

Troponin T isoforms alter the tolerance of transgenic mouse cardiac muscle to acidosis

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Abstract

Troponin T (TnT) is an essential protein in the Ca^{2+} regulatory system of striated muscle. Three fiber type-specific TnT genes have evolved in higher vertebrates to encode cardiac, slow and fast skeletal muscle TnT isoforms. To understand the functional significance of TnT isoforms, we studied the effects of acidosis on the contractility of transgenic mouse cardiac muscle that expresses fast skeletal muscle TnT. Contractility analysis of intact cardiac muscle strips showed that while no differences were detected at physiological pH, the transgenic cardiac muscle had significantly greater decreases in $+dF/dt_{\text{max}}$ at acidic pH than that of the wild-type control. Contractility of skinned cardiac muscles demonstrated that the presence of fast TnT resulted in significantly larger decreases in force and Ca^{2+} sensitivity at acidic pH than that of the wild-type control. The effect of TnT isoforms on the tolerance of muscle to acidosis may explain the higher tolerance of embryonic versus adult cardiac muscles. The results are consistent with the hypothesis that charge differences in TnT isoforms contribute to the contractility of muscle. The data further support a hypothesis that slow TnT is similar to the cardiac, but not fast, and TnT may contribute to the higher tolerance of slow muscles to stress conditions. Therefore, TnT isoform diversity may contribute to the compatibility of muscle thin filaments to cellular environments in different fiber types, during development and functional adaptation.

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Contractility of striated muscle can be altered by the cellular environment. A decrease of pH is a major factor affecting muscle contractility under stress conditions. Contraction of cardiac and skeletal muscle is activated by Ca^{2+} through the thin filament-associated troponin-tropomyosin system. Troponin T (TnT)³ is the tropomy-

osin-binding subunit of the troponin complex that is at a central position in the allosteric thin filament regulatory system [1–3]. The structure–function relationship of TnT is a determinant for muscle contractility and may affect responses.

A family of homologous genes has evolved in higher vertebrates encoding three muscle fiber type-specific (cardiac, slow skeletal muscle and fast skeletal muscle) TnT isoforms [4–6]. Alternative RNA splicing generates more protein isoforms from the three TnT genes [7]. The expression of TnT isoforms in cardiac and skeletal muscles is regulated during development and fiber type differentiation. Protein and cDNA sequence data have demonstrated that the structure of TnT isoforms is well conserved in the COOH-terminal and central regions. The main primary structure difference

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³ Abbreviations used: BSA, bovine serum albumin; mAb, monoclonal antibody; pI, isoelectric point; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TnC, troponin C; TnI, troponin I; TnT, troponin T.

among cardiac, slow and fast skeletal muscle TnTs is in the NH₂-terminal domain that is also highly variable among the alternative RNA splicing-generated isoforms. Structural variations of the NH₂-terminal region significantly alter the charge profile of TnT isoforms. NH₂-terminal alternative splicing produces high molecular weight to low molecular weight, acidic to basic, cardiac TnT isoform switches during embryonic and postnatal heart [8] and skeletal muscle [9] development.

The functional significance of the various TnT isoforms is not fully understood. Differences in the activation of actomyosin ATPase have been found between reconstituted thin filaments containing TnT isoforms with differences in the NH₂-terminal structure [10–12]. We have demonstrated that the structure of the NH₂-terminal variable region can modulate the global conformation of the TnT molecule and its binding affinities to troponin I (TnI), troponin C (TnC), and tropomyosin [13–15]. These data support a hypothesis that TnT isoform expression may play a role in modulating the contractility of different types of muscle fibers, during development and functional adaptation to physiological and pathological conditions.

Slow skeletal muscle exhibits a higher tolerance to fatiguing stimulation than that of fast skeletal muscle [16]. Mainly determined by the NH₂-terminal charge, the slow skeletal muscle TnT is more acidic in comparison to the adult fast TnT [17]. It has been demonstrated that the NH₂-terminal charge of TnT isoforms contributes to the tolerance of the thin filament regulatory system to acidosis [18]. We have shown that the durability of slow skeletal muscle correlates with the troponin isoform contents [19]. The greater fatigue resistance of slow skeletal muscle may be due to their greater tolerance to the inhibitory effects of the decrease in intracellular pH that occurs with fatigue. Therefore, the regulated expression of TnT isoforms during muscle development and physiological or pathological adaptations may contribute to the muscle's tolerance to stress.

Cardiac muscle has evolved to perform continuous contractile function similar to slow skeletal muscle. Consistently, cardiac TnT is also acidic similar to slow TnT [20]. In the present study, we characterized the effects of TnT isoforms on the tolerance of muscle to acidosis using transgenic mouse cardiac muscle expressing a fast skeletal muscle TnT. The results demonstrate that intact ventricular muscle strips and skinned trabeculae of the transgenic mice had a lower tolerance to low pH environment as compared to the control preparations from wild-type mice. The data provide new evidence that the structure and function of TnT isoforms may be a factor in determining the durability of the different types of striated muscle.

Materials and methods

Transgenic mice expressing fast TnT in the cardiac muscle

To investigate the functional differences between the cardiac and fast skeletal muscle TnTs in an integrated muscle cell environment, we have constructed transgenic mice expressing a chicken breast muscle fast skeletal muscle TnT under the control of cloned mouse cardiac α -myosin heavy chain gene promoter [21], generously provided by Dr. Jeffrey Robbins, University of Cincinnati. The construction and characterization of the transgenic mice on the C57BL/6 background have been described previously [22]. Post-natal expression of the chicken fast skeletal muscle TnT was obtained in the transgenic mouse cardiac muscle with proportional incorporation into the cardiac myofilaments and no alteration of cardiac TnI and tropomyosin contents [22]. The transgenic mouse cardiac muscle contained both cardiac and fast TnT at an approximately 50:50 ratio [23]. Cardiac muscle from wild-type C57BL/6 mice was used as controls in the contractility analysis.

Measurement of contraction of intact cardiac muscle under acidosis

Mice were euthanized by cervical dislocation. Intact cardiac muscle strip preparations were prepared as described previously [24,25]. Briefly, the heart was rapidly isolated and transferred to a dissecting dish containing a modified Ringer solution with the following composition (in mM): 142 NaCl, 4.0 KCl, 2.5 CaCl₂, 2.0 MgCl₂, 5.0 HEPES, 10 glucose, and continuously bubbled with 100% O₂ at room temperature. Butanedione-monoxime (BDM; 10mM) was added to the dissecting solution to prevent injury due to dissection and to reduce the consumption of O₂ and ATP [25–28]. Hearts were carefully washed to remove all residual blood; then, they were placed on its dorsal position and opened longitudinally. The ventricular free wall was cut into strips following the longitudinal orientation of the myocytes and mounted on a Radnoti muscle perfusion system (Radnoti, Monrovia, CA) between a force transducer and a stationary post. The output of the force transducer was digitized and stored in a computer for analysis. The resting tension and the stimulatory voltage (provided by a Grass digital stimulator) were adjusted to produce maximal isometric twitch force (F_{max}) at a frequency of 60 contractions per minute. The measurements were automated using a Powerlab/400 E series control system (ADInstruments, Mountain View, CA). The digitized force signals were electrically analyzed to yield $+dF/dt_{max}$, which was taken as an index of muscle contractility under control and test conditions.

The cardiac muscle strips were superfused at room temperature (22°C) with bathing media at physiological pH (7.41). After force stabilized for 30 min, the preparation was switched to lower pH media (7.0) to measure the changes in contractility. It is well established that decreasing extracellular pH (e.g., metabolic acidosis) decreases intracellular pH and inhibits myocardial contractility [29]. Therefore, this protocol allows measurements of contractility under normal conditions and the effects of acidosis.

Measurement of skinned fiber contractility

Transgenic and control mouse hearts were removed immediately after euthanasia and placed in a dissecting Petri dish, containing a modified Ringer solution without any added calcium. The heart was carefully washed to remove all residual blood and then placed in a dissecting solution with the following composition (in mM): 76.0 KMS, 10.0 EGTA–KOH, 1.0 MgATP, 1.0 DTT, and 50 imidazole–HCl, ionic strength 170.0, and pH 7.0. A cocktail of protease inhibitors was added (1.0 leupeptin, 0.1 PMSF, 1.0 benzamidin, and 0.01 aprotinin). Butanedione-monoxime (BDM; 10 mM) was added to the dissecting solution to prevent injury due to dissection and to reduce the consumption of O₂ and ATP [25–28]. The heart was placed on its dorsal position and opened longitudinally to expose semi-attached free trabeculae from the left ventricular wall. A trabecula was carefully dissected with a piece of the ventricular wall left on each end of the preparation. After the dissection, the trabecula was transferred to dissecting solution without BDM.

To control Ca²⁺ concentration in the vicinity of the myofilaments and directly examine the effect of pH on Ca²⁺ activation of contraction in the absence of cellular Ca²⁺ handling system, all membrane structures of the muscle cell needed to be disrupted. This was accomplished by exposing the isolated trabecula for 30 min to a skinning solution containing 1% (w/v) Triton X-100, a non-ionic detergent that permeabilizes the sarcolemmal membrane and all sub-cellular organelles. The trabeculae were used within 72 h after being skinned.

The trabecula was mounted by clamping the ends via the remaining ventricular wall tissue to a force transducer and a stationary post. The skinned trabeculae were exposed to solutions of varying Ca²⁺ concentrations (*p*Ca 8.5 to 4.0) to determine the force vs. *p*Ca relationship at pH 7.0 and 6.5. All experiments were bracketed, i.e., the force vs. *p*Ca data set was measured under control (pH 7.0) conditions, then under test (pH 6.5) conditions, and then under control (pH 7.0) again. In all trabeculae tested, the control curves generated before and after the test curve did not differ significantly, suggesting that no structural damage of the preparations

resulted from the low pH treatment. The force vs. *p*Ca relationship was analyzed as described previously [25]. Maximum calcium-activated force (F_{\max}) was recorded and normalized to the cross-sectional area of each trabecula or to the percentage of maximal force produced by each trabecula as described previously [25]. A computer program was used to fit the force vs. *p*Ca curve for each fiber to the Hill equation; i.e., $\%F_{\max} = 100 [Ca^{2+}]^n / ([Ca_{50}]^n + [Ca^{2+}]^n)$. Ca²⁺ sensitivity was evaluated from Ca₅₀ (the Ca²⁺ concentration producing half-maximal activation).

Western blotting

To verify the TnT isoform contents, the cardiac muscle of each of the transgenic and control mouse hearts used for contractility analysis was examined by Western blotting. Total protein was extracted from fresh ventricular muscle sampled next to the strips used in contractility analysis using a high-speed mechanical homogenization. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 50 mM Tris–HCl, pH 6.8, 10% glycerol, 0.1% bromophenol blue, 150 mM β-mercaptoethanol, and 1% SDS was used to extract muscle proteins to avoid proteolysis. The protein bands resolved by SDS–PAGE using 14% gel and Laemmli buffers were electrically transferred to nitrocellulose membrane using a Bio-Rad Lab semidry electrotransfer apparatus at 5 mA/cm² for 30 min. The nitrocellulose membrane was blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5). To examine the expression of TnT isoforms, the nitrocellulose membrane was incubated with a monoclonal antibody (mAb), 6B8, specific to chicken breast muscle TnT [13], and an anti-cardiac TnT mAb, CT3 [14], both were diluted in TBS containing 0.1% BSA. The expression of cardiac TnI was also examined by Western blot using a mAb against TnI (TnI-1) [30] to evaluate the expression of directly related myofibril proteins. The subsequent washing, incubation with alkaline phosphatase-labeled anti-mouse IgG second antibody (Sigma), and 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium substrate reaction were performed as described previously [13].

Data analysis

Densitometric analysis of the SDS–PAGE and Western blots used the NIH Image program, version 1.61, on images scanned at 600 dpi. The TnT molecular weight and isoelectric point (*pI*) were calculated from amino acid sequences by using programs from DNA-Star. Contractility data were analyzed by SigmaStat (Jandel) program for statistical significance. One-way ANOVA followed by Tukey's test was used to examine parametrically distributed data and Wilcoxon's signed

rank test was applied for non-parametrically distributed data.

Results and discussion

The primary structure of each muscle type-specific TnT isoform is conserved across species. In contrast, the cardiac, slow or fast skeletal muscle TnTs have significantly diverged [17]. Therefore, the differences between mouse cardiac and chicken fast skeletal muscle TnTs mainly reflect a muscle fiber type difference. Fig. 1 summarizes the structural difference between cardiac and fast skeletal muscle TnTs. Both cardiac and skeletal muscle TnTs undergo a developmentally regulated isoform switch that is an acidic to basic transition of the protein [8,9]. The developmentally regulated NH₂-terminal variable region is a predominant difference between the two TnT isoforms. One reason we chose the chicken breast muscle fast TnT for this investigation is that it is a large fast TnT isoform with a size close to that of adult cardiac TnT. While the size difference between the NH₂-terminal variable regions of the chicken breast muscle fast TnT and the adult mouse cardiac TnT is minimal, their difference in NH₂-terminal amino acid composition results in a significant difference in charge. Calculated from the amino acid sequences, the avian breast muscle fast TnT expressed in the transgenic mouse cardiac muscle has a higher isoelectric point than that of cardiac TnT (6.91 and 5.19, respectively). Similar to cardiac muscles, chicken breast muscle contains solely α -tropomyosin, unique to other fast skeletal muscles that express both α - and β -tropomyosins [31], providing comparable systems for studying the function of TnT that is the tropomyosin-binding subunit of troponin. Therefore, analyzing the effect of chicken breast muscle fast TnT on the function of cardiac muscle may provide relevant information regarding the functional significance of TnT isoforms in comparable environments. To understand the functional significance of acidic and basic TnT isoforms will help understanding TnT isoform regulation as well as the molecular mechanism of muscle contraction and adaptation under physiological and pathological conditions.

Western blots of the ventricular muscle protein extracts from each mouse heart used in contractility assays confirmed the expression of exogenous fast skeletal muscle TnT in all of the transgenic mice. The expression pattern was similar to the results of previous characterization of this transgenic mouse line [22]. As expected, the Western blots on the wild-type control mouse cardiac muscle samples detected only cardiac TnT. The expression of cardiac TnI was not different in the transgenic mouse cardiac muscle as compared with the wild-type controls, indicating a comparable background in the transgenic cardiac muscle for characterization of the functional effects of the exogenous fast TnT. Representative Western blots are shown in Fig. 2.

The stress analysis on intact muscle strips revealed differences between the effect of lowered pH on the contractility of wild-type cardiac muscle and transgenic mouse cardiac muscle containing fast skeletal muscle TnT. The results in Fig. 3 show that superfusion with pH 7.0 media that is acidic compared to normal plasma as represented by the superfusion with pH 7.4 media resulted in greater decreases in the contractility of the transgenic cardiac trabeculae than that of the wild-type control. Lowered extracellular pH is predicted to produce a correspondingly lowered intracellular pH [29]. Therefore, dropping extracellular pH from 7.4 to 7.0

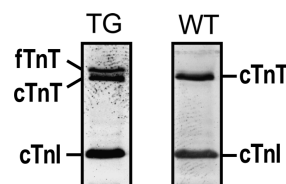


Fig. 2. Expression of fast skeletal muscle TnT in transgenic mouse cardiac muscle. Total protein extracts from transgenic (TG) and wild-type (WT) mouse hearts were analyzed by SDS-PAGE and Western blotting with a mixture of mAbs against chicken breast muscle TnT (6B8), mouse cardiac TnT (CT3), and TnI (TnI-1). The blots verified the expression of fast skeletal muscle TnT (fTnT) in the transgenic mouse cardiac muscle. The expression of endogenous cardiac TnT (cTnT) and cardiac TnI (cTnI) was not affected as compared with the normal mouse heart control. Although the calculated molecular weight of chicken fast TnT is slightly lower than those of adult mouse cardiac TnT (Fig. 1), it migrated slower than mouse cardiac TnT on the SDS-PAGE, presumably due to their amino acid compositions.

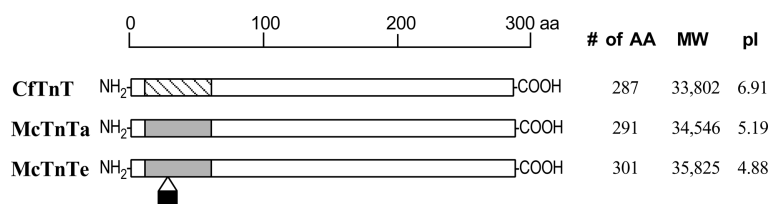


Fig. 1. N-terminus-based charge difference between cardiac and fast skeletal muscle TnTs. The primary structure maps of chicken breast muscle fast TnT (CfTnT) and mouse cardiac TnT (McTnTa, adult isoform; McTnTe, embryonic isoform) were deduced from sequence information [18,40]. The shaded region indicates the NH₂-terminal variable region. The solid box represents an acidic segment in McTnTe encoded by an alternatively spliced exon [40]. AA, amino acid; MW, molecular weight.

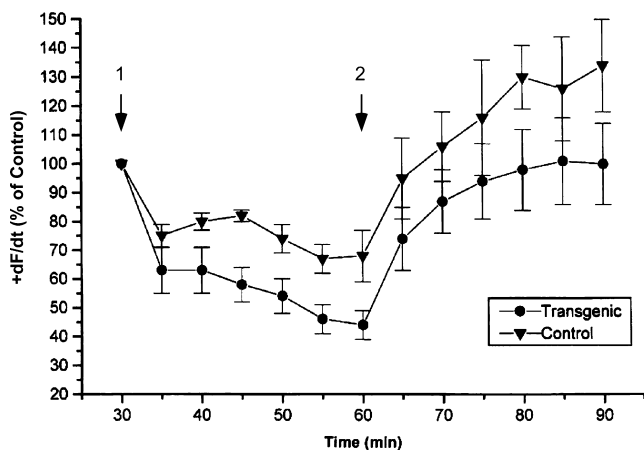


Fig. 3. Decreased tolerance of the transgenic mouse cardiac muscle to acidic pH. Contractility assays showed that the muscle strips from wild-type control hearts responded with a decreased $+dF/dt_{\max}$ after 30 min incubation in the low pH medium ($n = 3$). Cardiac muscle strips from the transgenic mice were found to be more susceptible to the same decrease in pH ($n = 4$; $P < 0.05$). The arrows indicate the time points when the muscle strip was switched from pH 7.4 bathing medium to pH 7.0 medium (arrow 1) and returned to pH 7.4 medium (arrow 2). The contractility was recovered in both wild-type and transgenic mouse cardiac muscles after returning to the physiological pH.

should lower the intracellular pH to ~ 6.5 – 6.6 . The changes in $+dF/dt_{\max}$ (Fig. 3) indicate that this approach was effective in decreasing intracellular pH. The muscle strips from control hearts responded to this decrease in pH with an average $\sim 30\%$ decrease in $+dF/dt_{\max}$ after 30 min superfusion with pH 7.0 media. In comparison, cardiac muscle strips from the transgenic mice demonstrated a higher susceptibility to the same decreased pH treatment, exhibiting an average decrease in $+dF/dt_{\max}$ of $\sim 55\%$ after 30 min superfusion with pH 7.0 media.

Because the two groups of muscle differ primarily in the TnT contents, the results indicate that the fast skeletal muscle TnT with a relatively basic isoelectric point in comparison to that of the cardiac TnT lowered the tolerance of the cardiac muscle contractile system to the acidic environmental pH (Fig. 3), supporting our hypothesis that charge differences among TnT isoforms may play significant roles in muscle contractility. This observation is in agreement with previous protein-binding studies in which the affinity of an acidic TnT to other thin filament regulatory proteins is less affected by acidic pH than that of a basic TnT isoform [18]. It is also in agreement with the previous observation of higher tolerances of embryonic versus adult cardiac muscles to acidosis [32–34], where the embryonic cardiac muscle expresses more acidic cardiac TnT (Fig. 1) than that of the adult cardiac muscle [8]. The data suggest that in cardiac muscle, the charge of different TnT isoforms mainly determined by the NH_2 -terminal structure con-

tributes to the tolerance of the contractile apparatus to environmental conditions.

Slow skeletal muscle has a significantly higher tolerance to stress conditions than fast skeletal muscle [16]. We have recently showed in a hind limb unloading rat model that the switching from slow to fast fiber contents in the soleus muscle resulted in a significant decrease in the tolerance to fatiguing stimulation [19]. A slow to fast TnT isoform switch was found in the unloaded soleus muscle, indicating a possible contribution to the functional changes. The role of TnT isoforms in the muscle's tolerance to acidosis shown in the present study further supports the contribution of TnT isoform regulation to the adaptation of muscle to stress conditions.

After returning to normal buffer at pH 7.4, the $+dF/dt_{\max}$ of cardiac muscle recovered from the depression due to acidosis (Fig. 3). The preserved contractile potential precludes significant structural damage resulting from the low pH treatment. In the later phase of recovery, control cardiac muscle strips produced higher $+dF/dt_{\max}$ as compared with the initial level. The mechanism for this overshoot remains to be investigated. The transgenic mouse cardiac muscle expressing fast TnT did not exhibit the overshoot, suggesting that the TnT isoform-based tolerance of muscle to acidosis also affects the recovery phase.

Skinned fiber experiments demonstrated that the maximum calcium-activated force and calcium sensitivity decreased with the drop in pH as expected (Table 1). Verifying the hypothesis that the presence of fast skeletal muscle TnT in the transgenic mouse cardiac muscle is partly responsible for the decreased tolerance to the decrease in environmental pH, we found that the decrease in force in the skinned transgenic cardiac muscle was greater than that of the wild-type mice (Fig. 4 and Table 1). Since the Triton treatment removed the plasma membrane and sarcoplasmic reticulum, these preparations represent the properties of myofilament proteins in the absence of the Ca^{2+} handling system. The pH 6.5 condition corresponds to the level predicted in the intact muscles superfused with pH 7.0 media, that also occurs in muscle under conditions such as ischemia, hypoxia, and fatigue.

Using the transgenic mouse cardiac muscle model, the present study demonstrates significant effects of TnT isoforms on the tolerance of muscle to acidosis. The charge variation of TnT isoforms may be an underlying mechanism for the physiological role of TnT isoform regulation in determining the tolerance of the thin filament regulatory system to pH changes [18]. The observation that the more acidic TnT isoforms confer a higher tolerance to acidosis supports the hypothesis that the alternatively spliced NH_2 -terminal domain of TnT functions as a functional tuning site for the fiber type-specific and developmentally regulated cardiac and skeletal muscle TnT isoforms [8,9]. This evidence

Table 1
Effects of pH on skinned fiber contractility

	Wild type		Transgenic	
	pH 7.0 (<i>n</i> = 3)	pH 6.5 (<i>n</i> = 4)	pH 7.0 (<i>n</i> = 4)	pH 6.5 (<i>n</i> = 4)
F_{\max} (kN/m ²)	17.1 ± 0.8	12.7 ± 2.9 ^a	16.6 ± 2.5	5.0 ± 0.8 ^{a,b}
Ca ₅₀ (μM)	2.51 ± 0.05	9.88 ± 0.025 ^a	2.16 ± 0.03	14.0 ± 0.18 ^{a,b}

Average ± SEM data for contractile properties of skinned trabecula. Ca₅₀, Ca²⁺ concentration required for generating 50% maximum force.

^a Significantly different from the pH7.0 control (*P* < 0.05).

^b Significantly different from the wild-type control (*P* < 0.05).

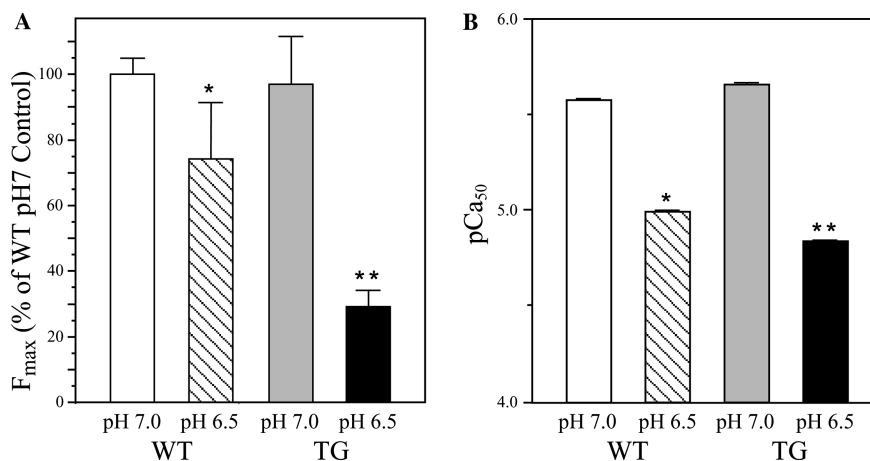


Fig. 4. Maximum force and Ca²⁺ sensitivity of Triton-skinned cardiac trabeculae from wild-type (WT) and transgenic (TG) mice. The data comparisons are shown as the means ± SEM. Contractile parameters are summarized in Table 1. (A) The maximum calcium-activated force (F_{\max} , shown as percentage of the WT control at pH 7.0) and (B) calcium sensitivity (shown as pCa₅₀) decreased with the drop in pH in both control and test muscles. However, the decreases in the transgenic cardiac muscle were greater than that of the wild-type mice. **P* < 0.05 compared with the data at pH 7.0; ***P* < 0.05 compared with the data at pH 7.0 as well as that of the WT cardiac muscle at pH 6.5.

also supports the hypothesis that the changes in fast and slow TnT isoform expression as well as the proportion of acidic and basic fast TnT isoforms in different muscle fibers may be a molecular mechanism in adaptation to physiological or pathological stresses [20]. The negative effect of the relatively more basic fast TnT on the tolerance of cardiac muscle to low pH further suggests a negative effect of the low molecular weight cardiac TnT that is relatively basic than the normal adult cardiac TnT expressed in failing human heart [35,36].

In addition to the charge difference, cardiac and fast TnTs do have other fine structural differences that may affect the function of TnT and contribute to the tolerance of myofilaments to environmental pH changes. The compatibility of thin filament function to changes in cellular environment may involve complex interactions among the regulatory proteins. Several previous studies have shown that TnI isoforms also contribute to the pH compatibility of striated muscle [37–39]. While transgenic expression of slow TnI in mouse cardiac muscle resulted in a higher tolerance of force generation to acidosis [38], our evidence that TnT isoform contents affect the pH tolerance of both F_{\max} and pCa₅₀ (Fig. 4) demonstrates another determinant in the thin filament

with a broader role in regulating the pH sensitivity of muscle. The data that both TnT and TnI isoforms contribute to the tuning of contractility are in agreement with the fact that the troponin complex functions as an allosteric machinery in the regulation and functional adaptation of striated muscle.

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