Contributions of Troponin I and Troponin C to the Acidic pH-Induced Depression of Contractile Ca^{2+} Sensitivity in Cardiotrabeculae†

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ABSTRACT: Acid pH diminishes the Ca^{2+} sensitivity for force generation in both cardiac and skeletal muscles, but the mechanisms for these remain undetermined. In permeabilized (skinned) single myofibers of fast-twitch skeletal muscle of the rat, we find that pCa_{50} of the pCa−force relationship was 5.73 in pH 7 and 5.02 in pH 6.2 ($\Delta pK_{skeletal}$ = pCa_{50} in pH 7 − pCa_{50} in pH 6.2 = 0.71 pCa unit); on the other hand, in skinned cardiotrabeculae, the pCa_{50} was 5.79 in pH 7 decreasing to 4.14 in pH 6.2 ($\Delta pK_{cardiac}$ = 1.65 pCa units). We have used this large differential between cardiac/skeletal ΔpKs to probe the mechanisms of the pH effects. Since troponin C (TnC) and troponin I (TnI) each have a central role in the Ca^{2+} switch, we exchanged these proteins in cardiac muscle with their skeletal counterparts and reinvestigated the pH effects. Firstly, with fast-twitch skeletal muscle (sTnC) substituting for 80% of the endogenous cardiac TnC (cTnC), the cardiac pH effect was decreased marginally (modified ΔpK = 1.39 pCa units). This TnC-mediated change was further probed with two distinct cardiac−skeletal TnC chimeras, c1/s and CBc1/s (the Ca^{2+}-binding c1/s), in which a majority of the N-terminal 41 amino acid residues was made cardiac and the rest skeletal [Gulati, J., & Rao, V. G. (1994) *Biochemistry 33*, 9052−9056]. The phenotype shift following sTnC/cTnC exchange in the trabeculae was blocked when c1/s was used in lieu of sTnC; on the other hand, interestingly, CBc1/s exactly mimicked sTnC. We conclude that cardiac 1−41 residues contain the effective pH sensor of cTnC. Next, to monitor the TnI effect, both cTnI and cTnC were exchanged with an sTnI + sTnC complex. This obliterated the cardiac/skeletal pH differential, suggesting that cTnI was dominant in specifying the cardiac type pH effect. The results of a hybrid complex (cTnI + sTnC) were also consistent with this. After accounting for the residual components following TnC and TnI exchanges, we estimated that 31% of the cardiac/skeletal pH differential was caused by TnC and a dominant 66% by TnI.

Acidity depresses the contractility of the heart, and since acidosis is an expected manifestation of myocardial ischemia, there is long-standing interest in elucidating the mechanisms underlying contractile inhibition (Gaskell, 1880; Katz & Hecht, 1969; Allen & Orchard, 1987; Marban & Kusuoka, 1987; Godt & Nosek, 1989; Gulati & Babu, 1989). The studies on permeabilized (skinned) cardiac muscle specimens have shown that acidic pH diminishes both the ability for maximal tension generation, as well as the Ca^{2+} sensitivity for force development (Fabiato & Fabiato, 1978; Donaldson et al., 1978). The release of H^+ ion is a fundamental event at various elementary steps in the cross-bridge cycle, and the pH-induced inhibition of maximal tension development in a fully activated contractile machinery is well explained by the possible retardation of cross-bridge steps (Koretz & Taylor, 1975; Cooke et al., 1988; Chase & Kushmerick, 1988; Godt & Kentish, 1989; Seow & Ford, 1993). On the other hand, diminished Ca^{2+} sensitivity of force development in acidotic milieu is a manifestation of proton action on the troponin−tropomyosin regulatory complex, but identities of the specific targets as well as the underlying molecular events in this mechanism of pH are unknown. The present investigation was initiated to examine the specific roles of TnC[1] and TnI in the pH regulation of Ca−force relation in heart muscle.

Previous comparisons of the overall pH effects on Ca−force relations of cardiac and skeletal muscle specimens indicated that cardiac muscle is significantly more susceptible to acidity (Gulati & Babu, 1989). The study of this disparity offered a useful approach into the investigations of the mechanisms of acidosis, and recent attention was directed to the differences between the cardiac/skeletal regulatory subunits, especially the TnCs. However, our initial study of this using sTnC exchange to replace cTnC in isolated hamster trabeculae indicated relatively little change in the pH effect (pH 7 → 6.5) on Ca−force relationship. A greater pH stimulus (pH 7 → 6.2) on myocytes from transgenic mice with cardiac-specific expression of sTnC revealed a more significant transformation (a mean reversal of 43%) of the pH effect from cardiac type to skeletal type (Metzger et al., 1993). The pH 6.2 effect is further explored presently by

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[1] Abbreviations: TnI, troponin I; sTnI, fast-twitch skeletal muscle TnI; cTnI, cardiac TnI; TnC, troponin C; c1/s and CBc1/s, cardiac−skeletal TnC chimeras; TnT, troponin T; TM, tropomyosin; LC1, myosin light chain 1; DTT, dithiothreitol; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; MOPS, 4-morpholinopropanesulfonic acid; BME, 2-β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; CrP, phosphocreatine; CPK, creatine phosphokinase; PAGE, polyacrylamide gel electrophoresis; P_o, maximal force of the skinned fiber in pCa 3.5; pCa, $-\log(Ca^{2+})$.

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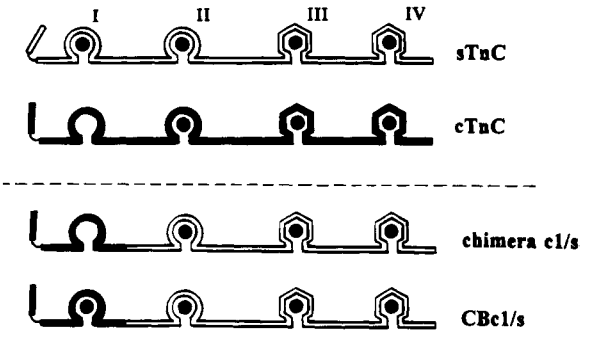

FIGURE 1: Wild-type TnC isoforms and cardiac−skeletal TnC chimeras. In c1/s the residues 1−41 of bovine cardiac TnC were spliced with residues 41−159 of rabbit sTnC. In CBc1/s, additional modification was made by replacing ^{27}VLGA^{30} by D-AD to restore Ca^{2+} binding in the cardiac EF-hand [modified from Rao et al. (1995)].

TnC exchange in vitro, using genetically derived TnC chimeras. These chimeras comprised different regions of cardiac and skeletal sequences (residues 1−41 cardiac and 42−160 skeletal in one chimera and with additional variations within the 41 cardiac residues for Ca competency for another chimera; Gulati & Rao, 1994), and the goal was to locate the pH-sensing domain in cTnC for this partial pH effect.

To explain the origin of the remaining major difference in the cardiac/skeletal pH phenotype, we have explored the role of TnI. As noted earlier (Gulati & Babu, 1989), the possible involvement of TnI was suggested also from the study of Solaro et al. (1988) on ventricular muscles from adult and neonatal rats. The neonates indicated diminished susceptibility to pH despite conserved cTnC. To obtain definitive evidence for the TnI role into the pH dependencies of force regulation, we have presently replaced sTnI for cTnI in adult cardiac muscle.

A preliminary report of these results was made at a recent Biophysical Society meeting (Ding et al., 1994a), where the replacement of sTnI + sTnC in cardiac muscle was reported to reverse the phenotype of the pH effect on force development. Subsequently, the similar finding on the regulation of Mg^{2+}-ATPase in skeletal muscle myofibrillar specimens was also published (Ball et al., 1994). The present studies sought specifically to delineate the individual contributions of TnC and TnI in the pH regulation of tension in the cardiac milieu, with the use of chimeric TnC as well as a hybrid complex (cTnI + sTnC). The findings manifest the most direct evidence for the dominance of cardiac TnI in this effect.

MATERIALS AND METHODS

1. TnC and TnI Purifications. The TnCs of both skeletal and cardiac muscle types were used interchangeably. The skeletal TnC (sTnC) used in this study was exclusively the recombinant protein, prepared by the use of a synthetic cDNA encoding rabbit skeletal TnC (Babu et al., 1992). The cTnC was isolated from bovine heart by the method of Szynkiewicz et al. (1985). A cardiac−skeletal TnC chimera (c1/s), genetically engineered with amino acid residues 1−41 containing the N-terminal EF-hand of the cardiac type and residues 42−160 skeletal, was the same as before [Figure 1; see also Gulati et al. (1992)]. An additional TnC construct (CBc1/s) in which the ^{27}VLGA^{30} quadruplet was replaced with D-AD triplet to induce Ca^{2+} binding within the cardiac EF-hand of the chimera was also used (Gulati & Rao, 1994).

For TnI purification, we used TnC affinity column chromatography according to Syska et al. (1974). The tissue extracts for affinity chromatography were prepared by homogenizing 8−10 g of rabbit white muscle (for sTnI) or beef heart (for cTnI) in five volumes of 8 M urea, 100 mM Tris-HCl buffer (pH 8.00), containing 15 mM BME and 2 mM $CaCl_2$. The homogenate was cleared of tissue debris by filtration through glass wool, prior to affinity chromatography.

1.1. TnC Affinity Column and TnI−TnC Complexes. The TnC Sepharose 4B affinity column was prepared by the manufacturer's recommended procedure (Pharmacia); 1.25 g of CNBr-activated Sepharose 4B (Pharmacia) was washed twice with 10 mL of 1 mM HCl at 4 °C. The TnC coupling was started by the addition of 8 mL of rabbit sTnC (1.5 mg/mL) dissolved in 100 mM $NaHCO_3$, containing 5 mM $CaCl_2$, and the mixture was rocked gently overnight at 4 °C. The binding efficiency was checked using the Coomassie spot test (Harlow & Lane, 1988).

TnC−Sepharose conjugate was washed with 1 M ethanolamine (pH 8.0), followed by a wash with acetate buffer (100 mM Na-acetate, 500 mM NaCl, pH 4.0), and then equilibrated with PBS (Harlow & Lane, 1988). The TnC−Sepharose was mixed with muscle extract for 2 h at room temperature and centrifuged (~800*g*), and the pellet was packed in a column (7 cm × 0.8 cm). The column was washed with the buffer containing 8 M urea, 50 mM Tris-HCl, pH 8.0, 15 mM BME, and 1 mM $CaCl_2$ until no protein was detected in the filtrate. TnI was eluted with 10 mM EGTA. The yield was about 2 mg from a 8 g sample of the muscle. The purified protein could be stored at −80 °C in 6 M urea, 50 mM Tris, 15 mM BME, and 100 mM EGTA, at pH 6.00.

To prepare a TnI−TnC complex for fiber assay (see below: skinned trabeculae), an aliquot of TnI was dialyzed overnight against 20 mM imidazole, 6 mM $MgCl_2$, 5 mM EGTA, and 220 mM K-propionate, pH 6.70, 4 °C, and mixed with 1.5× TnC for complexation. Finally, 5 mM ATP, 15 mM creatine phosphate, and 5 mM DTT were added to TnI−TnC complex, and the total ionic strength was diluted to 150 mM. The mixtures were designed cTnI + cTnC and sTnI + sTnC and hybrids sTnI + cTnC and cTnI + sTnC. The final concentrations were 1.0 mg/mL for TnI and 1.5 mg/mL for TnC.

1.2. TnT Purification. The cardiac TnT was purified from the bovine heart by the method of Jin and Lin (1988), except that a 60 °C heating step was omitted as a precautionary measure to preserve the functional integrity of the purified protein. The product indicated a single band on the gel (not shown). The purified TnT was dissolved in 250 mM KCl, 20 mM MOPS, 5 mM $MgCl_2$, 2 mM EGTA, 0.1 μg/mL pepstatin A, and 0.5 mM DTT.

2. Skinned Trabeculae: TnC and TnI Depletions and Reconstitution. Rats were used throughout. Skinned fibers were prepared as described previously for the hamster (Babu et al., 1987; Gulati & Babu,1989). Typically, thin right ventricular trabeculae (50−150 μm width, 1−2 mm long) were isolated as needed from the freshly excised heart from adult Wistar female rats. The skeletal fibers were also used

Table 1: Solutions for Skinned Fibers

solution	pH	buffer[a] (mM)	ATP (mM)	EGTA (mM)	$MgCl_2$ (mM)	CrP (mM)	potassium propionate (mM)	CPK (unit/mL)	$CaCl_2$ (mM)	vanadate (mM)	NaN_3 (mM)	potassium acetate (mM)	EDTA (mM)
normal solutions:													
relaxing	7.0	20[a]	5	5	6	15	90	300					
activating (pCa 3.5)[b]	7.0	20[a]	5.3	5	5.5	15	85	300	5.97				
extraction solution for TnC:													
TnC extraction	7.2	10											5
extraction solutions for TnI + TnC:													
0R	6.7	30	10	5	10	10		300			5	20	
pCa 3.5	6.7	30	10	5	10	10		300	6.14		5	20	
vanadate soln	6.7	30	10	5	10	10				10	5	15	

[a] Buffer was imidazole except for pH 6.2 where 10 mM imidazole and 10 mM 2-(*N*-morpholino)ethanesulfonic acid were combined. [b] The solutions for intermediate pCas contained lower $CaCl_2$, as ascertained by computation.

Table 2: Force Regulation in Cardiac Muscle with TnC and TnI Extraction and Reconstitution (pH 7.0)

		TnC exchange			TnI + TnC exchange			
	native i	TnC extraction ii	+cTnC iii	+sTnC iv	TnI + TnC extraction v	+(cTnI + cTnC) vi	+(sTnI + sTnC) vii	+(cTnI + sTnC) viii
EGTA force	0	0	0	0	92 ± 6%	10 ± 4%	9 ± 3%	9 ± 4%
Ca-activated force	100%	6 ± 2%	94 ± 4%	95 ± 4%	8 ± 3%	87 ± 5%	86 ± 6%	78 ± 6%
N	45	22	7	10	23	8	12	3

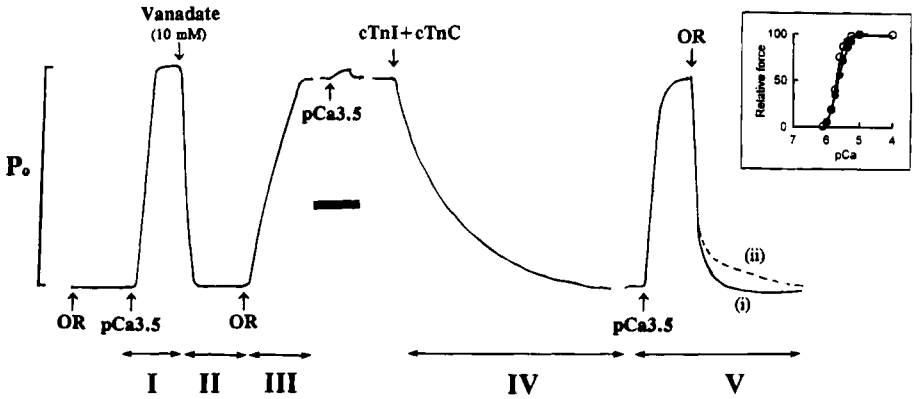

FIGURE 2: TnI extraction and reconstitution of a typical permeabilized trabeculum. Phase I: Tension response prior to vanadate treatment (P_0 = 118 kN/m^2). II: Vanadate treatment. The vanadate solution was the same as 0R solution in Table 1 with 10 mM vanadate. III: Ca^{2+}-free tension following vanadate treatment (0R, relaxing solution excluding vanadate). IV: Reversal of tension during reconstitution with cTnI+cTnC. V: pCa 3.5-induced tension following reconstitution. The time scale is indicated by the fat horizontal bar: 20 s in phases I and V, 20 min in phases II, III, and IV. The Ca^{2+}-dependent force recovery in the final phase V in this example was 97% P_0; the mean values for other specimens are indicated in Table 3. The temperature was 20 °C throughout. The tails of phase V indicate TnT effect: (i) when TnT (1 mg/mL) preincubation was included at the onset of phase IV prior to cTnI + cTnC loading and (ii) no TnT preincubation. Inset: Typical pCa–force relation on a fiber in pH 7 before (open circles) and after (filled circles) extraction–reconstitution with a TnC and TnI complex.

in the present study, and these were exclusively of the fast-twitch type from psoas muscles. The fiber type was checked routinely by measuring the Sr^{2+}-activation response in pSr5 [see Babu et al. (1986)]. The force transducer and skinning were as before. The skinned fiber solutions were modified from Gulati and Podolsky (1978; Babu et al., 1987) [see, also, Martyn and Gordon (1988)]. The compositions of the various solutions used in this study are depicted in Table 1. The normal solutions had the estimated ionic strength of 180 mM. The sarcomere length was adjusted at 2.3 μm using laser diffraction and was monitored throughout the experiment.

2.1. TnC Exchange. The TnC extractions and reconstitutions of the cardiac trabeculae were done by superfusing the fiber in TnC extraction EDTA medium, as described before (Babu et al., 1987; Gulati & Babu, 1989). This EDTA treatment protocol resulted in the exchange of 80% of the TnC as judged from the gels (Gulati et al., 1991). The Ca^{2+}-activated force levels of TnC-extracted and reconstituted fibers are listed in Table 2 (columns i–iv).

2.2. Vanadate Treatment: TnI + TnC Exchange. For TnI + TnC extractions, the vanadate treatment was employed, as modified from Strauss et al. (1992) and Van Eyk et al. (1993). The trabeculae were isolated freshly. All protein solutions contained 5 mM DTT, unless otherwise indicated. Moreover, the tissue specimen was checked for sarcomere homogeneity, and the activations with pCa3.5 were carried out after adjusting the sarcomere length at 2.3 μm (see above).

A typical cycle for the TnI + TnC extraction is depicted in Figure 2. Note that all solutions during this cycle (0R, pCa 3.5, vanadate) were of lower ionic strength (125 mM) than that of the normal solutions in Table 1. Following the final activation step in pCa 3.5 solution (Figure 2), the

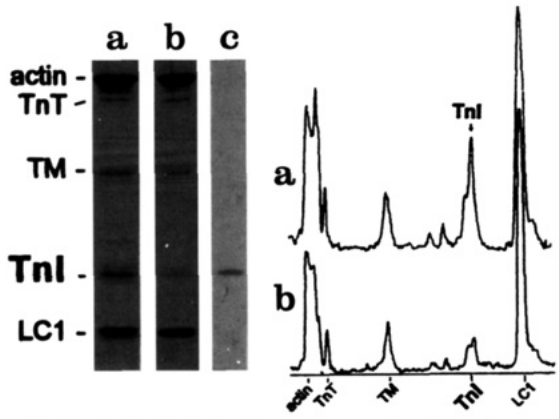

FIGURE 3: Typical gels indicating TnI extraction following vanadate treatment. a: Control trabecula. b: Vanadate-treated trabecula; 8% SDS−PAGE gel. TnC band was allowed to run off the gel. c: Purified cTnI.

Table 3: Quantitation of Protein Bands on the SDS−PAGE[a]

protein subunit	control	vanadate treated	unextracted (%)
TnI	0.430 ± 0.010	0.115 ± 0.028	27
TnC	0.153 ± 0.003	0.036 ± 0.027	23
TnT	0.129 ± 0.004	0.105 ± 0.015	81
TM	0.227 ± 0.018	0.227 ± 0.032	100

[a] All protein band intensities were normalized to the LC1 band intensity in the same gel lane.

trabeculae were transferred into the normal solution, and these were used in all subsequent operations. When force in pCa 3.5 had plateaued (phase I in Figure 2), the specimen was transferred to another relaxing solution containing additional 10 mM sodium vanadate and 5 mM DTT ('vanadate soln' in Table 1), which too was freshly made before using. The force level was close to base line throughout this phase II. After 8−10 min in 10 mM vanadate solution during phase II, the specimen was washed three to four times with the original (vanadate-free) relaxing solution ('soln 0R' in Table 1) with vigorous stirring. During this phase III (Figure 2), force developed despite the absence of Ca^{2+} (see also column v in Table 2), suggesting that TnI extraction was accomplished. This was further indicated by gels and the TnI + TnC reconstitutions (see below).

A typical SDS−PAGE run is depicted in Figure 3. By quantitative analysis, we ascertained that 77% of TnC and 73% of TnI were extracted (Table 3). A small amount (19%) of TnT was also deleted (Table 3), but evidently this did not affect the present results (see below).

TnI + TnC Repletions. The (TnI + TnC)-depleted fibers were reincubated in a fresh relaxing solution containing the purified rabbit skeletal sTnI + sTnC complex (1 mg/mL TnI + 1.5 mg/mL TnC) and the beef cardiac cTnI + cTnC complex or the hybrid cTnI + sTnC complex for 60−80 min at 20 °C (phase IV in Figure 2). The Ca^{2+}-independent force (EGTA force) became gradually inhibited during this incubation, presumably because TnC and TnI were being reinserted into the contractile machinery. At the end of the incubation period, the fiber was activated in pCa 3.5 to assess the recovery of Ca^{2+} regulation (97% force recovery in phase V in Figure 2; mean force recovery was 84%, with several fibers yielding 100% recovery; see columns vi−viii in Table 2). Fibers yielding less than 70% force recovery were omitted.

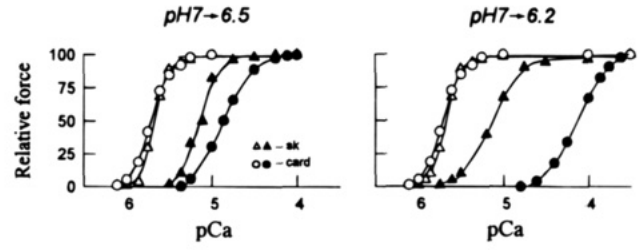

FIGURE 4: Effects of acidic pH 6.5 and 6.2 on Ca^{2+} sensitivity in cardiac (circles) and skeletal (triangles) skinned fibers. The open symbols are data at pH 7 and the closed symbols in the acidic pH milieus.

The pCa−force relation of a typical (TnI + TnC)-extracted−repleted trabeculum in comparison with the normal is also indicated in the inset to Figure 2. Normal force recoveries were consistently obtained with sTnI + sTnC and cTnI + cTnC complexes, as well as with a hybrid complex (cTnI + sTnC). However, the hybrid complex sTnI + cTnC gave incomplete force recovery (0.23 ± 0.02 P_o in four preparations) in cardiac muscle and was not further pursued. It is interesting that cTnC exchange in fast-twitch fibers had also produced down regulation (Babu et al., 1987; Gulati et al., 1989); on the basis of the present result, the reason for this would appear in part to be the inability of this hybrid to form a functional complex.

2.3. TnT Loading. Because of the accompanying decrease in TnT during TnI depletions (Table 3), we tested whether this had affected the fiber response. Therefore, the vanadate-treated fiber was loaded with purified TnT just prior to TnI and TnC repletions. The late relaxation phase of the reconstituted fiber was found to be accelerated with TnT (tail i in Figure 2), but as described below (Results) the TnT repletion had no further effect on pH dependence. This TnT effect on relaxation kinetics was not further investigated in the present study.

3. SDS−PAGE and Western Blots. All experimental specimens were stored for gel electrophoresis. The protocols for running the gels and silver staining were as described earlier (Babu et al., 1987; Gulati et al., 1991). The gels were analyzed by laser densitometry. For immunoblotting, unstained gel was transblotted to nitrocellulose membrane (0.45 μm pore size) using the BioRad transblot cell (Towbin et al.., 1979). The membrane was probed using ECL Kit RPN 2109 (Amersham). Dilution of 1:2000 was employed for TnI antibody (the monoclonal for cTnI was a gift of Dr. Schiaffino, Padova, Italy).

RESULTS

The principal objective of the present study was to delineate the TnC- and TnI-mediated effects of acid pH in depressing the Ca^{2+} sensitivity of force development in cardiac muscle. To accomplish this, we substituted either sTnC for cTnC or the complexed sTnI + sTnC or cTnI + sTnC for the endogenous cTnC and cTnI and investigated the pCa−force relations at pH 7, 6.5, and 6.2. These results were compared with findings on native (unextracted) cardiac and skeletal skinned fibers. In all experiments, cardiac specimens extracted and restored with either cTnC or cTnI + cTnC complex, as appropriate, were also studied.

1. Definition of the pH Effect on pCa−Force Relationship. The typical pCa−force curves for native (unextracted) skinned cardiac and skeletal fibers are shown Figure 4. The

Table 4: Effects of Acidosis on Ca-Sensitivity of Contraction

	native		cardiac muscle				
			TnC exchange		TnI + TnC exchange		
	cardiac i	skeletal ii	[cTnC] iii	[sTnC] iv	[cTnI + cTnC] v	[sTnI + sTnC] vi	[cTnI + sTnC] vii
pH 7.0 → 6.5	A		B		C		
pCa_{50} at pH 7.0	5.74 ± 0.04	5.71 ± 0.04	5.71 ± 0.05	5.66 ± 0.04	5.72 ± 0.03	5.67 ± 0.04	
pCa_{50} at pH 6.5	4.88 ± 0.06	5.14 ± 0.02	4.83 ± 0.03	4.85 ± 0.04	4.90 ± 0.04	5.04 ± 0.07	
N	7	4	4	6	4	6	
pH 7.0 → 6.2							
pCa_{50} at pH 7.0	5.79 ± 0.04	5.73 ± 0.04	5.75 ± 0.04	5.69 ± 0.02	5.71 ± 0.03	5.75 ± 0.05	5.70 ± 0.04
pCa_{50} at pH 6.2	4.14 ± 0.06	5.02 ± 0.02	4.12 ± 0.04	4.30 ± 0.07	4.10 ± 0.05	4.89 ± 0.07	4.41 ± 0.06
N	6	4	3	4	4	6	3

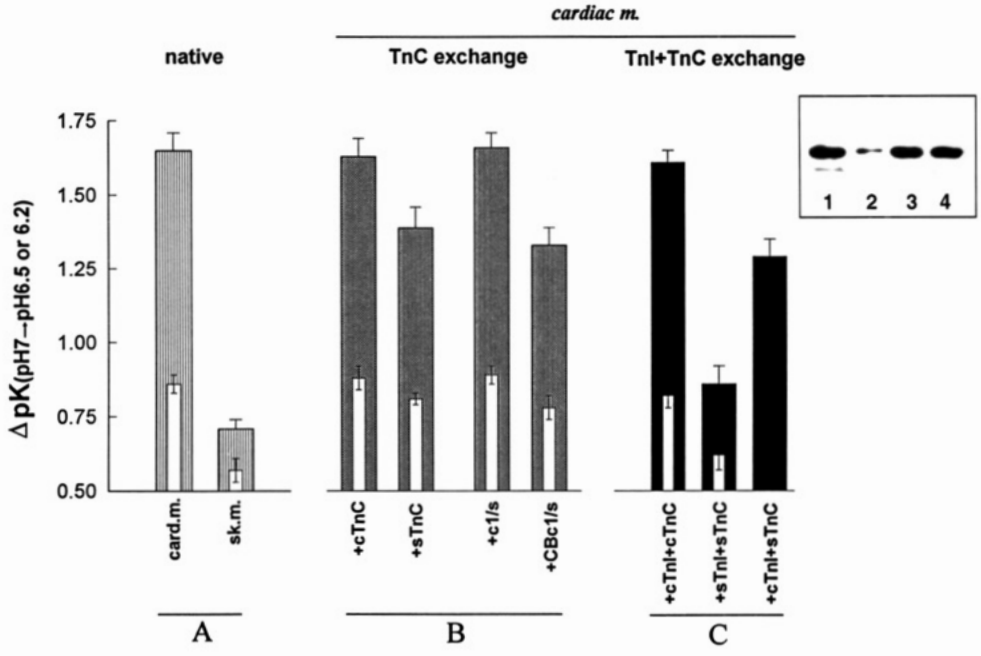

FIGURE 5: Acidotic (pH 7 → 6.5 and pH 7 → 6.2) shifts in Ca^{2+} sensitivity in native fibers (A), following TnC exchange (B), and following TnI + TnC exchange (C) in cardiac muscle. The open bars indicate the results for pH 6.5 and the shaded bars for pH 6.2. Inset: Western blots. 1: Native trabecula. 2: Trabecula following sTnI + sTnC exchange. 3: Trabecula following cTnI + cTnC exchange. 4: 20 ng of purified cTnI.

data were normalized to P_o in pCa 3.5 at the corresponding pH. The force ratio (i.e., P_o at pH 6.5/P_o at pH 7) was 0.68 ± 0.04 in cardiac muscle and 0.67 ± 0.02 in skeletal fibers. In pH 6.2, the force ratios were 0.41 ± 0.03 and 0.66 ± 0.05 for the cardiac and skeletal fibers, respectively [see, also, Metzger et al. (1993)].

The normalized pCa−force curves were shifted to the right in acid pH (Figure 4). The left hand panel in Figure 4 compares the typical results for cardiac and skeletal muscles at pH 6.5, and the right hand panel in Figure 4 shows the corresponding curves at pH 6.2. In both pH solutions, the cardiac curves are shifted much more than the skeletal muscle curves. The mean values are noted in Table 4.

To aid in further analysis, the pK shifts in acid pH (ΔpK = pCa_{50} at pH 7 − pCa_{50} at pH 6.5 or 6.2) are plotted in the bar diagram in Figure 5A. Each ΔpK value (bar height) directly indicates the decreases in Ca^{2+} sensitivities with acid pH (open inner bars, pH 6.5; shaded outer bars, pH 6.2). It is self-evident that the acidotic effect is greater in cardiac muscle compared with that in fast skeletal fiber ($\Delta pK_{cardiac}$ = 0.86 pCa unit at pH 6.5 and 1.65 pCa units at pH 6.2; $\Delta pK_{skeletal}$ = 0.57 pCa unit at pH 6.5 and 0.71 pCa unit at pH 6.2, respectively). We next investigated the effects of cTnC/sTnC exchange in myocardium to assess the TnC contribution for explaining the differences between cardiac and skeletal muscles.

2. pH Effect with TnC Exchange in Rat Cardiac Muscle. By following the established protocols [see also Babu et al. (1988) and Gulati et al. (1991)], we extracted the endogenous cTnC from cardiac specimens and then reconstituted these specimens with sTnC. The pCa−force relations were determined on these at pH 7 and 6.5 or pH 7 and 6.2.

The pCa_{50} values are listed in Table 4, and the corresponding ΔpK differences as defined above (ΔpK = pCa_{50} at pH 7 − pCa_{50} at pH 6.5 or 6.2) are plotted in the bar diagram in Figure 5B. The mean value for ΔpK (with +cTnC) is the same as in the unextracted cardiac muscle (compare the first bar in Figure 5A and the +cTnC-labeled bar in Figure 5B; also, columns i and iv of Table 4).

Following sTnC exchange (+sTnC-labeled bar in Figure 5B), pH dependence is decreased in comparison with +cTnC in Figure 5B (compare bar labeled +sTnC in Figure 5B with the cardiac and skeletal data in Figure 5A). This indicates that TnC could account for part of the pH effect. This contribution of TnC is derived as $\Delta\Delta pK$ = (ΔpK_{cTnC} − ΔpK_{sTnC})/($\Delta pK_{cardiac}$ − $\Delta pK_{skeletal}$) = 25 ± 11% for pH 7 →

6.2 and 24 ± 7% for pH 7 → 6.5. In light of these results, further analysis of the hamster data in Gulati and Babu (1989) was attempted by treating the results for pH 7 and 6.5 on each trabecula as pairs. This yielded $\Delta pK = 0.6$ pCa unit for untreated cardiac muscle and 0.52 pCa unit after sTnC exchange. The ΔpK for their skeletal fibers was 0.24 pCa unit. The $\Delta\Delta pK$ could thereby be estimated as 22%, which is close to the present value on the rat. Thus, the TnC contribution to the cardiac/skeletal pH differential ($\Delta\Delta pK$) is limited to approximately 25% of the total. The possible additional contribution from the residual TnC component will be considered below.

3. Substitution with Cardiac−Skeletal TnC Chimeras. The TnC effect was further investigated with a cardiac−skeletal chimera (called c1/s; see Figure 1; Gulati et al., 1992), in which 1−41 amino acid residues from cardiac TnC were spliced with 41−159 residues of skeletal TnC. The results in Figure 5B (see bar labeled c1/s) indicate that the chimera responded similarly to tissue cTnC.

A variant of the above chimera (called CBc1/s; see Figure 1) in which the cardiac EF-hand is further engineered to replace the ^{27}VLGA^{30} cluster with D-AD was also used. CBc1/s binds Ca^{2+} and also has the skeletal type Sr^{2+} phenotype in solution (Gulati & Rao, 1994). The pH effects in Figure 5 show that CBc1/s mimicked sTnC for the responsiveness to acidity as well.

These findings with the chimeras help locate the pH sensitivity domain within the residues 1−41 of the cardiac EF-hand and also define a critical role of Ca^{2+} binding in this mechanism. In efforts to explain the remaining (75%) difference in the pH responsiveness between cardiac and skeletal tissues, we next proceeded to investigate the specific influence of the TnI isoform.

4. Vanadate Treatment: TnI + TnC Exchange in Rat Cardiac Muscle. The vanadate treatment applied in this study extracted both TnI (77%) and TnC (73%) (Table 3). Approximately 19% of endogenous TnT was deleted as well. These extracted fibers were reconstituted with various TnI + TnC complexes (see methods and Figure 2). The fibers reconstituted with sTnI + sTnC, cTnI + cTnC, or cTnI + sTnC recovered close to full Ca^{2+}-dependent force (Table 2). It is interesting that despite the 25% residual cTnI + cTnC, there remained only 8% Ca-activated force (column v in Table 2). This might raise the possibility that there was a cooperative effect of these proteins on contractility or possibly selective extraction. These possibilities were not further elucidated in the present study.

The study of the effects of acidity on pCa−force relations of the trabeculae with various TnI + TnC treatments was the major aim of this investigation, and the various findings are summarized in Table 4C and Figure 5C. The specimens with sTnI+sTnC yielded a diminished pH effect on Ca sensitivity, and the ΔpKs at pH 6.5 and 6.2 were reduced relative to pH 7. The results at both acidic pHs mimicked the response on skeletal fibers (compare the middle bar in Figure 5C with the results in Figure 5A). The cTnI + cTnC restitution indicated normally restored cardiac type pH response (the first bar in Figure 5C), which rules out any extraction artifacts. Moreover, typical Western blots of the (cTnI + cTnC)-loaded (lane 3) and (sTnI + sTnC)-loaded (lane 2) fibers are also indicated in the inset to Figure 5. The antibody was cTnI-specific (Saggin et al., 1989), and the blots with cTnI loading were also normal (compare lanes 1 and 3).

To see if the results of (sTnI + sTnC)-reconstituted trabeculae were being modified by the relatively small TnT extraction, an additional control experiment was performed with TnT preloading (see above). For (sTnI + sTnC)-loaded specimen, we found that the pH effect (for pH 7 → 6.2) was indistinguishable from that determined without TnT preloading ($\Delta pK = 0.88$ pCa unit with TnT treatment preceding sTnI + sTnC loading and 0.86 pCa unit without TnT treatment).

Mean Comparisons of cTnI + cTnC Reconstitutions. The average values for the pH-induced shifts [estimated as $\Delta\Delta pK = (\Delta pK_{cTnC+cTnI} - \Delta pK_{sTnC+sTnI})/(\Delta pK_{cardiac} - \Delta pK_{skeletal})$] were 79 ± 10% at pH 6.2 and 66 ± 9% at pH 6.5. Thus a large part of the cardiac/skeletal pH-induced differential in Ca^{2+} sensitivity for force development is accounted for by specific TnC and TnI isoforms. However, whether the remaining 27% cardiac type response (mean of the corresponding values at pH 6.2 and 6.5) in the rat trabeculae in the presence of sTnC + sTnI must be accounted for by the residual cTnI and cTnC was not directly explored. Nonetheless, assuming that these residual proteins (25% mean residuals for cTnC + cTnI) would contribute to the pH effect analogously to the extracted counterparts, we estimated the TnC contribution as approximately 31%, and the combined contribution from TnI and TnC can be estimated as approximately 97% at acid pH. The putative TnI contribution is therefore 66%. Thus, TnC and TnI moieties together can account for nearly all of the difference between the pH effects on Ca^{2+} sensitivity for tension development in cardiac and skeletal muscles.

5. Hybrid Complex (cTnI + sTnC) Exchange. In an additional set of experiments, the endogenous rat cTnC and cTnI were exchanged for bovine cTnI + rabbit (recombinant) sTnC. The influence of pH in trabeculae following this hybrid substitution is indicated in the bar diagram in Figure 5C. The pH effect in this case was indistinguishable from the results of the experiment in which only sTnC was substituted for cTnC (Figure 5B, column labeled +sTnC). This agreement is the expected result and further strengthens the conclusion that TnI + TnC complex provides the target regions for the modification of Ca^{2+} sensitivity in tension generation by cardiac muscle. The possibility is raised that TnC and TnI are both modified in acidic pH.

DISCUSSION

By extracting endogenous cTnI + cTnC complex from cardiac trabeculae, using vanadate treatment (Strauss et al., 1992; Van Eyke et al., 1993), and reconstituting these fibers with a skeletal (TnI + TnC) complex, we have shown that the pH dependence of Ca−force relationship in cardiac muscle now mimics the response in skeletal muscle. Additionally, by extracting cTnC alone and substituting in its location either the fast-twitch skeletal TnC or the cardiac−skeletal chimera c1/s (Gulati et al., 1992) or CBc1/s (Gulati & Rao, 1994), we provide strong evidence that approximately 31% of the differential in pH effects on Ca^{2+} sensitivity between cardiac and skeletal muscles is due to TnC per se and the rest is mediated via TnI. Interestingly, these studies with the chimeras indicating that cardiac 1−41 amino acid residues are sufficient to transform the TnC-associated pH

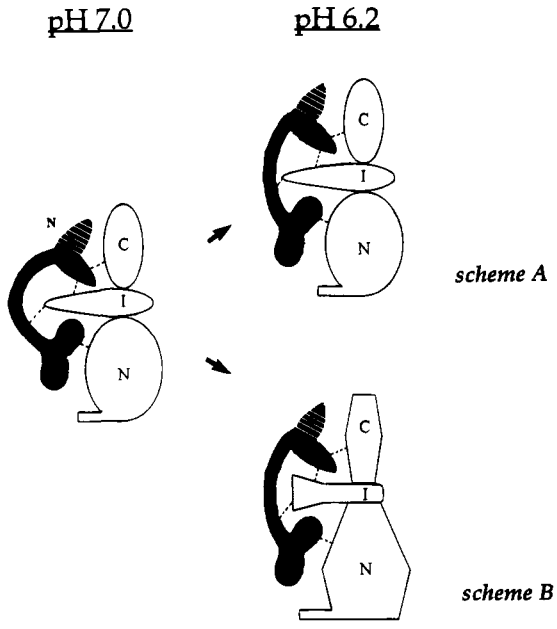

FIGURE 6: Cartoon representation of the pH effect on the cardiac (TnI + TnC) complex. The dumbbell-shaped TnC (shaded structure) is modeled after Herzberg et al. (1986), and the antiparallel TnI (unshaded structure) interaction is modeled after Farah et al. (1994). The dashed lines indicate the putative loci for TnC−TnI interactions. Scheme A assumes that the protons in the acidic solution affect TnC primarily at the pH-sensing domain. Scheme B indicates that TnC and TnI are both modified by pH and that these proteins act synergystically to command the acidotic effect. The putative pH-sensing domain of TnC is observed to reside within the cardiac domain comprising residues 1−41 (the interrupted structure in the TnC N-domain).

phenotype suggest also that the effective cardiac pH-sensor element of cTnC is located within the 41-residue domain. Mechanistically, furthermore, on the possibilities whether only TnC provides the target for H^+ ion (scheme A, Figure 6) or whether acidity influences TnC and TnI separately (scheme B, Figure 6), the present study with the hybrid complex favors scheme B. This is further discussed below.

1. Differences between Cardiac and Skeletal TnI + TnC Complexes. For the same pH change (i.e., pH 7 → 6.2), the cTnI + cTnC complex was strikingly more effective than the sTnI + sTnC complex in the suppression of Ca^{2+} sensitivity for myocardial contractility. Since cTnC → sTnC exchange accounted for only 31% of the difference in the pH responsiveness of cardiac and skeletal fibers, the contribution of TnI moiety is evidently dominant in the mechanism of contractile depression accompanying acidity. The findings with the hybrid complex cTnI + sTnC are in accord with this, since the pH effect in the cardiac trabeculae with hybrid complex varied from that of the normal myocardium by the amount expected from the TnC difference. Thus, the simplest possibility is that cTnI has a greater characteristic response to pH than sTnI and that this differential is maintained whether cTnC or sTnC was present in the complex. Whether cTnI responsiveness to pH is expressed equally well in isolation (i.e., in purified protein) or whether the effect is manifest only in complex with TnC, remains undetermined, however.

To understand how cTnI amplifies the TnC responsiveness, it is worthwhile scrutinizing differences between the primary structures of cardiac and skeletal TnIs. The most striking structural dissimilarity is in the N-terminus, which in the cardiac isoform contains an additional 26−33-residue unit (Wilkinson & Grand, 1978; Leszyk et al., 1988; Vallins et al., 1990), but this unit appears to be uninvolved because a mutant cTnI lacking the N-terminal unit produced no change in the pH effect of cardiac myofibrillar ATPase (Guo et al., 1994). Additional differences between cardiac and skeletal TnI isoforms occur in regions interfacing with TnC and/or actin [residues 30−80 and 130−150; residue numbers corresponding to bovine cardiac TnI, according to Leszyk et al. (1988)]. Future studies testing genetically engineered cTnI/sTnI chimeras should help define the pH-sensitive domains.

For instance, a candidate region comprising residues 30−80 includes the actin-binding domain and is also the most variable between cardiac and skeletal TnIs (40% invariant residues). The variant clusters deserve to be probed in future studies for the pH responsiveness. On the other hand, residues 130−150 (corresponding to skeletal residues 100−120) are nearly completely conserved, but in this case there is a significant substitution of Pro to Thr at residue 144 [see, also, Ball et al. (1994)]. The rabbit skeletal TnI, as well as the cardiac TnI (excluding the N-terminal unit), each has four turns predicted in the overall structures (Leavis & Gergely, 1984; Liao et al., 1992; Olah & Trewhella, 1994), and the Pro/Thr substitution in cTnI occurs at one of the putative turns at residues 143−146. Because of the high propensity of proline for turn formation in the protein structure (Levitt, 1978; MacArthur & Thornton, 1991; Nicholson et al., 1992), the Pro/Thr substitution at this critical juncture in sTnI could manifest an essential functional property of the loop.

Another possibility, that histidines are involved in the pH effects, should also be considered. For instance, the comparison of the human and bovine cTnI and rabbit sTnI primary sequences (Armour et al., 1993; Vallins et al., 1990) indicates three histidines in the cardiac (H33, H100, H171) versus only two in the skeletal isoform (H100, H162). Typically, the p*K* of histidines is close to 6.0, and the differences in the relative abundances and locations of histidines between the two isoforms could be significant. Finally, as noted above, because of the question related to the differences in the pH responsiveness between free cTnI or complexed cTnI, future studies of the target regions should also consider the possibility that properties of the histidines may vary under different conditions.

2. Relation to Other Studies. While the present study was near completion [see Ding et al. (1994a,b)], Ball et al. (1994) published their findings on Ca^{2+}-regulated ATPase in skeletal myofibrils. The exchange of sTnI + sTnC for cTnI + cTnC modified the ATPase response from skeletal to cardiac type. This correspondence with the present results on force development in cardiac trabeculae provides reasonable certainty that TnC and TnI moieties can explain the pH effect on Ca^{2+} sensitivity in both cardiac and skeletal milieus. However, in contrast to present findings on the myocardium, the skeletal myofibrils had indicated little change with the hybrid (cTnI + sTnC) exchange, and this deviation of the skeletal specimens is difficult to reconcile as yet.

Relative to the differences between cTnC and sTnC, further comparison with Metzger et al. (1993) may be worthwhile. Metzger et al. (1993) also studied the pH 6.2 effects but used cardiocytes from the Parmacek−Leiden−Field transgenic mouse embodying cardiac-specific expres-

sion of sTnC. Their mean pH response indicated about 43% conversion to the skeletal type compared with 25% estimated presently on the rat. After accounting for the residual TnC (approximately 20% in the present study), the present TnC contribution is increased to 31%. This discrepancy may represent a bona fide species difference. This too deserves to be checked with TnC exchange on mice. Remarkably, however, the published figure in Metzger et al. indicated a striking 81.5% conversion in phenotype (compare their Figure 5 with their Figure 1), which raises another interesting possibility that transgenic manipulation of sTnC in cardiac muscle was coupled with compensatory expression of sTnI.

In summary, the results of Gulati and Babu (1989) at pH 6.5 are extended to the rat at a lower pH of 6.2. The study reinforces the overall conclusion that TnC moiety is only partially responsible for the pH effect on Ca^{2+} sensitivity for force development in cardiac muscle. We have now also indicated that the pH-sensing element for this TnC-mediated regulation of Ca^{2+} sensitivity resides within the N-terminal 41 residues. The additional striking finding is the firm identification of a dominant contribution of the TnI moiety to the overall acidotic mechanism.

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