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Roles of Troponin Isoforms in pH Dependence of Contraction in Rabbit Fast and Slow Skeletal and Cardiac Muscles[1]

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Skinned fibers prepared from rabbit fast and slow skeletal and cardiac muscles showed acidotic depression of the Ca^{2+} sensitivity of force generation, in which the magnitude depends on muscle type in the order of cardiac > fast skeletal > slow skeletal. Using a method that displaces whole troponin-complex in myofibrils with excess troponin T, the roles of Tn subunits in the differential pH dependence of the Ca^{2+} sensitivity of striated muscle were investigated by exchanging endogenous troponin I and troponin C in rabbit skinned cardiac muscle fibres with all possible combinations of the corresponding isoforms expressed in rabbit fast and slow skeletal and cardiac muscles. In fibers exchanged with fast skeletal or cardiac troponin I, cardiac troponin C confers a higher sensitivity to acidic pH on the Ca^{2+} sensitive force generation than fast skeletal troponin C independently of the isoform of troponin I present. On the other hand, fibres exchanged with slow skeletal troponin I exhibit the highest resistance to acidic pH in combination with either isoform of troponin C. These results indicate that troponin C is a determinant of the differential pH sensitivity of fast skeletal and cardiac muscles, while troponin I is a determinant of the pH sensitivity of slow skeletal muscle.

Key words: acidosis, calcium sensitivity, isoform, muscle contraction, troponin.

Contraction of vertebrate striated muscles, *i.e.* skeletal (fast and slow) and cardiac muscles, is regulated by specific regulatory protein complexes, troponin (Tn) and tropomyosin (TM), distributed at regular intervals along the entire thin filament (*1, 2*). Tn is a complex of three different proteins, troponin T (TnT; TM-binding component), troponin I (TnI; inhibitory component), and troponin C (TnC; Ca^{2+}-binding component). Upon Ca^{2+} binding to TnC, a Ca^{2+}-sensitive interaction between TnC and TnI dis-inhibits the action of TnI on the thin filament and enables the force-generating interaction between actin and myosin. The Ca^{2+} sensitivity of contraction is thus determined by the Ca^{2+} affinity of TnC, which is dynamically altered through the interaction with TnI in the myofilament lattice (*3-6*).

Protons, which are believed to contribute to the inhibited contractility during skeletal muscle fatigue and myocardial ischemia, have a profound depressant effect on the Ca^{2+} sensitivity of skinned fibres prepared from vertebrate striated muscles depending on muscle type in the order of cardiac > fast skeletal > slow skeletal (*7-9*). Recent studies indicate that the muscle-type dependent effect of acidic pH on the Ca^{2+} sensitivity is due to the different isoform expressions of Tn components in these muscles (*10, 11*). The ectopic expression of the fast skeletal isoform of TnC in the heart of transgenic mice has been shown to reduce significantly the depressant effect of acidic pH on the Ca^{2+}-sensitivity of cardiac muscle (*10*), indicating that TnC plays an important role in determining the sensitivity of cardiac muscle to acidic pH. Metzger *et al.* (*10*) also showed that in fast skeletal skinned fibres, the extraction of endogenous TnC and reconstitution with purified cardiac TnC reciprocally increase the depressant effect of acidic pH on Ca^{2+}-sensitivity. The depressant effect of acidic pH on the Ca^{2+}-sensitivity of cardiac muscle is known to be significantly less in the neonatal heart than in the adult heart (*12, 13*), and this phenomenon has been shown to be closely associated with the developmental expression of the slow skeletal isoform of TnI in the heart during the fetal and early neonatal periods (*11*). Using techniques for TnI and/or TnC exchange in myofibrils, the contribution of the fast skeletal and cardiac isoforms of TnI and TnC to the pH dependence of Ca^{2+} sensitivity have been studied in both fast skeletal and cardiac muscles (*14, 15*). Ding *et al.* (*15*) showed that while the replacement of both TnI and TnC with fast skeletal isoforms within cardiac muscle modifies the pH dependence of Ca^{2+} sensitivity from the cardiac to fast skeletal type, the replacement of TnC alone with the fast skeletal isoform alters the pH dependence marginally. They concluded that cardiac TnI plays a dominant role in determining the cardiac type pH dependence. Ball *et al.*

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Abbreviations: CDTA, *trans*-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pCa, $-\log [Ca^{2+}]$; TM, tropomyosin; Tn, troponin.

(*14*), however, showed that while the replacement of both TnI and TnC with cardiac isoforms within the fast skeletal muscle modifies the pH dependence of the Ca^{2+} sensitivity from fast skeletal to cardiac type and the replacement of TnC alone with the cardiac isoform increases the pH sensitivity moderately, the replacement of TnI alone with the cardiac isoform does not alter the pH dependence, a finding in conflict with the idea proposed by Ding *et al.* (*15*) that the cardiac TnI isoform plays a dominant role in the cardiac type pH effect. Ball *et al.* (*14*) proposed that isoform-specific interactions between TnI and TnC play an important role in the differential pH sensitivity of fast skeletal and cardiac muscles.

In the present study, we address these uncertainties regarding the contributions of the TnI and TnC isoforms to the differential pH dependence of Ca^{2+} sensitivity in striated muscles, by exchanging endogenous TnI and TnC in cardiac skinned fibres with all possible combinations of the corresponding isoforms prepared from fast and slow skeletal and cardiac muscles. The results provide evidence that, unlike the findings of early studies (*14, 15*), the difference in the pH sensitivities of the fast skeletal and cardiac muscles depends solely on the TnC isoforms and also demonstrate that the resistance of slow skeletal muscle to acidic pH is a manifestation of the unique properties of the slow skeletal TnI isoform.

MATERIALS AND METHODS

Preparations of Skinned Fibers—Male albino rabbits were anaesthetized with sodium pentobarbital (40 mg kg^{-1}, i.v.) and killed by bleeding. The skinned fibres were prepared according to a modification of the procedure for disrupting the sarcolemma and sarcoplasmic reticulum of the psoas muscle described by Diffee *et al.* (*16*). Small bundles (0.5-1 mm wide and 5-7 mm long) were dissected from the back muscle and the left ventricular trabeculae of young male albino rabbits (~2 kg) and from the soleus muscle of an adult male albino rabbit (~4 kg). The bundles tied to glass capillary tubes were skinned with relaxing solution containing 0.5% (w/v) Brij-58 for 30 min at 25°C and stored for up to 3 weeks at −20°C in relaxing solution containing 50% (v/v) glycerol.

Preparation of Troponin Subunits—Fast skeletal, slow skeletal, and cardiac Tns were prepared from the back muscle, the soleus muscle, and the left ventricular myocardium of young male albino rabbits (~2 kg) according to the methods of Ebashi (*17*), Kohama (*18*), and Tsukui and Ebashi (*19*), respectively. The Tn subunits were separated in the presence of 6 M urea, 5 mM *trans*-1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid (CDTA), 15 mM 2-mercaptoethanol and with a linear gradient of 0-0.5 M NaCl using FPLC ion exchange columns, RESOURCE S and Q (6 ml) (Pharmacia Biotech) equilibrated with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH (pH 6.0) and 10 mM Tris-HCl (pH 8.0), respectively. The separated Tn subunits were further purified by Mono Q and S HR 5/5 (Pharmacia Biotech). TnC was dialyzed against 1 mM $NaHCO_3$ and 0.5 mM DTT. TnI and TnT were first dialyzed against 0.4 M KCl, 50 mM KH_2PO_4/KOH (pH 7.0), 15 mM 2-mercaptoethanol and then against 0.4 M KCl, 2 mM $NaHCO_3$, 15 mM 2-mercaptoethanol. The dialyzed proteins were then divided into aliquots and stored at −80°C. The purity of the Tn subunit isoforms obtained is shown in Fig. 1.

SDS-PAGE—SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (*20*) with 4% stacking and 12% separating gels. The gels were stained with Coomassie Brilliant Blue R-250 or silver using a staining kit (Pharmacia Biotech), and an optical densitometric scan was made using Phoretix gel analysis software (Phoretix International) calibrated by a photographic step tablet (21 steps·density range 0.05-3.03, Eastman Kodak).

Troponin Subunit Exchange in Skinned Fibers—TnI and TnC were extracted from the rabbit skinned cardiac muscle fibers by replacing the endogenous Tn-complex with exogenously added TnT by the method of Hatakenaka and Ohtsuki (*21, 22*) with slight modifications. The rabbit cardiac muscle fibers mounted to a mechanical apparatus were bathed at 25°C with continuous stirring in a solution of 50 mM MES/KOH (pH 6.0), 250 mM KCl, 4 mM EDTA, 0.5 mM DTT, and 1 mg/ml purified rabbit cardiac TnT for 60-min. The extracted fibres were then reconstituted with various isoforms of TnI and TnC at 25°C in relaxing solutions containing 0.5 mg/ml of each protein for 40 min.

Force Measurements—A small fiber (~120 μm in diameter) dissected from a bundle of skinned trabecula or a single fiber isolated from a bundle of skinned back muscle fibres or soleus fibers was mounted in a thermostatically controlled chamber with a capacity of 0.2 ml; both ends were attached to stainless steel wire hooks as described by Brandt *et al.* (*23*) and then further fixed with a fast-setting glue. The fiber length between the hooks was about 1 mm. The resting sarcomere lengths were set at 2.3-2.4 μm using laser diffraction. The force was measured with a strain gauge (UL-2GR, Minebea). The relaxing solution consisted of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)/KOH (pH 7.0), 100 mM KCl, 6 mM $MgCl_2$, 5 mM Na_2ATP, 4 mM EGTA, 0.5 mM dithiothreitol, and 10 mM creatine phosphate, as well as 35 units/ml creatine kinase. Activating solutions with the desired free Ca^{2+} concentrations at

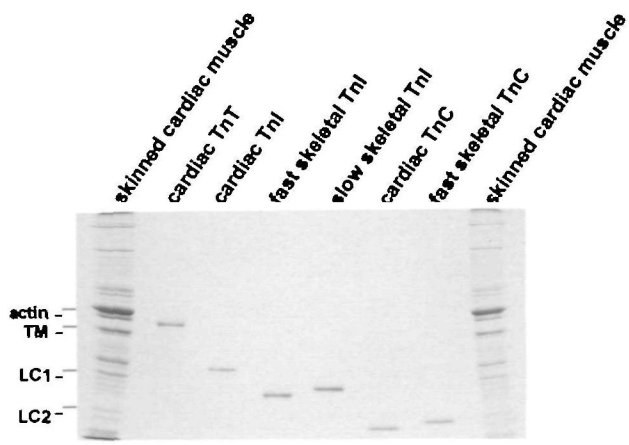


Fig. 1. **SDS-PAGE of various troponin components purified from cardiac and fast and slow skeletal muscles.** Cardiac TnT and all isoforms of TnI and TnC were prepared from rabbit muscles. Electrophoresis was performed as described in "MATERIALS AND METHODS." The gel was stained with Coomassie Brilliant Blue R-250. TM, tropomyosin; LC1 and 2, myosin light chains 1 and 2, respectively.

pHs 7.0, 6.5, and 6.2 were prepared by adding appropriate amounts of $CaCl_2$, calculated as described previously (*24*), to the relaxing solution containing 50 mM MOPS as a buffer at pH 7.0 and 6.5 or 50 mM MES at pH 6.2. All force measurements were performed at 25°C. The force-pCa ($-\log[Ca^{2+}]$) relationships were sequentially determined at pH 7.0, 6.5, 6.2, and 7.0 using the same fiber by cumulatively increasing the $[Ca^{2+}]$ level at each pH, and the responses at pH 7.0 were averaged; falls in the maximum force at pH 7.0 were 10.4 ± 2.9, 7.6 ± 2.9, and 9.3 ± 1.1% (means$\pm$SE) in cardiac, fast skeletal, and slow skeletal muscle fibres, respectively, and were no more than 10% in fibres reconstituted with various isoforms of TnI and TnC, with no significant changes in the Ca^{2+} sensitivity being observed. The midpoint (pCa_{50}) and Hill coefficient (n_H) of the force-pCa relationships were determined by fitting the data to the following form of the Hill equation by means of the Marquardt nonlinear least-squares method:

$$\text{relative force } (\%) = 100/\{1+10^{(pCa-pCa_{50})\,n_H}\}.$$

Statistical Analysis—All values are expressed as means$\pm$SE. The statistical significance of the differences between multiple groups were determined by one-way analysis of variance (ANOVA) followed by a *post-hoc* test (Newnan-Keuls multiple-comparison test) of all pairs of groups. Two-way ANOVA was used to determine how a response (*i.e.*, pH sensitivity) was affected by two different factors, TnI and TnC. When no interaction between the two factors was indicated by two-way ANOVA, the Newnan-Keuls multiple-comparison test was done on a factor with a significant effect to determine which variable in the factor (*i.e.*, isoform) has a significant effect. When an interaction between the two factors was indicated by two-way ANOVA, all combinations of two factors were compared by one-way ANOVA followed by the *post-hoc* Newnan-Keuls multiple-comparison test. P values less than 0.05 were considered significant.

RESULTS

Effect of Acidic pH on Ca^{2+}-Induced Force Generation in Cardiac, Fast Skeletal, and Slow Skeletal Muscles—Figure 2A compares the force-pCa relationships determined at pH 7.0, 6.5, and 6.2 in skinned fibers prepared from cardiac, fast skeletal, and slow skeletal muscles. At pH 7.0, the Ca^{2+} sensitivity evaluated by the half-maximally activating pCa (pCa_{50}) was the highest (6.16 ± 0.04, $n=5$) in slow skeletal muscle, intermediate (5.91 ± 0.03, $n=5$) in fast skeletal muscle, and the lowest (5.83 ± 0.03, $n=6$) in cardiac muscle ($p<0.01$, one-way ANOVA followed by Newnan-Keuls multiple-comparison test). As demonstrated by the rightward shifts in the force-pCa relationships, the acidic

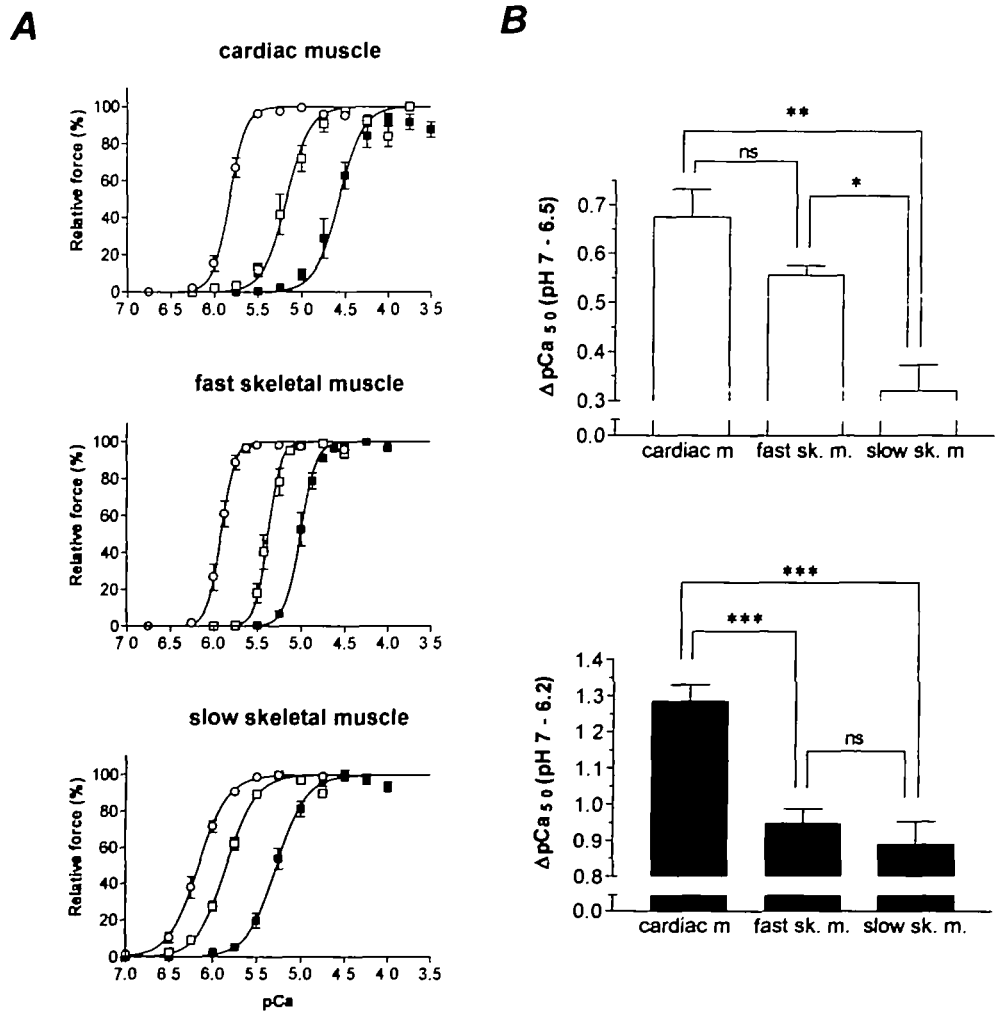


Fig. 2. **Effects of pH on force generation in rabbit skinned cardiac, fast skeletal, and slow skeletal muscle fibres.** A: Force-pCa relationships of skinned cardiac muscle (left ventricular trabeculae), fast skeletal muscle (back muscle), and slow skeletal muscle (soleus muscle), determined at pH 7.0 (○), 6.5 (◐), and 6.2 (●) as described in "MATERIALS AND METHODS." The data represent the means$\pm$SE of measurements on 6 cardiac, 5 fast skeletal, and 5 slow skeletal muscle fibres. The force generated by the individual fibres was normalized to the maximal force at each pH. B: Effects of acidic pH on the Ca^{2+} sensitivities in skinned cardiac, fast skeletal, and slow skeletal muscle fibres. The changes in Ca^{2+} sensitivity (ΔpCa_{50}) with the pH decreasing from 7.0 to 6.5 and 6.2 were calculated by fitting the force-pCa relationship data of individual fibers summarized in panel A to the Hill equation and expressed as means$\pm$SE.*,**,*** Significantly different at $p<0.05$, $p<0.01$, $p<0.001$, respectively (Newnan-Keuls multiple comparison *post-hoc* test). ns, not significant.

pH decreased the Ca^{2+} sensitivity in all types of muscles. The decrease in the Ca^{2+} sensitivity (ΔpCa_{50}) was the smallest in slow skeletal muscle when the pH decreased from 7.0 to 6.5, while no significant difference in ΔpCa_{50} was detected between the cardiac and fast skeletal muscles (Fig. 2B). On the other hand, cardiac muscle showed the largest ΔpCa_{50} when the pH decreased from 7.0 to 6.2, while the difference between the fast and slow skeletal muscles was not significant for this pH interval (Fig. 2B). These results indicate that the magnitude of acidotic depression of Ca^{2+} sensitivity depends on the muscle type in the order of cardiac > fast skeletal > slow skeletal as originally proposed by Donaldson *et al.* (*7*). Acidic pH also decreased the maximum force in all types of muscles, but no significant differences were found for these three types of muscles (Fig. 3).

Troponin Subunit Exchange in Skinned Cardiac Muscle Fibres—It has been demonstrated that the endogenous TnT·I·C-complex in skinned fibres is displaced by exogenous TnT when added in excess amounts under acidic and high ionic strength conditions (*21, 22, 25*). Figure 4 shows the effects of excess TnT treatment followed by incubation with TnI and TnC on the contractile response to Ca^{2+} in skinned cardiac fibres. After treatment with excess amounts of purified cardiac TnT, the Ca^{2+} sensitivity in

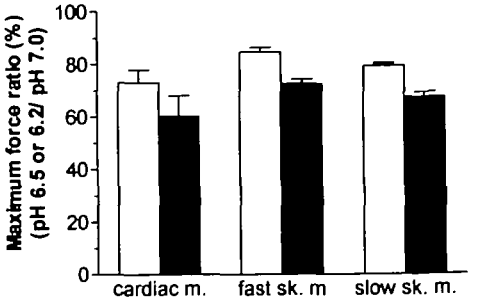
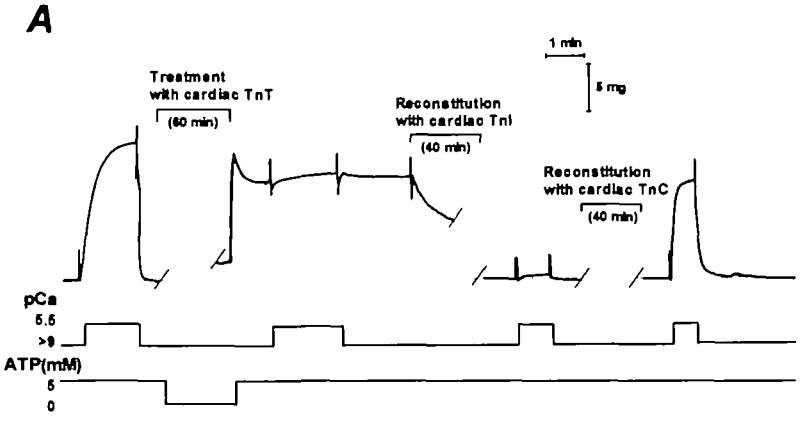
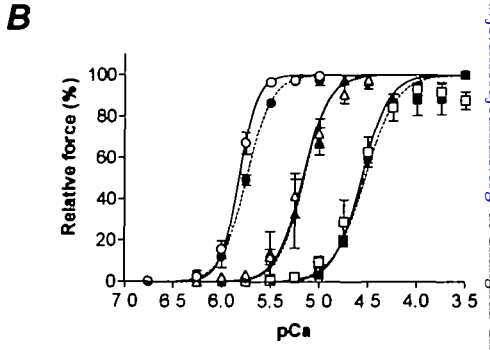
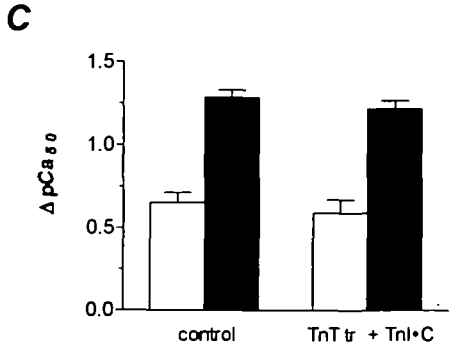


Fig. 3. **Effects of acidic pH on the maximum force in skinned cardiac, fast skeletal, and slow skeletal muscles.** The bars represent the mean values (±SE) of the maximum force generated by the fibers in Fig. 2 at pH 6.5 (□) and 6.2 (■) normalized to the maximum force at pH 7.0 in the same fiber. One-way ANOVA indicated no statistically significant differences among cardiac, fast skeletal, and slow skeletal muscles at either pH.







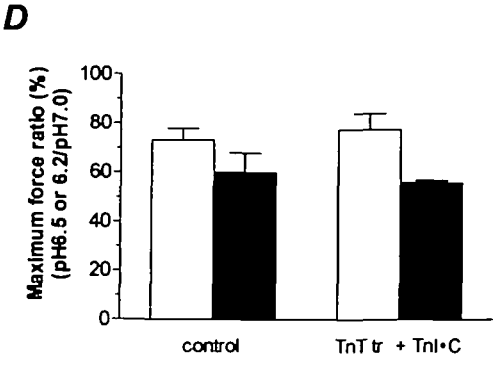


Fig. 4. Treatment of rabbit skinned cardiac muscle fibers with excess rabbit cardiac TnT and reconstitution with rabbit cardiac TnI and TnC. A: Force record showing the changes in the contractile response to Ca^{2+} in one skinned cardiac muscle fiber. B: Force-pCa relationships determined at pH 7.0 (○, ●), 6.5 (△, ▲), and 6.2 (□, ■) in skinned cardiac muscle fibres before (open symbols) and after (closed symbols) treatment with cardiac TnT and reconstitution with cardiac TnI and TnC. The data represent the means±SE of measurements on 3 fibers. The force generated by the individual fibers was normalized to the maximal force at each pH. C: Effects of acidic pH on the Ca^{2+} sensitivities in skinned cardiac muscle fibres before (control) and after treatment with cardiac TnT and reconstitution with cardiac TnI and TnC (TnT tr.+TnI·C). The ΔpCa_{50} values at the pH decreases from 7.0 to 6.5 (□) and 6.2 (■) were calculated by fitting the force-pCa relationship data of individual fibres summarized in panel B to the Hill equation. Date are expressed as means±SE. D: Effects of acidic pH on the maximum force in skinned cardiac muscle fibres before (control) and after treatment with cardiac TnT and reconstitution with cardiac TnI and TnC (TnT tr.+TnI·C). The maximum force generated by the fibres in panel B at pH 6.5 (□) and 6.2 (■) were normalized to the maximum force at pH 7.0 in each fiber and are expressed as means±SE. The paired *t*-test indicated no statistically significant differences in the ΔpCa_{50} values (C) or the maximum force ratios (D) at either pH interval between the control and reconstituted fibers.

force development was completely lost due to a marked inhibition of relaxation in the absence of Ca^{2+} (Fig. 4A). SDS-PAGE analysis demonstrated that the two subunits of the Tn-complex, TnI and TnC, in skinned fibres was almost completely lost after TnT treatment (Fig. 5, lane b). After incubation with purified cardiac TnI, the force development was inhibited regardless of Ca^{2+} concentration, while the Ca^{2+}-sensitive force development was restored by subsequent incubation with purified cardiac TnC (Fig. 4A). These observations indicate that endogenous TnI and TnC were almost completely removed after TnT treatment as a consequence of the displacement of endogenous Tn-complexes by the added TnT. SDS-PAGE analysis indicated that the reincorporation of TnI and TnC after subsequent incubation with purified cardiac TnI and TnC was nearly stoichiometric (Fig. 5, lane c).

The level of maximum force at pH 7.0 after TnT treatment followed by reconstitution with cardiac TnI and TnC was an average of 60% of the pre-TnT treatment value (Fig. 4A), indicating that this procedure had some non-specific deteriorative effect on the maximal force-generating capability. This is probably due to a structural disorder in the myofilament lattice of skinned fibres that could occur under the acidic and high-ionic-strength conditions used for TnT treatment. Nevertheless, the normalized force-pCa relationships in the treated fibers at pH 7.0, 6.5, and 6.0 were almost superimposable on those determined before TnT treatment (Fig. 4B), and no significant differences in the acidic pH-induced decreases in Ca^{2+} sensitivity (ΔpCa_{50}) and maximum force were detected between pre-treated and treated fibres (Fig. 4, C and D). These results indicate that the treatment does not alter the normal myofilament

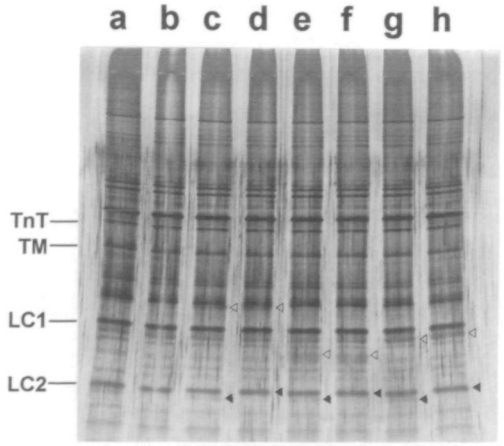


Fig. 5. **SDS-PAGE of skinned rabbit cardiac muscle fibres after treatment with rabbit cardiac TnT and after subsequent reconstitution with various isoforms of TnI and TnC prepared from rabbit muscles.** Lane a, an untreated control fiber. Lane b, a fiber treated with cardiac TnT. Lane c, a fiber reconstituted with cardiac TnI and cardiac TnC. Lane d, a fiber reconstituted with cardiac TnI and fast TnC. Lane e, a fiber reconstituted with fast TnI and cardiac TnC. Lane f, a fiber reconstituted with fast TnI and fast TnC. Lane g, a fiber reconstituted with slow TnI and cardiac TnC. Lane h, a fiber reconstituted with slow TnI and fast TnC. The positions of TnI and TnC reconstituted into fibers in lanes c-h are indicated by open (<) and closed (◂) triangles, respectively. Electrophoresis was performed as described in "MATERIALS AND METHODS" and the gel was stained with silver. LC1 and LC2, myosin light chains 1 and 2, respectively.

functions reflected in these parameters and that the displacement of endogenous Tn-complex in the skinned fibres due to excess TnT and subsequent reconstitution with TnI and TnC is an effective approach for investigating the role of troponin isoform subunits in the differential pH dependence of contraction in vertebrate striated muscle contraction.

Figure 5 shows an SDS-PAGE analysis of protein content in skinned cardiac muscle fibers treated with cardiac TnT and reconstituted with six possible combinations of all isoforms of TnI and TnC expressed in vertebrate striated muscles. The TnI/TM ratios estimated by gel scan were 0.49 and 0.05 in untreated control (lane a) and TnT-treated (lane b) fibers, respectively, and the TnC/TM ratios in these fibers were 0.21 and 0, respectively, indicating that more than 90% of endogenous TnI and TnC in the skinned fiber was removed by TnT treatment. Densitometric scans of the fibers reconstituted with various isoforms of TnI and TnC (lanes c-h) indicated TnI/TM ratios of 0.48, 0.48, 0.45, 0.43, 0.38, and 0.40 in lanes c, d, e, f, g, and h, with TnC/TM ratios in these lanes of 0.22, 0.22, 0.21, 0.21, 0.22, and 0.22, respectively; note that slow TnI co-migrated with an unknown band (lanes g and h) and fast TnC co-migrated with myosin light chain 2 (LC2) (lanes d, f, and h) so that the ratios of slow TnI and fast TnC to TM were estimated from the increased intensities of these bands. These results indicate that all isoforms of TnI and TnC were exchanged nearly stoichiometrically into the skinned fibres. Figure 6 shows the force-pCa relationships determined at pH 7.0, 6.5, and 6.2 in these skinned cardiac muscle fibres. At pH 7.0, consistent with the differential Ca^{2+} sensitivity observed in cardiac, fast skeletal, and slow skeletal muscles (Fig. 2A), the native combination in fast skeletal muscle (*i.e.*, fast TnI+fast TnC) conferred a higher Ca^{2+} sensitivity ($pCa_{50}=5.89\pm0.02$, $n=5$) than the native combination in cardiac muscle (cardiac TnI+cardiac TnC) ($pCa_{50}=5.75\pm0.02$, $n=3$), and the native combination in slow skeletal muscle (slow TnI+cardiac TnC) conferred a higher Ca^{2+} sensitivity ($pCa_{50}=5.99\pm0.05$, $n=4$) than the native combinations in either cardiac or fast skeletal muscles ($p<0.05$, one-way ANOVA followed by Newnan-Keuls multiple-comparison test). This provides strong evidence that the differential Ca^{2+} sensitivity in the three types of muscle is determined by the TnI and TnC isoforms and that the TnI and TnC isoforms have effectively been substituted into the skinned cardiac muscle fibers. No significant differences in the acidic pH-induced decrease in the maximum force were found among these reconstituted fibers (Fig. 7), suggesting that the effect of pH on the maximum force is determined independently of the TnI and TnC isoforms.

To determine how pH sensitivity is affected by two different factors (*i.e.*, TnI and TnC), the extents of the decrease in Ca^{2+} sensitivity (ΔpCa_{50}s) for all combinations of TnI and TnC isoforms were compared by two-way ANOVA (Fig. 8). When the pH decreased from 7.0 to 6.5, this test showed no interaction between the two factors and that the effect of the TnC isoform on ΔpCa_{50} is not significant, while the effect of the TnI isoform is very significant ($p=0.004$), indicating that the TnI isoform affects the pH sensitivity independently of the TnC isoform (Fig. 8A). Thus, all combinations of the three groups categorized by the TnI isoform were then compared by Newnan-Keuls

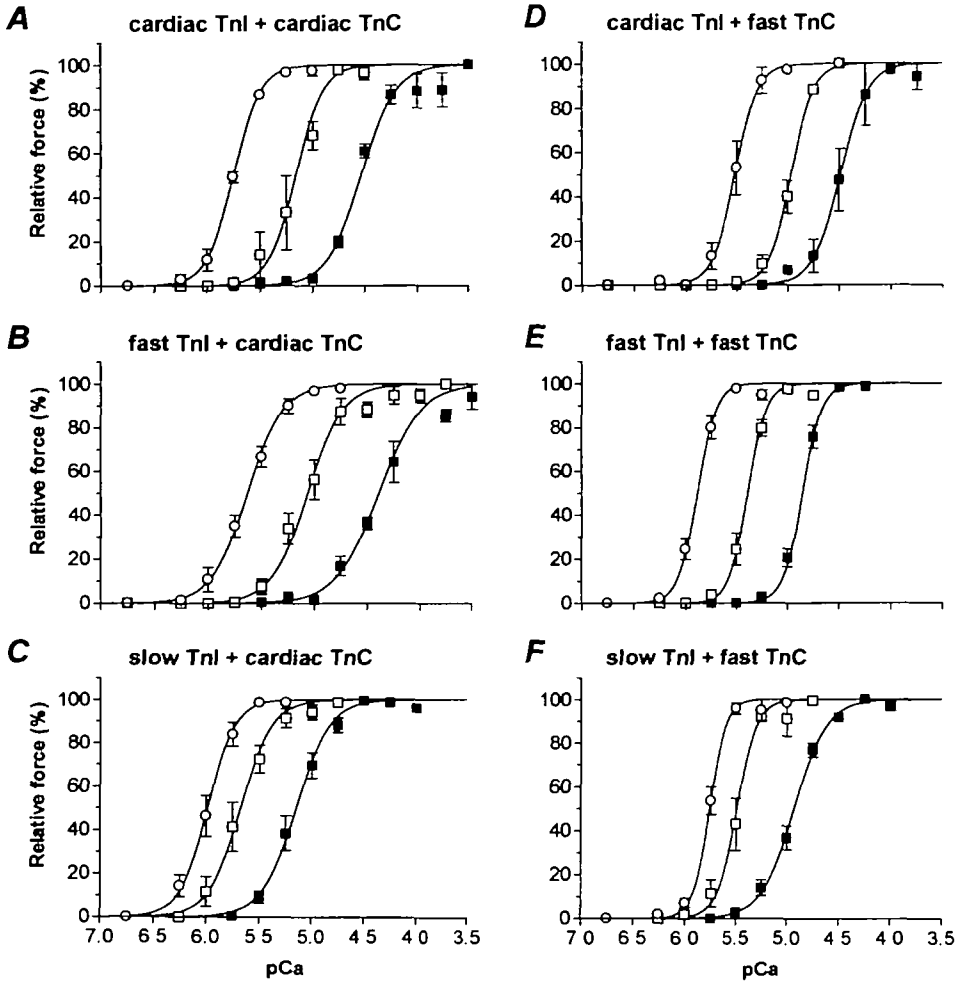


Fig. 6. **Effects of pH on the force-pCa relationships in skinned cardiac muscle fibres reconstituted with various combinations of TnI and TnC isoforms.** Skinned cardiac muscle fibres were treated with excess cardiac TnT and reconstituted with various isoforms of TnI and TnC prepared from fast and slow skeletal and cardiac muscles as shown in Fig. 4A, and the force-pCa relationships were sequentially determined at pH 7.0 (○), 6.5 (□), and 6.2 (■) using the same fibers as described in "MATERIALS AND METHODS." The data represent the means±SE. A, reconstitution with cardiac TnI and cardiac TnC ($n=3$); B, reconstitution with fast skeletal TnI and cardiac TnC ($n=5$); C, reconstitution with slow skeletal TnI and cardiac TnC ($n=4$); D, reconstitution with cardiac TnI and fast skeletal TnC ($n=3$); E, reconstitution with fast skeletal TnI and fast skeletal TnC ($n=5$); F, reconstitution with slow skeletal TnI and fast skeletal TnC ($n=3$). The force generated by each individual fiber was normalized to the maximal force at each pH. The levels of maximum force at pH 7.0 after TnT treatment followed by reconstitution with various TnI and TnC isoforms were 50-60% of the pre-TnT treatment value.

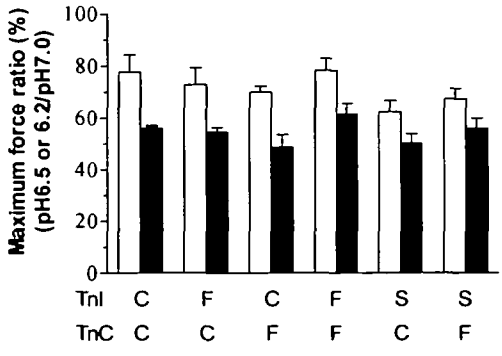


Fig. 7. **Effects of acidic pH on the maximum force in skinned cardiac muscle fibres reconstituted with various combinations of TnI and TnC isoforms.** The bars represent the mean values (±SE) of the maximum force generated by the fibres in Fig. 6 at pH 6.5 ( ) and 6.2 (■) normalized to the maximum force at pH 7.0 in the same fiber; C, cardiac; F, fast skeletal; S, slow skeletal. One-way ANOVA indicated no statistically significant differences among these six fiber groups at either pH.

multiple-comparison test to determine which TnI isoform has a significant effect on ΔpCa_{50} (Fig 8B). This *post-hoc* test revealed that slow skeletal TnI confers a significantly lower ΔpCa_{50} than cardiac and fast skeletal TnIs, while cardiac and fast skeletal TnIs do not confer significantly different ΔpCa_{50}s. When the pH decreased from 7.0 to 6.2, two-way ANOVA indicated a significant interaction between the two factors and that the effects of the TnI and TnC isoforms on ΔpCa_{50} are both significant ($p<0.0001$ and $p=0.006$, respectively) (Fig. 8C). Since it is difficult to interpret the effects of the two factors separately when a significant interaction is indicated by two-way ANOVA, the ΔpCa_{50}s for all combinations of TnI and TnC isoforms were then compared by one-way ANOVA. This test demonstrated that there is a significant difference in ΔpCa_{50}s among the six combinations. The *post-hoc* Newnan-Keuls multiple-comparison test of all pairs revealed that the slow skeletal TnI confers the lowest ΔpCa_{50} irrespective of the TnC isoform, as in the case of the pH being decreased from 7.0 to 6.5, while the cardiac and fast skeletal TnIs both confer a higher ΔpCa_{50} when combined with cardiac TnC than with fast skeletal TnC (Fig. 8C). Furthermore, two-way ANOVA restricted to the four combinations of cardiac and fast skeletal isoforms of TnI and TnC at this pH interval showed no significant interaction between the two factors and that the effect of the TnI isoform on ΔpCa_{50} is not significant while the effect of TnC isoform is extremely significant ($p=0.0005$), indicating that the TnC isoform determines the pH sensitivity independently of the TnI isoform in these combinations. These results indicate that

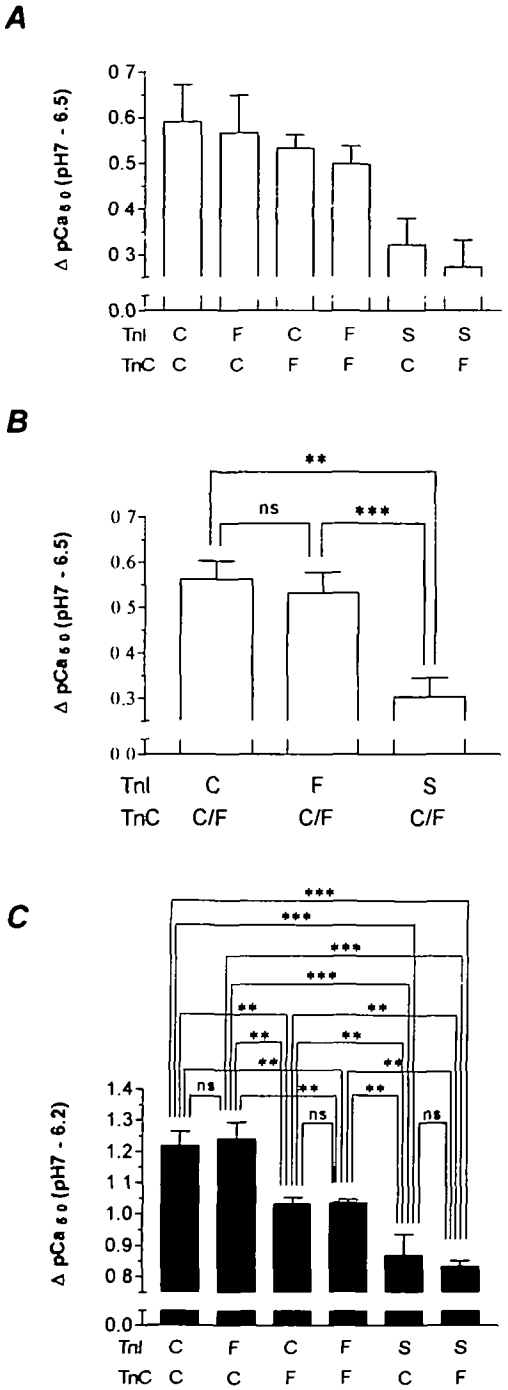


Fig. 8. **Effects of acidic pH on the Ca^{2+} sensitivities of skinned cardiac fibers reconstituted with various combinations of TnI and TnC isoforms.** The mean changes (±SE) in Ca^{2+} sensitivity with the pH decreasing from 7.0 to 6.5 (A and B) or 6.2 (C) were calculated by fitting the force-pCa relationship data of individual fibres summarized in Fig. 6 to the Hill equation; C, cardiac; F, fast skeletal; S, slow skeletal. All pairs of columns in panels B and C were compared by Newnan-Keuls multiple-comparison test. *,**,***Significantly different at $p<0.05$, $p<0.01$, $p<0.001$, respectively. ns, not significant.

the highest resistibility of the Ca^{2+} sensitivity to acidic pH in slow skeletal muscle is conferred by its TnI isoform and that the differences in the pH sensitivities of cardiac and fast skeletal muscles are conferred by the TnC isoforms.

DISCUSSION

The vertebrate striated muscles express three distinct isoforms of TnI, *i.e.*, fast and slow skeletal and cardiac TnIs, and two distinct isoforms of TnC, *i.e.*, fast skeletal and cardiac (or slow skeletal) TnCs. In the present study, we investigated the roles of TnI and TnC isoforms in the differential pH dependence of Ca^{2+} sensitivity in striated muscles by exchanging the endogenous TnI and TnC in cardiac skinned fibres with all possible combinations of the corresponding isoforms expressed in vertebrate striated muscles, since comparisons made between a limited combination may lead to conclusions of only limited validity. We found that the slow skeletal isoform of TnI confers the highest resistibility to acidic pH irrespective of the TnC isoform present. On the other hand, the fast skeletal and cardiac isoforms of TnI were found to be equivalent in terms of their contributions to pH sensitivity and always confer a higher acidic pH sensitivity in combination with cardiac TnC than in combination with fast skeletal TnC.

There are two apparently conflicting hypotheses regarding the roles of TnI and TnC isoforms in specifying the differential pH effects on the Ca^{2+} sensitivities of fast skeletal and cardiac muscles. Ball *et al.* (*14*) proposed that the individual isoforms of TnI and TnC may play no specific roles in determining the pH sensitivity, and that the differential pH sensitivities in these muscles are determined only by the isoform-specific interactions between TnI and TnC. On the other hand, Ding *et al.* (*15*) proposed that TnI and TnC independently contribute to the pH sensitivity and that TnI plays a dominant role in the differential pH effects on the Ca^{2+} sensitivities of fast skeletal and cardiac muscles.

Ball *et al.* (*14*) determined the contributions of TnI and TnC to the pH sensitivity by altering their isoform populations in fast skeletal myofibrils by the same procedure for TnI and/or TnC subunit replacement used in the present study. They found that while the substitution of cardiac TnI alone into fast skeletal myofibrils did not alter the pH dependence of the Ca^{2+} sensitive myofibrillar ATPase activity, substitution of cardiac TnC alone increased the depressant effect of acidic pH. The pH sensitivity conferred by substitution of TnC alone, however, was not as great as that of native cardiac myofibrils. The substitution of both TnI and TnC further increased the depressant effect of acidic pH, thus conferring a pH sensitivity similar to that of cardiac myofibrils. Their results indicate that fast skeletal and cardiac TnIs are equivalent in terms of their contributions to the pH sensitivity when combined with fast skeletal TnC, consistent with the results of the present study, but not equivalent when combined with cardiac TnC in contrast to our results. While this discrepancy is a serious problem in interpreting the role of TnI in the pH sensitivity of fast skeletal and cardiac muscles, both studies show that cardiac TnC always confers a higher pH sensitivity than fast skeletal TnC irrespective of the TnI isoform, thus indicating that the TnC isoform plays an important role in the differential pH sensitivity in fast skeletal and cardiac muscles. This conclusion is consistent with studies showing that the ectopic expression of fast skeletal TnC in the heart of transgenic mouse significantly reduces the depressant effect of acidic pH on Ca^{2+} sensitivity, and that the ex-

change of cardiac TnC into fast skeletal skinned fibres reciprocally increases the depressant effect of acidic pH on the Ca^{2+} sensitivity (*10*).

Based on studies using skinned cardiac muscle fibres, Ding *et al.* (*15*) proposed that the difference in the pH sensitivities of fast skeletal and cardiac muscles is caused mainly (66%) by TnI and less (31%) by TnC. However, there is a notable discrepancy between their results and those of Ball *et al.* (*14*) as well as ours as to the pH sensitivities conferred by cardiac and fast skeletal TnI isoforms in combination with fast skeletal TnC. While Ball *et al.* (*14*) and we detected no differences in the pH sensitivities conferred by TnI isoforms in these combinations, Ding *et al.* (*15*) reported that the two TnI isoforms have remarkably different effects on the pH sensitivities when combined with fast skeletal TnC and concluded that the cardiac TnI isoform dominates the phenotype of cardiac muscle. However, this conclusion cannot explain the results of Ball *et al.* (*14*) and our findings in which "fast skeletal TnI" in combination with cardiac TnC was found to confer a more characteristic phenotype of cardiac muscle than "cardiac TnI" in combination with fast skeletal TnC.

We previously demonstrated that the exchange of whole Tn in fast and slow skeletal myofibrils with slow and fast skeletal Tn, respectively, changed the pH dependence of their Ca^{2+} sensitivities of ATPase activity to slow and fast skeletal muscle types, showing the Tn isoform to play a critical role in the differential pH dependence of fast and slow skeletal myofibrils (*26*). The present study further demonstrates that the slow skeletal TnI isoform is a critical determinant that confers a resistibility to acidic pH, which is the highest in vertebrate striated muscles, on slow skeletal muscle. It is known that the slow skeletal isoform of TnI is expressed in the heart during fetal and early neonatal periods, and this isoform switching of TnI is correlates well with the relative insensitivity to acidic pH exhibited by cardiac muscles in these developmental stages (*11*). Recently, experiments using an adenovirus-mediated gene transfer technique demonstrated directly that the ectopic expression of slow skeletal TnI confers resistibility to acidic pH on the Ca^{2+} sensitivity of adult cardiac myocytes (*27*). The present study also demonstrates directly that developmental isoform switching of TnI in the heart brings about a dramatic change in the resistance of cardiac muscle to acidic pH from the highest to the lowest.

Myosin crossbridge interaction with actin increases the Ca^{2+} affinity of TnC (*5, 6, 28-30*), and acidic pH has a depressant effect on the maximum force of skinned fibres prepared from vertebrate striated muscles (*7, 8, 31*), suggesting the contribution of crossbridges to the acidic pH-induced depression of Ca^{2+} sensitivity. However, it has recently been demonstrated that the acidic pH-induced depression of myofilament response to Ca^{2+} is primarily related to a direct change in the Ca^{2+} affinity of TnC and not to the indirect change in the Ca^{2+} affinity of TnC induced by changes in the number and/or strength of crossbridge attachment in both fast skeletal and cardiac muscles (*32*). The results of the present study were made based on experiments using preparations of the same type (cardiac) of muscle and no significant changes were observed in the effect of pH on the maximum force after reconstitution with various isoforms of TnI and TnC. This precludes the possibility that crossbridge attachment plays an important role in the differential pH dependence of Ca^{2+} sensitivity in striated muscles.

The present study provides the first evidence that TnC is the most crucial determinant of pH sensitivity in cardiac and fast skeletal muscles, whereas TnI is the most crucial determinant in slow skeletal muscle. The difference in the pH sensitivity between fast skeletal and cardiac muscles is therefore expected to be due specifically to some difference(s) in the functional properties and thus in the structures of the two TnC isoforms. Using a fluorescent probe-labeled purified TnC, Palmer and Kentish (*33*) showed that reducing the pH from 7.0 to 6.2 decreases the Ca^{2+} affinity of both cardiac and fast skeletal TnC, and the decrease in the Ca^{2+} affinity of cardiac TnC is twice that of fast skeletal TnC. This suggests that a direct effect of H^+ on TnC may play an important role in the differential pH sensitivity of cardiac and fast skeletal muscles. However, they also showed that the extent of the decrease in the Ca^{2+} affinity of purified TnC can explain only about one-half of the decrease in the Ca^{2+} sensitivity observed in skinned fibres, suggesting an additional indirect mechanism determining the differential pH sensitivity of cardiac and fast skeletal muscles. It should be noted that histidine has an electrical charge that is drastically affected in the pH range of 7.0 to 6.2 and that cardiac TnC contains no histidine while fast skeletal TnC has only one histidine at residue-125. The crystal structure of TnC in complex with the N-terminal fragment of TnI (residues 1-47) revealed that His-125 in the C-terminal lobe of fast skeletal TnC interacts electrostatically with Arg-13 in fast skeletal TnI (*34*). Since the C-terminal lobe of TnC is considered to play a structural role in anchoring TnC to the N-terminal region of TnI (residues 1-40) (*35, 36*), protonation of His-125 in the C-terminal lobe of fast skeletal TnC might affect the structural integrity of the TnC-TnI complex and indirectly cause the Ca^{2+} sensitivity of skinned fibres to be more resistant to acidic pH. On the other hand, the slow skeletal TnI is expected to have some unique structure(s) that confers the highest resistibility to acidic pH on slow skeletal muscle. One candidate is a region comprising five residues, 27-31 (CQQEH), corresponding to residues 29-33 (LEKEE) and 61-65 (LEREA) in fast skeletal and cardiac TnI, respectively. Interestingly, only slow skeletal TnI has a histidine residue in this region. Further studies to specify the sites or regions in the TnC and TnI molecules that determine pH sensitivity will contribute to a better understanding of the mechanisms involved in the pathophysiological effects of pH as well as physiological Ca^{2+} regulation in vertebrate striated muscle contraction.

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