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Effects of Missense Mutations Phe110Ile and Glu244Asp in Human Cardiac Troponin T on Force Generation in Skinned Cardiac Muscle Fibers[1]

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The functional effects of two missense mutations in human cardiac troponin T, Phe110Ile and Glu244Asp, associated with familial hypertrophic cardiomyopathy were examined by exchanging the bacterially expressed and purified mutant troponin T into rabbit cardiac skinned muscle fibers. Both mutations significantly increased the maximum force without affecting the cooperativity. The Glu244Asp mutation also increased the Ca^{2+} sensitivity of the force generation, as in the case of other mutations associated with a poor prognosis. On the other hand, the Phe110Ile mutation, associated with a favorable prognosis, had no effect on the Ca^{2+} sensitivity. The results strongly support the hypothesis that increased Ca^{2+} sensitivity is responsible for the pathogenesis of hypertrophic cardiomyopathy with a poor prognosis caused by mutations in troponin T.

Key words: calcium sensitivity, familial hypertrophic cardiomyopathy, force, skinned fiber, troponin.

In vertebrate striated muscles, the contraction of myofibrils is regulated by troponin, which is distributed at regular intervals along the entire thin filament (*1, 2*). Troponin is a protein complex consisting of three different components; a Ca^{2+}-binding component, troponin C (TnC), an inhibitory component, troponin I (TnI), and a tropomyosin (TM)-binding component, troponin T (TnT). The contractile interaction between myosin and actin-TM is inhibited by TnI, and this inhibition by TnI is abolished by the activating or neutralizing action of TnC. TnT has no apparent function such as inhibition or neutralization of the contractile interaction between myosin and actin-TM. However, TnT plays a critically important role in the Ca^{2+} regulation of contraction, since the neutralizing action of TnC is hardly sensitive to Ca^{2+} in its absence (*1*).

Recently, mutations in the genes for various cardiac myofibrillar proteins, including troponin T, were shown to cause familial hypertrophic cardiomyopathy (HCM); at least 13 different mutations in the TnT gene have been identified; 11 missense mutations, a mutation involving a deleted codon and a splice donor site mutation (*3*). It has previously been shown that the missense mutations, Ile79Asn, Arg92Gln, and Arg278Cys, and the splice donor site mutation have a Ca^{2+}-sensitizing effect on the contraction of cardiac muscle (*4-7*), suggesting that enhancement of the myofilament response to Ca^{2+} might be a common functional defect associated with mutations in TnT. In the present study, we examined the functional consequences of two other missense mutations, Phe110Ile and Glu244Asp, under physiological conditions using skinned cardiac muscle fibers in order to gain further insights into the molecular mechanism underlying the pathogenesis of HCM caused by mutations in TnT.

Human cardiac TnT cDNA was amplified by RT-PCR of human heart mRNA, and constructed in the pET3-d vector for expression and mutagenesis. Mutagenesis was carried out by PCR according to the method described by Horton (*8*); the oligonucleotides employed were 5′-GGGCTCACATTGAGAACAGG-3′ and 5′-TATAACTTGGATGCAGAGAAG-3′ (the changed bases are underlined) for mutations Phe110Ile and Glu244Asp, respectively. The recombinant human cardiac TnTs were expressed in *Escherichia coli* BL21(DE3) and purified as described previously (*4*). The purity of the proteins was assessed by SDS-PAGE (Fig. 1). These recombinant human cardiac TnTs were partially exchanged into rabbit skinned cardiac muscle fibers by means of a previously developed troponin exchange technique (*4-6, 9, 10*). Briefly, endogenous TnT·TnI·TnC complexes in the skinned fibers were first replaced by the recombinant TnT, which was added in an excess amount under acidic and high-ionic-strength conditions, and then the fibers were reconstituted with purified rabbit native cardiac TnI and TnC. Determination of the decreased amount of endogenous TnI, which had been demonstrated to be directly proportional to the extent of TnT exchanged

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Abbreviations: MOPS, 3-(*N*-morpholino)-propanesulfonic acid; pCa, $-\log [Ca^{2+}]$; TM, tropomyosin; Tn, troponin.

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into the fibers (4, 6), on an SDS-PAGE gel indicated that the wild-type and mutant TnTs were exchanged into the fibers to almost the same extent, ranging from 50 to 55%. Skinned fibers were prepared from the left ventricular trabeculae of young male albino rabbits (~3 mo old), and force measurements were performed as described previously (4). The relaxing solution consisted of (in mM) 50 MOPS/KOH (pH 7.0), 100 KCl, 6 $MgCl_2$, 5 ATP, 4 EGTA, 0.5 DTT, and 10 creatine phosphate, as well as 35 U/ml creatine kinase. Activating solutions with desired free Ca^{2+} concentrations at pH 6.5-7.5 were prepared by adding appropriate amounts of $CaCl_2$, calculated as described previously (11), to the relaxing solution containing 50 mM MOPS (pH 7.0 and 6.5) or Tris (pH 7.5) as a pH buffer. All force measurements were performed at 25°C.

Figure 2 compares the force-pCa relationship determined at pH 7.0 in the fibers exchanged with the missense mutant TnT with that in the fibers exchanged with the human wild-type cardiac TnT. Table I summarizes the Ca^{2+} sensitivity (pCa_{50}) and cooperativity (n_H) in the force-pCa relationships, as well as the maximum force. Both mutant TnTs significantly increased the maximum force up to 120-130% of the level attained by wild-type TnT without affecting the cooperativity. The Glu244Asp mutant also shifted the force-pCa relationship to the left, as evidenced by a significant increase in pCa_{50}, indicating that this mutant has a Ca^{2+}-sensitizing effect. Before TnT exchange, there were no statistically significant differences in the Ca^{2+} sensitivity and cooperativity between these three fiber groups (data not shown). The effects of pH on these parameters were then examined by sequentially determining the force-pCa relationships in respective fibers at pH 7.5, 7.0 and 6.5 (Fig. 3). The Ca^{2+}-sensitizing effect (*i.e.*, an increase in pCa_{50}) of the Glu244Asp mutation seemed to be more evident at an acidic pH, 6.5, but was not significant at an alkaline pH, 7.5, as in the case of mutations Ile79Asn and Arg92Gln (4, 7). On the other hand, the Phe110Ile mutation had no Ca^{2+} sensitizing effect at any pH examined (Fig. 3A). Both mutations increased the maximum force by 20-30% at all pHs without affecting the cooperativity of the force-pCa relationship (Fig. 3, B and C).

In a previous study involving isolated cardiac myofibrils (7), the Phe110Ile and Glu244Asp mutations in human cardiac TnT were found to increase the maximum ATPase activity, consistent with the increase in the maximum force of skinned cardiac muscle fibers observed under more physiological conditions in the present study. In the present study, the Glu244Asp mutation was also found to increase the Ca^{2+} sensitivity of the force generation in skinned cardiac muscle fibers; this Ca^{2+}-sensitizing effect was not observed on the myofibrillar ATPase activity in the previous study, probably due to the differences in the experimental conditions or the accuracy of the measurements.

We have previously shown that the HCM-causing missense mutations, Ile79Asn, Arg92Gln, and Arg278Cys, and a splice donor site mutation in human cardiac TnT increase the Ca^{2+} sensitivity of the force generation in skinned fibers, ΔpCa_{50} (pCa_{50} in fibers exchanged with mutant TnT-pCa_{50} in fibers exchanged with wild-type TnT)

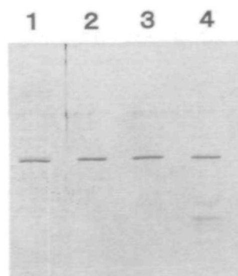
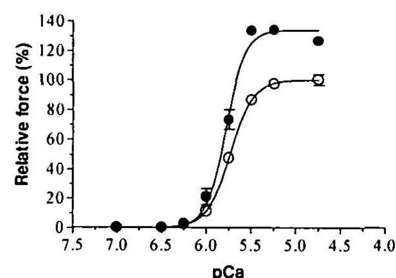


Fig. 1. **SDS-PAGE of purified recombinant wild-type, Phe110-Ile, and Glu244Asp human cardiac TnTs.** Lane 1, rabbit cardiac tissue-derived (native) TnT. Lane 2, human cardiac wild-type TnT. Lane 3, human cardiac Phe110Ile mutant TnT. Lane 4, human cardiac Glu244Asp mutant TnT. The gel was stained with Coomassie Brilliant Blue R-250.



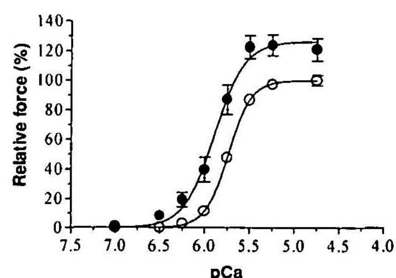


Fig. 2. **Effects of exchanging Phe110Ile (A) or Glu244Asp (B) mutant TnT into skinned cardiac muscle fibers on force-pCa relationships.** Force-pCa relationships determined after the exchange of recombinant wild-type (○) and mutant (●) human cardiac TnT were compared; forces were normalized as to the averaged maximum force in the fibers exchanged with wild-type TnT, and are expressed as means±SE of measurements on 3 to 6 fibers.

TABLE I. **Maximum force, Ca^{2+} sensitivity, and cooperativity in recombinant human cardiac TnT-exchanged fibers.** Ca^{2+} sensitivity [pCa at half-maximum force generation (pCa_{50})] and cooperativity [Hill coefficient (n_H)] were calculated by fitting the data for individual fibers summarized in Fig. 2 to the Hill equation as described previously (6, 17). Values are means±SE of measurements on *n* fibers.

TnT	pCa_{50}	n_H	Maximum force[a]	*n*
Wild-type	5.73±0.01	3.37±0.18	64.6±2.3 (100)	4
Phe110Ile	5.78±0.01	4.13±0.67	86.8±1.2 (134)**	3
Glu244Asp	5.89±0.03**	2.99±0.31	79.9±4.5 (124)*	6

[a]Maximum force was expressed as a percentage of the maximum force developed in the same fiber before TnT exchange; values in parentheses are % of the maximum force developed in the wild-type TnT-exchanged fibers. *$p<0.05$, **$p<0.01$ *vs.* wild-type TnT-exchanged fibers (Dunnett's multiple comparison test).

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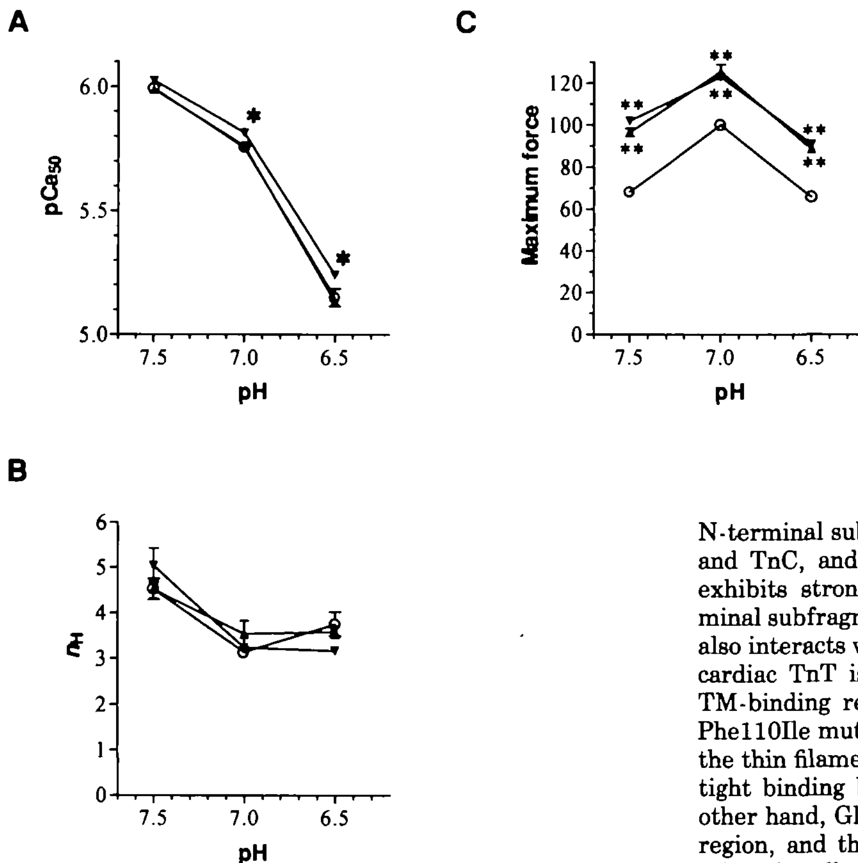

Fig. 3. **Effects of pH on Ca^{2+} sensitivity (pCa_{50}) (A), cooperativity (n_H) (B), and maximum force (C) of fibers exchanged with wild-type (○), Phe110Ile (▲), or Glu244Asp (▼) human cardiac TnT.** Force-pCa relationships were sequentially determined at pH 7.0, 7.5, 6.5, and 7.0 using the same fiber, and the responses at pH 7.0 were averaged to compensate for any rundown of the fiber. pCa_{50} and n_H were then calculated by fitting the data for individual fibers to the Hill equation as described previously (*6, 17*). Maximum force was expressed as a percentage of the maximum force developed in the wild-type TnT-exchanged fibers at pH 7.0. All data are means ± SE of measurements on three fibers. $^*p < 0.05$, $^{**}p < 0.01$ *vs.* wild-type TnT-exchanged fibers (Dunnett's multiple comparison test).

being 0.12, 0.17, 0.07, and 0.23-0.25, respectively, at pH 7.0 (*4-6*). Of interest are the clinical data showing that patients with the Ile79Asn, Arg92Gln, and splice donor site mutations have a similar clinical phenotype of a poor prognosis characterized by a high risk of sudden death (*12*), whereas patients with the Phe110Ile mutation have a favorable prognosis (*13*). The present study shows that the Phe110Ile mutation increases the maximum force but does not affect the Ca^{2+} sensitivity of the force generation, strongly suggesting that an increase in the Ca^{2+} sensitivity is a common functional defect involved in the pathogenesis of HCM with a poor prognosis. Although the clinical characteristics and prognosis of patients with the Glu244-Asp mutation are not known, its Ca^{2+}-sensitizing effect ($\Delta pCa_{50} = 0.16$ at pH 7.0) is comparable in magnitude to the Ca^{2+}-sensitizing effects of the Ile79Asn and Arg92Gln mutations (*4, 7*), suggesting that this TnT mutation is associated with a poor prognosis. It should be noted that the increase in the maximum force caused by the Phe110Ile mutation also results in an apparent increase in the Ca^{2+} sensitivity due to a scale-up of the overall force (Fig. 3C). However, the resultant increase in the sub-half-maximum force is much smaller than those in the cases of the other TnT mutations that cause an increase in pCa_{50} (*4-6*); this may account for the favorable prognosis of the patients associated with the Phe110Ile mutation, since intact cardiac muscle is never activated beyond the half-maximum level (*14*).

Previous studies on the chymotryptic subfragments of the rabbit fast skeletal TnT molecule have shown that the N-terminal subfragment, TnT_1, does not interact with TnI and TnC, and its α-helical region of about 80 residues exhibits strong TM-binding ability, whereas the C-terminal subfragment, TnT_2, interacts with TnI and TnC, and also interacts weakly with TM (*15, 16*). Phe-110 of human cardiac TnT is in the regions homologous to the strong TM-binding region in the TnT_1 subfragment. Thus, the Phe110Ile mutation may potentiate the maximum level of the thin filament activation by altering the strength of the tight binding between the TnT_1 region and TM. On the other hand, Glu-244 of human cardiac TnT is in the TnT_2 region, and thus the Glu244Asp mutation may exert its effects by affecting the interaction between the TnT_2 region and TnI or TnC as well as the strength of the loose binding between this region and TM. The skinned fibers exchanged with the Phe110Ile or Glu244Asp mutant TnT developed a slightly but significantly higher maximum force than those exchanged with the wild-type TnT even before being reconstituted with TnI and TnC (data not shown), suggesting that these mutants directly potentiate the maximum level of the thin filament activation. The increase in the maximum force, however, was more significant after reconstitution with TnI and TnC, indicating that these mutations in TnT also affect the regulatory function of a whole troponin complex in the thin filament and lead to further potentiation of the thin filament activation. Further studies to clarify the mechanisms underlying the potentiation of the maximum force and Ca^{2+}-sensitization associated with these HCM-causing mutations in cardiac TnT will shed some light on the molecular mechanisms of the thin filament regulation by Ca^{2+} in striated muscles.

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