcium, cardiac muscle, hypertrophy, troponin.

Troponin (Tn) is a specific Ca^{2+} receptor protein located on thin filaments for regulation of striated muscle contraction. This protein is a complex of three subunits, troponin C (TnC), troponin I (TnI), and troponin T (TnT). TnC is a Ca^{2+} binding component. TnI has an inhibitory effect on the interaction between myosin and actin-tropomyosin, which is reversed by Ca^{2+} binding to TnC. TnT is a tropomyosin-binding component and integrates the entire Tn complex into the thin filament.

Familial hypertrophic cardiomyopathy (HCM) is an autosomal dominant heart disease associated with a high risk of sudden death. This disease has recently been demonstrated to be caused by mutations in several cardiac sarcomeric proteins including TnT (*1*, *2*), TnI (*3*), β-myosin heavy chain (*4*), actin (*5*), α-tropomyosin (*1*), myosin-binding protein C (*6*), and myosin light chains (*7*). At least thirteen point mutations and one splice donor site mutation in the TnT gene have so far been identified (*8*). A number of biochemical and physiological studies of the functional consequences of HCM-linked TnT mutations have been reported (*9–27*). However, the exact mechanisms by which these TnT mutations cause this disease remain unclear.

We have previously reported that five HCM-linked TnT missense mutations (Ile79Asn, Arg92Gln, Phe110Ile, Glu244Asp, Arg278Cys) have two different effects on the ATPase activity of isolated myofibrils and/or the force development of skinned fibers; *i.e.*, Ca^{2+}-sensitization (Ile79Asn, Arg92Gln, Glu244Asp, Arg278Cys) and potentiation of maximum contractile activity (Phe110Ile, Glu244Asp) (*24–27*). In the present study, we examined the effects of four HCM-linked TnT mutations (Arg92Gln, Phe110Ile, Glu244Asp, Arg278Cys) on the functions of the other troponin subunits, TnI and TnC, to gain insight into the molecular mechanisms of the patho-physiological effects exerted by these TnT mutations. It was found that these TnT mutations impair the inhibitory action of TnI and enhance the neutralizing action of TnC, but the effect is highly variable depending on the mutation.

MATERIALS AND METHODS

Cloning and Mutagenesis of Human Cardiac Tn cDNAs—The cloning and mutagenesis of human cardiac Tn cDNAs (TnC, TnI, and TnT) were carried out as described previously (*24*). Briefly, human cardiac Tn cDNAs were cloned by RT-PCR from human heart mRNA and the obtained wild-type Tn cDNAs were then constructed into a pET-3d vector for expression. To obtain mutant TnTs, mutagenesis was carried out by PCR. The results of the mutations in TnT cDNA were confirmed by DNA sequencing.

Expression and Purification of Recombinant Tns—The

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Abbreviations: Tn, troponin; TnT, troponin T; TnI, troponin I; TnC, troponin C; HCM, familial hypertrophic cardiomyopathy; 2-ME, 2-mercaptoethanol; pCa, $-\log[Ca^{2+}]$.

wild-type Tn and mutant TnTs were expressed in *Escherichia coli* BL21(DE3) and purified using an FPLC system as described previously (*24*).

Preparation of Myofibrils—Porcine cardiac myofibrils were prepared and stored in a solution comprising 50% glycerol, 100 mM KCl, 20 mM MOPS (pH 7.0), and 0.5% 2ME at −20°C as described previously (*24*).

Tn Exchange in Myofibrils—Tn exchange was performed according to the previously reported method (*28*). Briefly, myofibrils (500 μg/ml) were incubated in a solution of 20 mM MOPS/KOH (pH 6.2), 265 mM KCl, 5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM 2-ME, and 35 μg/ml of recombinant human cardiac TnT at 25°C for 1 h with shaking. These myofibrils were washed with a solution of 0.5% Briji-58, 20 mM MOPS (pH 7.0), 265 mM KCl, 5 mM $MgCl_2$, 0.5 mM 2-ME, and the myofibrils were resuspended in a solution (500 μg/ml) containing 60 mM KCl and 1 mM sodium bicarbonate after washing with the same solution. The myofibrils were then reconstituted with recombinant TnI and TnC on ice for 1 h.

ATPase Activity Measurement—The reaction mixture (200 μl) for the ATPase assay consisted of 90 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS (pH 7.0), 1 mM Ca^{2+}-EGTA, 4 mM ATP, and 40 μg myofibrils. The reaction was started by adding ATP at 25°C and terminated by adding 1.6 ml of a mixture of 50% acetone, 2.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 1.25 N H_2SO_4 after 15 min incubation, a period in which the ATPase activity was linear with time. The contents were mixed carefully, 160 μl of 1 M citric acid was added to the tubes, and the yellow color was measured at OD_{355} (*29*). The Ca^{2+} concentrations in the reaction mixtures were calculated as described previously (*30*).

Electrophoresis—SDS/PAGE was performed according to the procedure of Laemmli with an acrylamide concentration of 12%. The gel was stained with Coomassie Brilliant Blue R-250 and an optical densitometric scan was obtained using the Phoretix gel analysis software package (Phoretix International).

RESULTS AND DISCUSSION

Exchange of Troponin in Porcine Cardiac Myofibrils with Recombinant Human Cardiac TnT—The three subunits of human cardiac Tn (TnT, TnI, TnC) were expressed in *E. coli* and purified. Recombinant human cardiac TnI and TnT had somewhat faster electrophoretic mobilities than porcine TnI and TnT, respectively, in 12% SDS/PAGE gels, while TnC from both species showed exactly the same mobility (Fig. 1A, lanes 1 and 2).

Endogenous TnT·TnI·TnC-complexes in isolated porcine cardiac myofibrils were exchanged with the recombinant human cardiac TnT by treating the myofibrils with an excess amount of TnT under slightly acidic (pH 6.2) and high ionic strength conditions (*28*). SDS/PAGE analyses demonstrated an incorporation of recombinant TnT into the myofibrils accompanied by decreases in endogenous TnT, TnI, and TnC after treatment with recombinant human cardiac TnT (Fig. 1A, lanes 3 and 4). There were no significant differences in the amounts of endogenous TnI and TnC displaced by treatment with recombinant human cardiac wild-type or mutant TnTs (Fig. 1B). Densitometric scans indicated that about 65% of the endogenous Tn was displaced, consistent with the previous study using rabbit cardiac myofibrils (*24*).

Effects of TnI and TnC on the ATPase Activity of Myofibrils Exchanged with Human Cardiac Mutant TnTs—Myofibrils treated with TnT showed a high ATPase activity, even in the absence of Ca^{2+}, due to the loss of TnI. The ATPase activities of wild-type and mutant TnT-treated myofibrils in the absence of Ca^{2+} increased up to approximately 65% of the activity in the presence of Ca^{2+}. There were no significant differences in the ATPase activities in the absence or presence of Ca^{2+} between wild-type and mutant TnT-treated myofibrils. To test the effects of mutations in TnT on the inhibitory activity of TnI, we measured the decrease in the ATPase activity of mutant TnT-treated myofibrils caused by the addition of TnI in the absence of TnC. As shown in Fig. 2, the recombinant human cardiac TnI completely inhibited the ATPase activities of the myofibrils treated with wild-type and mutant TnTs at a concentration of 9.45 μg/ml. At lower concentrations (0.95–3.15 μg/ml), the inhibitory activity of TnI was significantly weaker in myofibrils treated with Arg92Gln, Phe110Ile, and Glu244Asp mutant TnTs than in myofibrils treated with wild-type TnT. A similar impairment of the inhibitory activity of TnI was seen in myofibrils treated with the Ile79Asn mutant TnT (*24*). The inhibitory activity of TnI decreased only slightly in myofibrils treated with Arg278Cys mutant TnT, although no statistically significant differences were detected. The amounts of TnI required for 50% inhibition (IC_{50}) are summarized in Table I.

To test the effects of mutations in TnT on the neutralizing activity of TnC, we next measured the increase in the ATPase activity of mutant TnT-treated myofibrils caused by addition of TnC in the presence of TnI (Fig. 3). The neutralizing activity of TnC increased slightly in myofibrils treated with Arg92Gln and Arg278Cys mutant TnTs, although we could detect no statistically significant differ-

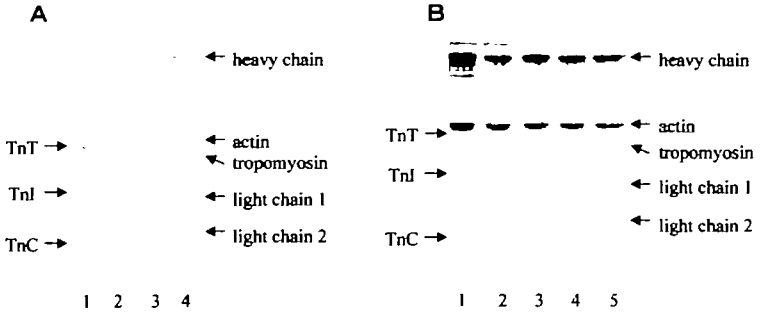

Fig. 1. **SDS-PAGE patterns of porcine cardiac myofibrils treated with recombinant human cardiac TnT.** A: Porcine cardiac myofibrils were treated with wild-type TnT. Lane 1, purified porcine cardiac TnT·TnI·TnC-mixture; lane 2, recombinant human cardiac TnT·TnI·TnC-mixture; lane 3, untreated intact myofibrils; lane 4, myofibrils treated with wild-type TnT. B: Porcine cardiac myofibrils were treated with mutant TnTs. Lane 1, untreated intact myofibrils; lane 2, myofibrils treated with Arg92Gln TnT; lane 3, myofibrils treated with Phe110Ile TnT; lane 4, myofibrils treated with Glu244Asp TnT; lane 5, myofibrils treated with Arg278Cys TnT. All data are representative of four other experiments.

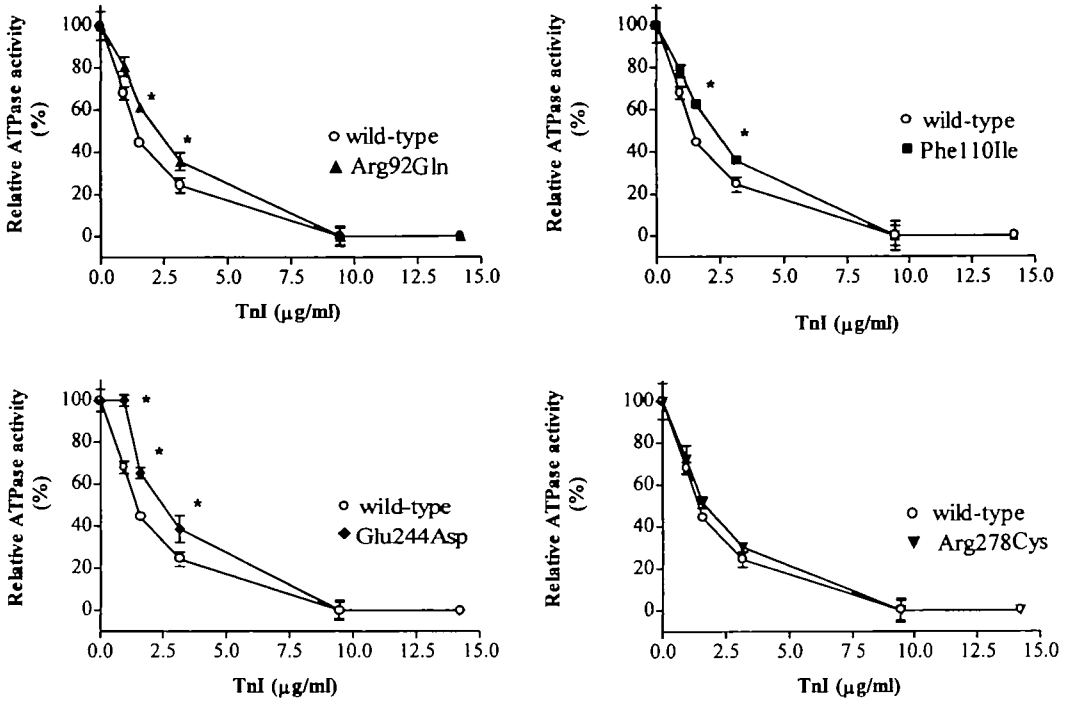

Fig. 2. **The effects of recombinant human cardiac TnI on the ATPase activity of myofibrils treated with wild-type or mutant TnTs.** Myofibrils were treated with wild-type or mutant TnTs, and then reconstituted with various amounts of recombinant human cardiac TnI. The ATPase activity was measured in the absence of Ca^{2+} (pCa 7.05). The results are expressed as percentages of ATPase activity before the addition of TnI [33.6 ± 1.2, 35.5 ± 1.9, 34.1 ± 2.0, 33.8 ± 2.0, and 34.1 ± 1.7 nmol P_i/mg/min (means ± SE of three experiments performed in duplicate) in myofibrils treated with wild-type, Arg92Gln, Phe110Ile, Glu244Asp, and Arg278Cys TnTs, respectively]. Asterisk (*), $p < 0.05$ compared with wild-type TnT-treated myofibrils (*t* test).

TABLE I. **Effects of HCM-linked TnT mutations on the inhibitory effect of TnI and the neutralizing effect of TnC.** Myofibrils were treated with wild-type or mutant TnTs, and then reconstituted with various amounts of TnI to examine the effect of TnI. To examine the effect of TnC, the TnT-treated myofibrils were reconstituted with 9.45 μg/ml of TnI, and then reconstituted with various amounts of TnC. The ATPase activity was measured as described in "MATERIALS AND METHODS." IC_{50} is the amount of TnI required for 50% inhibition of the ATPase activity in the absence of Ca^{2+}. EC_{50} is the amount of TnC required for 50% activation of the maximum ATPase activity in the presence of Ca^{2+}. Maximum ATPase activity is the net ATPase activity recovered with a saturating amount of TnC in the presence of Ca^{2+}. The data represent the means ± SE of three experiments performed in duplicate.

TnT type	IC_{50} for TnI (μg/ml)	EC_{50} for TnC (μg/ml)	Maximum ATPase activity (nmol P_i/mg/min)
Wild-type	1.51 ± 0.23	1.57 ± 0.12	35.9 ± 0.8
Arg92Gln	2.45 ± 0.21*	1.24 ± 0.23	37.5 ± 2.0
Phe110Ile	2.44 ± 0.23*	0.89 ± 0.15*	42.0 ± 1.5*
Glu244Asp	2.78 ± 0.30*	0.84 ± 0.13*	44.2 ± 0.6*
Arg278Cys	1.73 ± 0.22	1.20 ± 0.21	32.3 ± 0.4

*$p < 0.05$ compared with wild-type TnT (*t* test).

ences compared to wild-type TnT-treated myofibrils. In the case of myofibrils treated with Phe110Ile and Glu244Asp mutant TnTs, the neutralizing activity of TnC increased markedly, and a potentiation of the maximum ATPase activity was observed (Table I). The amounts of TnC required for 50% maximum neutralization (EC_{50}) are summarized in Table I.

Based on the above findings, the HCM-linked mutations in TnT examined in the present study can be classified into three groups as follows: (i) Arg92Gln, which markedly weakens the inhibitory activity of TnI with slight or no enhancement of the neutralizing activity of TnC, (ii) Phe110Ile and Glu244Asp, which markedly weaken the inhibitory activity of TnI and also markedly enhance the neutralizing activity of TnC, and (iii) Arg278Cys, which marginally weakens the inhibitory activity of TnI and also marginally enhances the neutralizing activity of TnC. Previously, we reported that the Ile79Asn mutation impairs the action of TnI without affecting the action of TnC, effects similar to those of the Arg92Gln mutation observed in the present study. According to the effects on myofibrillar ATPase activity, the Ile79Asn, Arg92Gln and Arg278Cys mutations can be classified into the same group that causes Ca^{2+} sensitization (*24*). This suggests that impairment of the inhibitory activity of TnI is closely related to the Ca^{2+} sensitization caused by these mutations. Although the Arg278Cys mutation has only marginal effects on the actions of TnI and TnC, this may account for the relatively smaller Ca^{2+} sensitizing effect of this mutation (*26*). Another interesting finding in this study is that the Phe110Ile and Glu244Asp mutations, which potentiate the maximum ATPase activity and force (*24, 27*), both enhance the neutralizing action of TnC. This suggests that enhancement of the neutralizing action of TnC is closely related to the potentiation of the maximum contractile activity caused by these mutations. The Phe110Ile and Glu244Asp mutations also markedly impaired the inhibitory activity of TnI as well as the Ile79Asn and Arg92Gln mutations. A previous study, how-

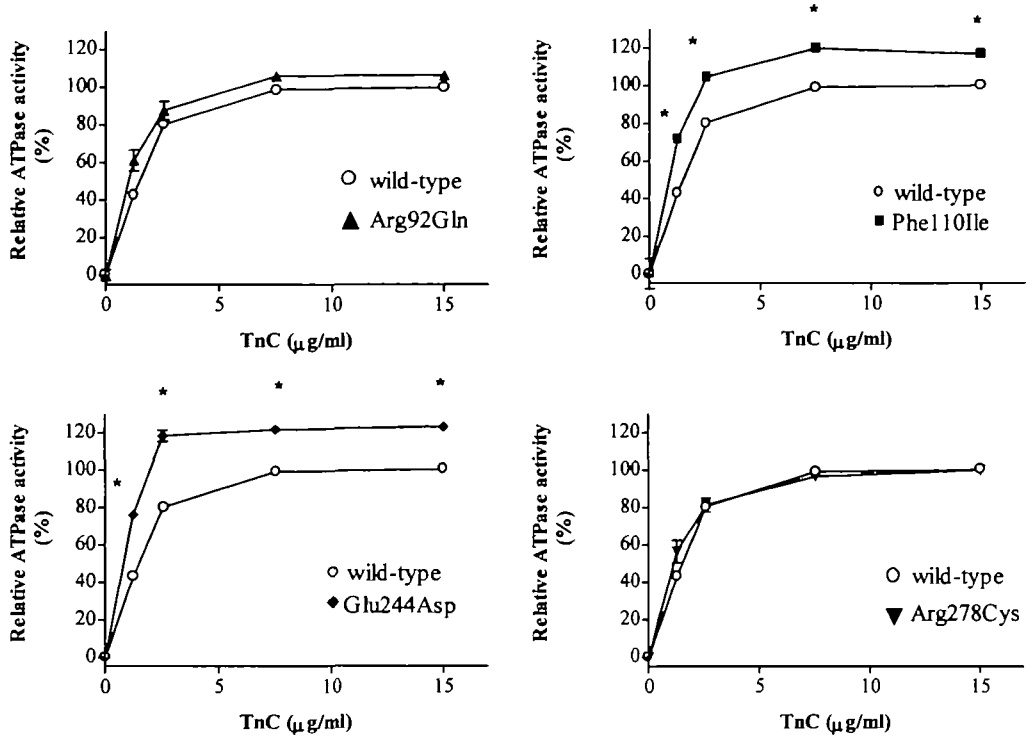

Fig. 3. **The neutralizing effect of TnC on the ATPase activity of myofibrils reconstituted with TnI after TnT treatment.** Myofibrils treated with wild-type or mutant TnTs were reconstituted with TnI (9.45 μg/ml) and the effect of various amounts of TnC on the ATPase activity were determined in the presence of Ca^{2+} (pCa 5.18). The results are expressed as the percentage of the maximum ATPase activity of myofibrils treated with wild-type TnT (means ± SE of three experiments performed in duplicate). Asterisk (*), $p < 0.05$ compared with wild-type TnT-treated myofibrils (*t* test).

ever, showed that the Phe110Ile and Glu244Asp mutations had no significant Ca^{2+} sensitizing effect on myofibrillar ATPase activity (*24*). This might be explained by the simultaneous marked effects of these two mutations on the neutralizing action of TnC.

Finally, the present study reveals that HCM-linked TnT mutations alter multiple processes in Ca^{2+} regulation involving inhibition by TnI and neutralization by TnC. The degree of alteration, however, varies markedly from mutation to mutation. This may explain the differential effects exerted by these TnT mutations on the Ca^{2+} regulation of ATPase activity and force generation in cardiac muscle (*24–27*), and on the phenotype in patients with differing mutations (*31*).

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