Isoform Specific Interactions of Troponin I and Troponin C Determine pH Sensitivity of Myofibrillar Ca^{2+} Activation†

Karen L. Ball, Mary D. Johnson, and R. John Solaro*

Department of Physiology and Biophysics, University of Illinois College of Medicine, Chicago, Illinois 60612-7342

Received January 13, 1994; Revised Manuscript Received May 9, 1994®

ABSTRACT: We investigated whether differences in isoforms of troponin I (TnI) and troponin C (TnC) can account for the greater inhibition of Ca^{2+}-dependent MgATPase activity by acidic pH in cardiac (c) than in fast skeletal (fs) myofilaments. We studied fast skeletal myofibrils from which whole Tn was extracted by displacement with excess fsTnT (the tropomyosin binding subunit of Tn) followed by reconstitution with TnC–TnI. Exchange of fsTnI with cTnI did not alter the effect of a drop in pH from 7.0 to 6.5 on the relation between pCa (–log [Ca^{2+}]) and MgATPase activity of fast skeletal myofibrils. Exchange of fsTnC with cTnC did, however, induce an increase in the effect of this same pH change on Ca^{2+} activation. Yet, the pH sensitivity of Ca^{2+} activation of fast skeletal myofibrils containing cTnC was not as great as that of native cardiac myofibrils. However, when both fsTnC and fsTnI of fast skeletal myofibrils were replaced by cTnC–cTnI, there was a pH-induced shift in Ca^{2+} sensitivity similar to that of cardiac myofibrils. In studies using fluorescent probes, both pure fsTnC and pure cTnC showed decreased Ca^{2+} binding as pH was lowered. This decrease was potentiated in the fsTnC–fsTnI and cTnC–cTnI complexes. However, the effect of acidic pH was the same in fsTnC and the hybrid complex, fsTnC–cTnI, and in cTnC and the hybrid complex, cTnC–fsTnI. Thus, isoform specific interactions between TnI and TnC appear important in the differential response of skeletal and cardiac myofilaments to acidosis.

In the present study our aim was to investigate the role of thin filament protein interactions in deactivation of striated myofilaments by decreased pH. Under acidotic conditions, there is a marked reduction in maximal force generation, maximal unloaded shortening velocity, and Ca^{2+} sensitivity of striated myofilaments (Donaldson & Hermansen, 1978; Metzger & Moss, 1987, 1988, 1990; Robertson & Kerrick, 1979; Solaro et al., 1988). Alterations in maximum force and shortening velocity have been accounted for by effects of acidic pH on cross-bridge function (Metzger & Moss, 1987, 1988, 1990), yet the potential mechanism(s) underlying a reduction in Ca^{2+} sensitivity are more complex and have yet to be fully defined.

An interesting aspect of the effect of decreased pH that has provided initial insight into the underlying mechanism(s) is the observation that the response to altered pH depends on fiber type (Donaldson & Hermansen, 1978; Metzger & Moss, 1987, 1988, 1990). For example, under acidic conditions skinned fiber preparations from fast skeletal muscle demonstrate a greater reduction in maximum force generation and Ca^{2+} sensitivity than slow skeletal fibers (Metzger & Moss, 1987, 1988, 1990). Metzger and Moss (1987, 1988, 1990) have proposed that differences in myosin isoforms present in various striated muscle types could account for altered susceptibility to deactivation by decreased pH. Work from our laboratory, however, suggests a significant role of the thin filament as well. We have shown that neonatal rat cardiac myofilaments are resistant to the effects of acidosis, decreases in Ca^{2+} sensitivity being much less than that of adult cardiac myofilaments (Solaro et al., 1988). This developmental difference in pH sensitivity is also apparent in dog heart myofibrils, which demonstrate no change in myosin isoform populations with development (Solaro et al., 1986). Inasmuch as troponin C (TnC)[1] also demonstrates no changes in isoform composition during myocardial development, we proposed that this differential response to acidic pH is due to isoform switching of TnI (Martin et al., 1991; Solaro et al., 1986). However, there are differences in TnC isoforms between fast skeletal and cardiac muscle that might account for differential responses of these fiber types to acidosis (Parmacek & Leiden, 1991).

It has been proposed that acidosis leads to interference of Ca^{2+} binding to TnC and/or transmission of the Ca^{2+} signal through the thin filament (Blanchard et al., 1984; Blanchard & Solaro, 1984; El-Saleh & Solaro, 1988). Due to the critical nature of Ca^{2+} binding to TnC and subsequent interaction with TnI in myofilament activation, it could be hypothesized that isoform specific differences in the structure/function of either protein will affect how the myofilaments respond to increased H^+. To address this hypothesis, we have systematically studied the contributions of TnC and TnI in the response of skeletal myofibrils to decreased pH. By selectively altering the TnC and/or TnI populations within fast skeletal preparations, we demonstrated that when both fsTnC and fsTnI were replaced with the corresponding cardiac isoforms, fast skeletal myofibrils appeared similar to cardiac myofibrils in terms of their sensitivity to deactivation of Ca^{2+}-dependent ATPase

† Supported by NIH Grants R01-HL-22231 (R.J.S.) and T32-HL-07692 (K.L.B.).

* Address correspondence to this author at the Department of Physiology and Biophysics, University of Illinois at Chicago, 901 South Wolcott Ave., Room E202, Chicago, IL 60612-7342. Phone: (312) 996-7620. Fax: (312) 996-1414.

® Abstract published in *Advance ACS Abstracts,* June 15, 1994.

[1] Abbreviations: TnC, troponin C; TnI, troponin I; fsTnC, fast skeletal troponin C; fsTnI, fast skeletal troponin I; fsTnT, fast skeletal troponin T; PMSF, phenylmethanesulfonyl fluoride; BME, β-mercaptoethanol; DTT, DL-dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DANZ, 5-dimethylaminonaphthalene-1-sulfonyl aziridine; IAANS, 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid; DMSO, dimethyl sulfoxide; $fsTnC_{DANZ}$, danzylaziridine-labeled fast skeletal troponin C; $cTnC_{IA}$, IAANS-labeled cardiac troponin C; pCa, –log molar free calcium concentration; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/94/0433-8464$04.50/0

activity by acidic pH. Through use of fluorescent probes, we further showed that whereas cTnI and fsTnI induced an increase in the effect of acidic pH on their respective isoforms of TnC, there was no effect of either cTnI or fsTnI in hybrid complexes with fsTnC and cTnC. It is apparent therefore that effects of acidic pH on transmission of the TnC–Ca^{2+}-binding signal depends on the isoforms of TnI present.

EXPERIMENTAL PROCEDURES

Preparations

Isolation of Myofibrillar Proteins. Myofibrils were isolated from bovine ventricular and rabbit psoas tissue as described previously (Pagani & Solaro, 1984). Tissue was processed by homogenization, Triton X-100 extraction, and extensive washing in the standard buffer composed of 60 mM KCl, 30 mM imidazole, pH 7.0, and 2 mM $MgCl_2$ containing the protease inhibitors leupeptin (0.5 μg/mL), pepstatin A (0.5 μg/mL), and PMSF (0.2 mM). Isolated myofibrils were resuspended in the standard buffer, and protein concentration was determined by the method of Lowry et al. (1951).

Isolation of Cardiac and Skeletal Troponin Subunits. Crude troponin was initially prepared from bovine cardiac and rabbit back skeletal muscle ether powder preparations (Potter, 1982). Final purification of cardiac and skeletal troponin subunits was performed by ion exchange column chromatography as detailed by Potter (1982).

Displacement of Troponin by Excess TnT and Reconstitution with the TnC–TnI Complex. TnC and TnI were extracted from isolated rabbit psoas myofibrils by slight modification of the protocol described by Shiraishi et al. (1992). Isolated myofibrils were resuspended to a concentration of 6 mg/mL in extracting solution which was composed of 250 mM KCl, 20 mM MOPS, pH 6.5, 5 mM EGTA, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.1 μg/mL pepstatin A. Skeletal TnT (suspended in extracting solution) was added to the myofibrils to produce fsTnT concentration in the myofibrillar suspension of 0.35 mg/mL. The mixture was then incubated at 25 °C for 60 min with gentle shaking every 2–3 min. Following centrifugation at 2000*g*, extracted myofibrils were resuspended to a concentration of 12 mg/mL in the reconstitution buffer which was composed of 10 mM KCl, 20 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.1 μg/mL pepstatin A. Following addition of the TnC–TnI complex (final concentration of 3 mg/mL), the preparation was incubated at 25 °C for 75 min with gentle shaking. Protein was collected by centrifugation at 2000*g* for 15 min, and reconstituted myofibrils were resuspended in standard buffer for measurement of myofibrillar ATPase activity.

Preparation of the TnC–TnI complex for reconstitution was modified from the protocol of Johnson et al. (1980). Skeletal and cardiac isoforms of TnC and TnI were dissolved in 6 M urea (concentration of about 6 mg/mL) and equimolar amounts of TnC and TnI (0.2 mmol) mixed together. The TnC–TnI mixture was then sequentially dialyzed against the following: once against 6 M urea, 2.5 mM $CaCl_2$, 50 mM Tris, pH 8.0; one time against 6 M urea, 100 mM KCl, 10 mM Tris, pH 8.0, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM DTT; and two times versus the reconstitution buffer described above. Each dialysis was against a 1-L volume for at least 8 h.

Fluorescence Labeling of Skeletal and Cardiac TnC and Formation of the TnC–TnI Complex. Skeletal TnC was labeled with danzylaziridine (DANZ), as detailed by Johnson et al. (1978). A 2-fold molar excess of DANZ was added to fsTnC (about 2 mg/mL) dissolved in 10 mM MOPS, pH 7.0, 90 mM KCl, 2 mM EGTA, and 2.5 mM $CaCl_2$. After incubation at room temperature for 24 h, free label was removed by exhaustive dialysis against 10 mM MOPS, pH 7.0, 90 mM KCl, and 2 mM EGTA. Following this protocol, incorporation of the probe was 1.1 mol DANZ/mol fsTnC. Cardiac TnC was labeled with the fluorophore 2-(4′-iodoacetamidoanilino)napthalene-6-sulfonic acid (IAANS). The protocol for labeling was as described by Johnson et al. (1980) with the following modifications. The IAANS stock (10 mM) was dissolved in DMSO and incubated in 5-fold molar excess to cTnC. Labeling conditions were 6 M urea, 90 mM KCl, 10 mM MOPS, pH 7.0, 2 mM EGTA, and 2.6 mM $CaCl_2$. The reaction proceeded for 8 h at room temperature and was followed by gel filtration on Sephadex G-50 to remove excess free label. Incorporation of the probe with this protocol was 0.57 mol/mol cTnC.

Following labeling, $fsTnC_{DANZ}$ and $cTnC_{IA}$ were complexed with TnI as described (Johnson et al., 1980), with the following modifications. Cardiac or skeletal TnI was dissolved in 9 M urea and an equimolar amount mixed with the labeled TnC. The mixture was then dialyzed sequentially against the following: 500 mL of 6 M urea, 10 mM MOPS, pH 7.0, 0.5 mM $CaCl_2$, 0.5 mM DTT; 2 L of 0.25 M KCl, 10 mM MOPS, pH 7.0, 3 mM $MgCl_2$, 0.1 mM DTT; 2 L of 90 mM KCl, 10 mM MOPS, pH 7.0, 3 mM $MgCl_2$, 0.1 mM DTT. Samples were then centrifuged to remove any uncomplexed TnI.

Procedures

Myofibrillar ATPase Activity. Ca^{2+}-dependent Mg^{2+}-ATPase activity of isolated myofibrils was assayed by determination of inorganic phosphate release during incubation at 30 °C. Assay conditions were 2 mM Mg^{2+}, 60 mM imidazole, 5 mM $MgATP^{2-}$, 120 mM ionic strength, at pH 7.0 or 6.5. Ca^{2+} concentrations were varied over a range of pCa (–log of molar free calcium) values from 8.0 to 4.875. Total concentrations of $CaCl_2$, EGTA, KCl, $MgCl_2$, and ATP required were calculated by a computer program generated from the dissociation constants as reported by Fabiato (1981). Enzyme activity was initiated by addition of ATP and terminated during the linear phase of the reaction with 10% trichloroacetic acid at 5 min for skeletal preparations and at 10 min for cardiac myofibrils. Following centrifugation for 10 min at 3000 rpm to remove precipitable material, supernatant fractions were assayed for inorganic phosphate by the method of Carter and Karl (1982). Nonlinear least-squares regression was used to fit pCa-ATPase activity relationships to the Hill equation:

$$\text{relative activity} = [Ca^{2+}]^n/(K + [Ca^{2+}]^n)$$

Shifts in pCa_{50} values were analyzed for significance by an unpaired *t*-test with the criteria for significance set at $p < 0.05$.

Polyacrylamide Gel Electrophoresis. Myofibrillar proteins were analyzed on 12.5% polyacrylamide gels in the presence of sodium dodecyl sulfate, as described by Laemmli (1970). Separated proteins were visualized by Coommassie blue staining. To analyze TnC content, myofibrils were separated by alkaline urea gel electrophoresis as described previously (Blanchard & Solaro, 1984).

Fluorescence Measurements. Fluorescence measurements of Ca^{2+}-binding to TnC were all made on a Perkin-Elmer LS-5B luminescence spectrometer. For the $cTnC_{IA}$ measurements, the excitation wavelength was 330 nm with fluorescence

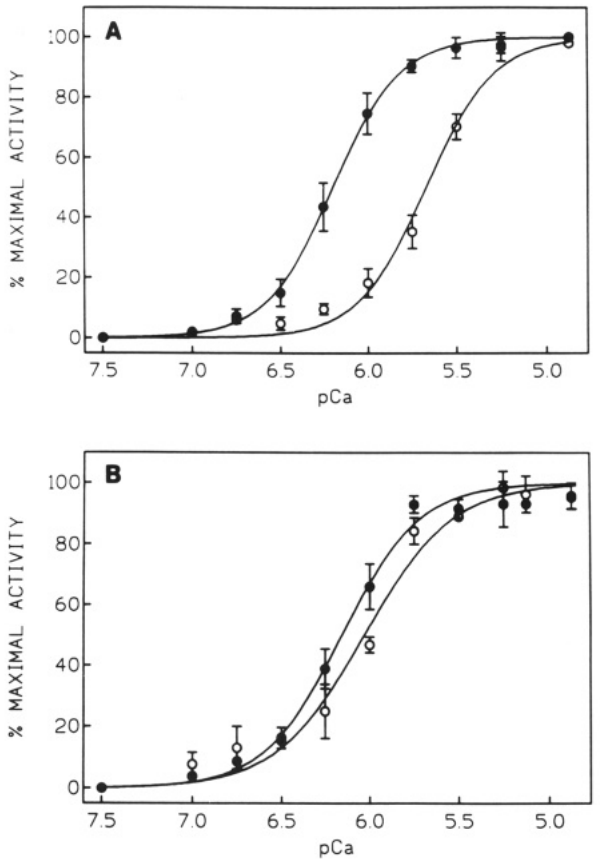

FIGURE 1: Effect of decreased pH on the pCa-ATPase activity relationship of cardiac (A) and fast skeletal (B) myofibrils. Isolated myofibrils from bovine ventricular and rabbit psoas muscle were assayed for Ca^{2+}-dependent Mg^{2+}-ATPase activity at pH 7.0 (closed circles) and 6.5 (open circles) as described under Experimental Procedures. Other conditions were 2 mM Mg^{2+}, 60 mM imidazole, 5 mM $MgATP^{2-}$, and 120 mM ionic strength. Data were fit to the Hill equation by nonlinear least-squares regression and are means ± SE. Shifts in pCa_{50} were 0.52 ± 0.03 (n = 5) for cardiac and 0.10 ± 0.08 (n = 3) for skeletal preparations.

emission monitored at a wavelength of 445 nm. Excitation and emission wavelengths for $fsTnC_{DANZ}$ measurements were at 340 and 520 nm, respectively. The wavelengths chosen for measurements were the maximum wavelengths obtained from excitation and emission spectra run prior to Ca^{2+} titrations. Measurements were made with protein concentrations of about 1 μM, and Ca^{2+} titrations were performed in 90 mM KCl, 10 mM MOPS, 2 mM EGTA, and 3 mM $MgCl_2$. The pH of the measurements was at either 7.0 or 6.5, and the appropriate free Ca^{2+} concentrations were achieved by sequential addition of $CaCl_2$ as calculated using the binding constants reported by Fabiato (1981). Conditions were such that pH did not change over the range of pCa's examined. Normalized changes in fluorescence intensities were fit to the Hill equation as described above, shifts in pCa_{50} values analyzed by a paired t-test, and differences between shifts analyzed by an unpaired t-test with significance set at $p < 0.05$.

RESULTS

Native and Reconstituted Myofibrillar Preparations. Data depicted in Figure 1 show differences in the effect of decreased pH on the relationship between pCa and Ca^{2+}-dependent Mg^{2+}-ATPase activity of cardiac and skeletal myofibrils. In panel A, myofibrils isolated from bovine cardiac tissue demonstrate a decrease in Ca^{2+} sensitivity as pH was decreased from 7.0 to 6.5. This desensitization, as evidenced by a

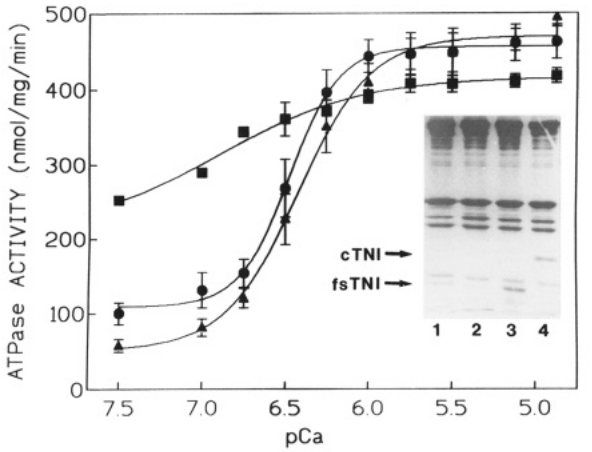

FIGURE 2: Extraction of rabbit psoas myofibrils with excess fsTnT and reconstitution with TnI–TnC complex. See Experimental Procedures for details. The Ca^{2+} dependence of myofibrillar ATPase activity is shown for control (closed circles), extracted (closed squares), and myofibrils reconstituted with the fsTnC–fsTnI complex (closed triangles). Data are mean ± SE, n = 3. ATPase activity was measured at pH 7.0. Other conditions are as given in the legend to Figure 1. (Inset) 12.5% SDS–PAGE profile of myofibrillar preparations. Lane 1, controls; lane 2, extracted myofibrils; lane 3, reconstituted with fsTnC–fsTnI; lane 4, reconstituted with fsTnC–cTnI.

rightward shift in the pCa-ATPase activity relationship, corresponded to a shift in pCa_{50} (half-maximally activating pCa) of 0.52 ± 0.03 (n = 5) pCa units. In contrast, myofibrils isolated from rabbit psoas tissue (panel B) were relatively resistant to the effects of decreased pH, with only a 0.10 ± 0.08 (n = 3) pCa unit shift being observed.

To investigate the role of thin filament protein isoforms in these observed differences, we systematically exchanged skeletal and cardiac isoforms of TnI and TnC into fast skeletal myofibrillar preparations. We took advantage of a method which uses excess TnT to displace the Tn complex from myofibrils (Shiraishi et al., 1992). These preparations, which show no loss of other myofilament proteins, can then be reconstituted with various TnC–TnI complexes. The effectiveness of this protocol is shown in Figure 2. Analysis of protein content by SDS–PAGE is shown in the inset (proteins with mobilities faster than TnI were run off the gel to get better separation of higher molecular weight components). Comparison of lanes containing proteins from control (lane 1) and fsTnT-treated preparations (lane 2) shows that, although extraction was not complete, there was a marked reduction in content of fsTnI (lane 2) and fsTnC (data with alkaline urea gels not shown). We could detect no significant change in any of the other myofibrillar proteins. TnI and TnC levels were restored with reconstitution (Figure 2, lanes 3 and 4). The plot of ATPase activity versus pCa, depicted in Figure 2, demonstrated that, following treatment of skeletal myofibrils with excess fsTnT, myofibrillar ATPase activity was markedly enhanced at low Ca^{2+} concentrations, with a loss of activity at maximally activating Ca^{2+} levels. These data indicate the loss of both TnI and TnC from the myofibrillar preparations. Following incubation with the fsTnC–fsTnI complex, activity at relatively high pCa values was once again inhibited and there was a concomitant restoration of maximal activity.

Myofibrils were reconstituted with one of four TnC–TnI combinations: fsTnC–fsTnI, fsTnC–cTnI, cTnC–fsTnI, or cTnC–cTnI. The effectiveness of reconstitution with all four combinations is shown in Figure 3. Reconstitution with fsTnC–fsTnI (panel A) and fsTnC–cTnI (panel C) resulted in restoration of both inhibition of ATPase activity at low

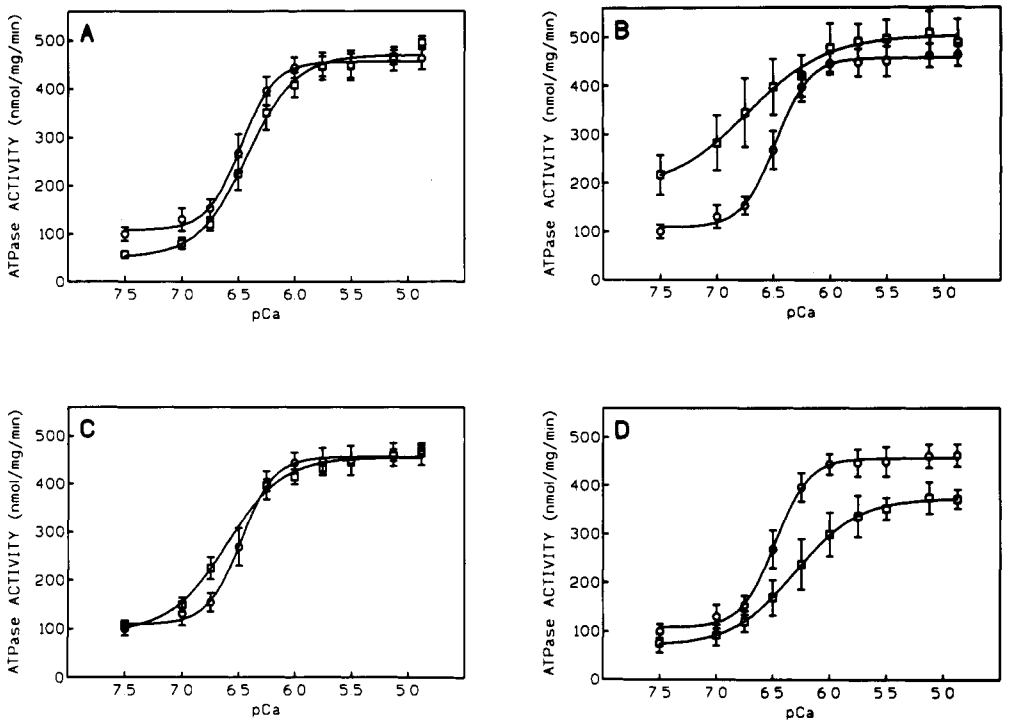

FIGURE 3: pCa-ATPase activity relationships of psoas myofibrils reconstituted with four combinations of TnC–TnI. In all panels native psoas myofibrils (open circles) and reconstituted preparations (open squares). (A) fsTnC–fsTnI; (B) cTnC–cTnI; (C) fsTnC–cTnI; and (D) cTnC–fsTnI. ATPase activity was measured at pH 7.0, and data were fit to the Hill equation by nonlinear least-squares regression. Other conditions are as given in the legend to Figure 1. Data are means ± SE, $n = 3$.

Ca^{2+} concentrations and maximal Ca^{2+} activation. In contrast, exchange of cTnC for endogenous fsTnC (panel D) resulted in a small depression of maximal activity consistent with results from Moss et al. (1991) in which replacement of cTnC for fsTnC in skinned fibers resulted in a 10% reduction in maximal force generation. Reconstitution with cTnC–cTnI (panel B) resulted in restoration of maximal ATPase activity but inhibition at low Ca^{2+} concentrations to a lesser extent than controls. This is consistent with reports that, when substituted into skeletal preparations, cTnI is less effective than fsTnI in inhibition of ATPase activity (Talbot & Hodges, 1981b) and force generation (Rüegg et al., 1989). Reconstituted preparations in which fsTnC was replaced with cTnC (panels B and D of Figure 3) demonstrate a reduced cooperativity in Ca^{2+} activation as shown by a reduced steepness of the pCa–activity curves. We think the difference in slope reflects the presence of cTnC in these preparations. This interpretation agrees with previous work of Moss et al., (1986), who showed that substitution of cTnC for fsTnC in skinned skeletal muscle fibers alters the pCa_{50} for Ca^{2+} activation and reduces the cooperativity. This is apparently related to the fact that there is a single regulatory binding site in cTnC and two in fsTnC, which reduces cooperative activation of the thin filament by Ca^{2+} and by cross-bridges.

After reconstitution, ATPase activity was measured at pH 7.0 and 6.5, and the pCa–ATPase activity relationships are shown in Figure 4. Panel A demonstrates that Ca^{2+} activation of myofibrils reconstituted with fsTnC–fsTnI was resistant to decreased pH (pCa_{50} shift = 0.09 ± 0.07, $n = 3$), in a manner similar to that of native skeletal preparations (Figure 1). Substitution of only cTnI for fsTnI did not change the pH resistance of skeletal myofibrils (panel B) (pCa_{50} shift = 0.12 ± 0.06, $n = 3$). When cTnC was substituted for fsTnC, however, the myofibrils demonstrated an increased response to acidic pH with a 0.25 ± 0.02 unit shift in the pCa_{50} (panel C) ($n = 3$; $p < 0.05$ vs fsTnC–fsTnI or fsTnC–cTnI). Interestingly, it was only when the cTnC–cTnI complex was reconstituted into the skeletal preparation (panel D) that the maximal rightward shift in pCa_{50} was observed (pCa_{50} shift = 0.47 ± 0.04, $n = 3$; $p < 0.05$ vs fsTnC–fsTnI, fsTnC–cTnI, or cTnC–fsTnI). Thus, substitution of both cTnC and cTnI into fast skeletal myofibrils was required to produce a change in pCa_{50} with acidic pH similar to that shown in Figure 1 for native cardiac myofibrils.

Fluorescence Measurements of TnC–TnI Interactions. Results with myofibrillar preparations indicated that the effect of acidosis on the affinity of TnC for Ca^{2+} is influenced by the specific isoform of TnI with which it reacts. Using fluorescently labeled TnC, we did a series of experiments to determine the effect of acidic pH on TnC alone and TnC complexed with the two isoforms of TnI. We did not expect these experiments to provide quantifiable comparisons with the more complex myofibrillar assembly splitting ATP. Activation and potentially TnC Ca^{2+}-binding of thin filaments in the myofibrils is dependent on cross-bridge binding, which is not present in the isolated regulatory proteins. Yet, as will be shown, our data from studies using fluorescent probes demonstrate isoform specific interactions between TnI and TnC with potential significance to pH responsiveness.

Our first approach was to label fsTnC with danzylaziridine ($fsTnC_{DANZ}$), a fluorophore known to report Ca^{2+}-binding to the regulatory Ca^{2+}-specific binding sites (Johnson et al., 1978). As shown in Figure 5A and Table 1, a decrease in pH resulted in a drop in Ca^{2+} binding as indicated by a decrease in the pCa required for half-saturation (0.33 ± 0.02 unit shift in pCa_{50}, $n = 3$; $p < 0.05$). Previous reports using fsTnC labeled with danzylaziridine have demonstrated that this rightward shift is potentiated when fsTnC is complexed with fsTnI (El-Saleh & Solaro, 1988). Similarly, we observed a potentiation of the decreased Ca^{2+} affinity when $fsTnC_{DANZ}$ was complexed with fsTnI (panel B), the shift in pCa_{50} being 2.3-fold greater (pCa_{50} shift = 0.77 ± 0.03, $n = 3$; $p < 0.05$

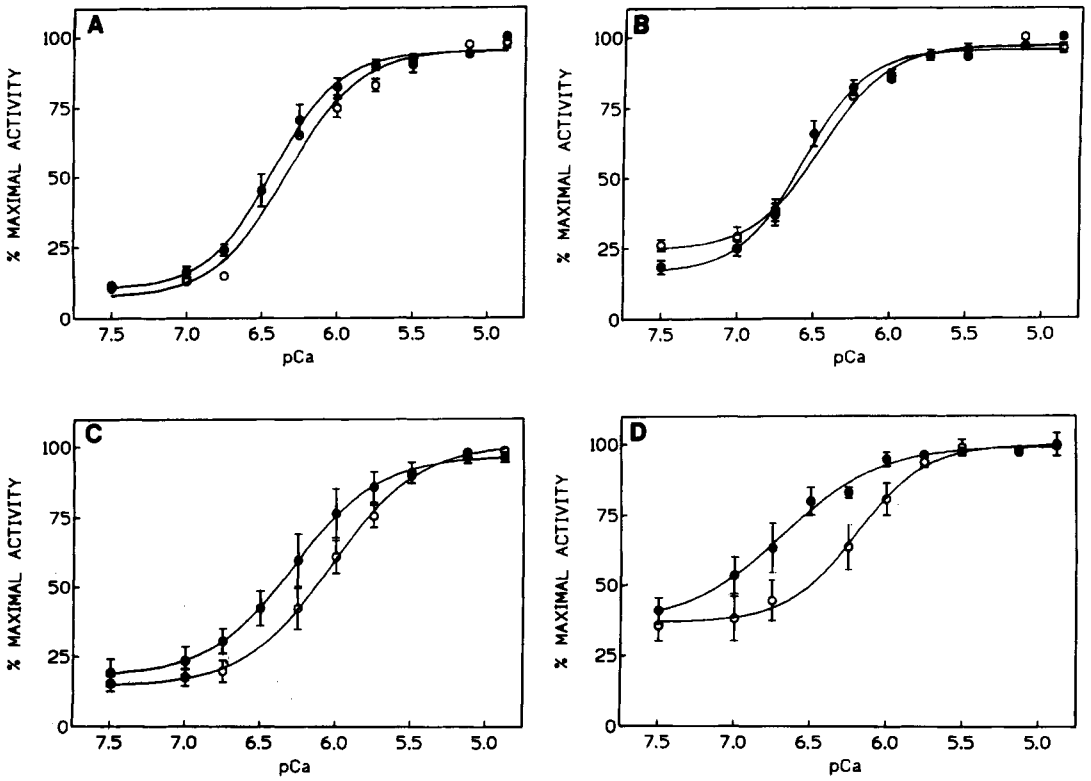

FIGURE 4: Effects of pH on Ca^{2+} activation of psoas myofibrils reconstituted with various isoforms of TnC and TnI. Conditions are as given in the legend to Figure 1; measurements were made at pH 7.0 (filled circles) and at pH 6.5 (open circles). (A) fsTnC–fsTnI; (B) fsTnC–cTnI; (C) cTnC–fsTnI; and (D) cTnC–cTnI. Shifts (mean ● SE; $n = 3$) in pCa_{50} values were 0.09 ± 0.07 for fsTnC–fsTnI, 0.12 ± 0.06 for fsTnC–cTnI, 0.25 ± 0.02 for cTnC–fsTnI, and 0.47 ± 0.04 for cTnC–cTnI. See the text for statistical comparisons.

Table 1: Summary of Fluorescence Titrations

	pCa_{50}[a]		
	pH 7.0	pH 6.5	change in pCa_{50}[b]
$cTnC_{IA}$[c]	5.89 ● 0.01	5.68 ± 0.01	0.21
$cTnC_{IA}$–cTnI	7.17 ± 0.02	6.86 ± 0.04	0.33[d]
$cTnC_{IA}$–fsTnI	6.42 ± 0.01	6.21 ± 0.03	0.20
$fsTnC_{DANZ}$	6.25 ± 0.01	5.92 ± 0.01	0.33
$fsTnC_{DANZ}$–fsTnI	6.87 ± 0.01	6.10 ± 0.02	0.77[e]
$fsTnC_{DANZ}$–cTnI	6.75 ● 0.02	6.46 ● 0.02	0.29

[a] pCa_{50}, half-maximally saturating Ca^{2+} concentration. [b] pCa_{50}, change in pCa_{50}. [c] $n = 3$ for all titrations. [d] Significantly different from $cTnC_{IA}$ and $cTnC_{IA}$–fsTnI, $p < 0.05$. [e] Significantly different from $fsTnC_{DANZ}$ and $fsTnC_{DANZ}$–cTnI, $p < 0.05$.

vs $fsTnC_{DANZ}$). When complexed with cTnI (Figure 5C), however, there was no potentiation of the pH effect, the shift in pCa_{50} being 0.29 ± 0.07 ($n = 3$) pCa units. Calcium titration of the regulatory sites of $fsTnC_{DANZ}$ alone or complexed with fsTnI resulted in an increase in the fluorescence signal. When $fsTnC_{DANZ}$ was complexed with cTnI, however, the fluorescence signal was quenched upon binding of Ca^{2+}. These data, together with the different effects of decreased pH on Ca^{2+} binding to the $fsTnC_{DANZ}$–fsTnI and $fsTnC_{DANZ}$–cTnI complexes, indicate that reaction of fsTnI and cTnI with TnC_{DANZ} produces different conformational changes associated with Ca^{2+} binding to $fsTnC_{DANZ}$.

We also labeled cTnC with IAANS ($cTnC_{IA}$) and measured the effects of decreased pH on Ca^{2+} binding to the single regulatory site. As shown in Figure 6 (panel A) and Table 1, $cTnI_{IA}$ demonstrated a decrease in Ca^{2+}-binding to the Ca^{2+}-specific site with a 0.5 pH unit drop (pCa_{50} shift = 0.21 ± 0.01, $n = 3$; $p < 0.05$). This decrease in Ca^{2+} affinity was potentiated when $cTnC_{IA}$ was complexed with cTnI (panel B), the change in pCa_{50} being 64% greater (0.33 ± 0.03, $n = 3$; $p < 0.05$ vs $cTnC_{IA}$). Complex formation of $cTnC_{IA}$ with fsTnI, however, did not elicit an enhancement of the pH effect (panel C), the shift in pCa_{50} being 0.20 ± 0.01 ($n = 3$) compared to 0.21 ± 0.01 pCa units for $cTnC_{IA}$ alone.

DISCUSSION

The question we have addressed in the present paper is, Why is there a difference among the various types of striated muscle in susceptibility to deactivation by acidosis? This question must be considered in the context of the two apparently independent mechanisms by which acidosis could deactivate striated myofilaments. One mechanism involves direct or indirect effects of acidosis on the affinity of TnC for Ca^{2+}. The other involves inhibition of the effect of strongly attached cross-bridges on thin filament activation, a mechanism we will discuss first.

It is now clear that the reaction of the cross-bridge with the thin filament can itself disinhibit the thin filament (Bremel & Weber, 1972). This disinhibition is a cooperative process involving near-neighbor cross-bridges (Brandt et al., 1984; Bremel & Weber, 1972; Moss, 1992). Inhibition of the actin–myosin reaction as pH falls could, therefore, have an effect on myofilament response to Ca^{2+} with no change in Ca^{2+} bound to TnC. In fact, evidence that acidic pH has a bigger effect on maximum force developed by fast as compared to slow skeletal muscle myofilaments provides an example of how different effects of acidosis on actin–myosin interactions might account for the more pronounced shift in pCa_{50} in slow versus fast skeletal myofilaments. In this case, as pH decreases, relatively fewer force generating cross-bridges engage the thin filament in fast muscle myofilaments (Metzger & Moss, 1987, 1988, 1990), and the ability of cross-bridges to disinhibit the thin filaments would therefore be less in fast versus slow myofilaments. It follows that as pH falls the level of thin filament activation and steady-state force production at a given

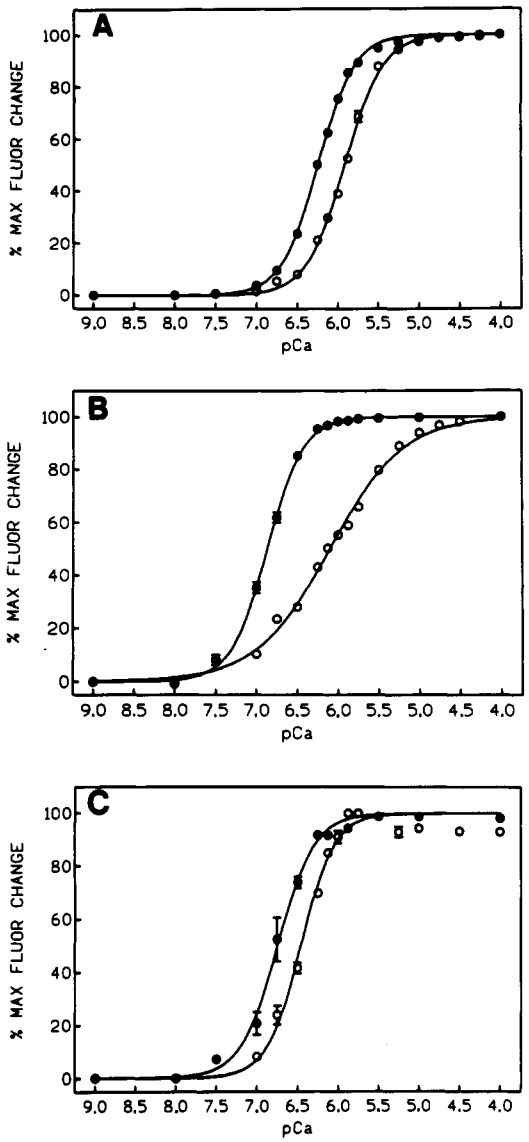

FIGURE 5: Comparison of the effects of decreased pH on Ca^{2+} binding to the regulatory sites of $fsTnC_{DANZ}$ alone or complexed with TnI. Calcium-dependent fluorescence changes were measured in 10 mM MOPS, 90 mM KCl, 2 mM EGTA, and 3 mM $MgCl_2$ as detailed under Experimental Procedures. Normalized pCa-fluorescence changes are shown as obtained at pH 7.0 (closed circles) and 6.5 (open circles) for (A) $fsTnC_{DANZ}$ alone, (B) $fsTnC_{DANZ}$–fsTnI, and (C) $fsTnC_{DANZ}$–cTnI. Data are means ± SE; $n = 3$. SE measurements not apparent were smaller than symbol size. See Table 1 for statistical comparisons.

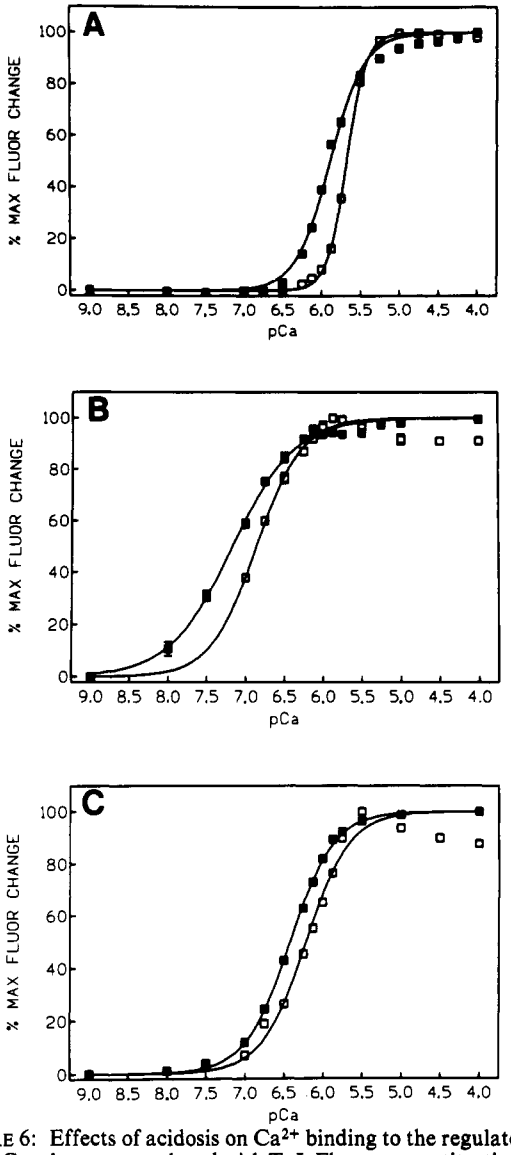

FIGURE 6: Effects of acidosis on Ca^{2+} binding to the regulatory site of $cTnC_{IA}$ alone or complexed with TnI. Fluorescence titrations were performed as detailed in the legend to Figure 5 and under Experimental Procedures. Normalized pCa–fluorescence relationships at pH 7.0 (closed squares) and 6.5 (open squares) are shown for (A) $cTnC_{IA}$ alone, (B) TnC_{IA}–cTnI, and (C) TnC_{IA}–fsTnI. Data are means ± SE; $n = 3$. See Table 1 for statistical comparisons.

pCa value would be less in fast skeletal myofilaments, resulting in a larger rightward shift of the relative force–pCa relation.

Although this mechanism might well account for some of the differences in effects of acidic pH on striated myofilaments, results presented here and earlier work (Solaro et al., 1986, 1988; Martin et al., 1991) indicate that there are additional mechanisms that may be especially important in the differences between skeletal and cardiac myofilaments. In particular, studies on the developing myocardium argue against thick filament alterations as the most prominent mechanism for a differential response to acidosis. For example, despite the fact that neonatal and adult rat heart preparations contain different myosin isoforms and both display equivalent reductions in relative maximum force generation upon decreasing pH from 7.0 to 6.5 (Solaro et al., 1988), neonatal cardiac myofilaments are relatively resistant to deactivation of Ca^{2+} sensitivity by acidic pH (Solaro et al., 1986, 1988; Martin et al., 1991). Similar developmental studies in the dog myocardium demonstrate resistance of neonatal myofilaments to acidosis despite the same myosin isoform profile as the adult (Solaro et al., 1986).

From evidence discussed above, it is apparent that mechanisms involving differential effects of the affinity of TnC for Ca^{2+} may be the most prominent reason for the different effects of acidosis on Ca^{2+} activation of skeletal and cardiac myofilaments. This difference appears to be due not only to TnC itself but also to other thin filament proteins with effects on transmission of the Ca^{2+}-binding signal (Blanchard et al., 1984; Blanchard & Solaro, 1984; El-Saleh & Solaro, 1988). Perhaps the most compelling evidence for this comes from studies showing differences in effects of acidosis on cardiac myofilament TnC Ca^{2+}-binding and force or ATPase activity despite the expression of the same TnC isoform (cTnC) throughout development (Solaro et al., 1986, 1988). Data in the present study argue further for the specific importance of the TnC–TnI interaction in pH responsiveness. The data further imply that various isoforms of TnC and TnI interact

differently such that interference with transmission of the Ca^{2+} signal by acidic pH is modulated differently in various striated muscles. The exact mechanism whereby acidosis might interfere with the TnC–TnI interaction remains unclear owing to a lack of detailed information on the structure of TnI and its interaction with TnC, especially in the case of the cardiac variants. There is, however, enough structural and functional information available to permit speculation.

Using transgenic animals overexpressing fsTnC in the cardiac compartment, Metzger et al. (1993) recently showed that substitution of fsTnC for cTnC in myofibrils was associated with a smaller pH-induced shift in the pCa_{50} for activation of force. Our results demonstrating that substitution of fsTnC for cTnC in fast skeletal myofibrils is associated with a bigger pH-induced shift in Ca^{2+} activation support their findings and provide further evidence for different effects of protons on TnC isoforms. Protonation of the carboxyl groups of fsTnC produces large conformational changes as evidenced by an enhancement of tyrosine fluorescence due to increased distance between the tyrosine residue and neighboring quenching groups (Lehrer & Leavis, 1974). Ingraham and Swenson (1983) further confirmed these findings by demonstration that titration of the carboxyl groups within fsTnC results in a stabilization of the N-terminal domain. It was suggested that this stabilization occurs as a result of cancellation of the repulsive electrostatic interactions between the clustered carboxylates within the Ca^{2+}-binding domains. Distinct differences are apparent between fsTnC and cTnC in the carboxylate clustering within Ca^{2+} binding site I. Due to the fact that these carboxyl groups are protonated in fsTnC, significant differences in titratability and conformational alterations might be expected.

In our studies, the greatest change in the fast skeletal myofibrillar response to acidic pH occurred with substitution of both cTnC and cTnI. These results indicated differences in effects of pH on the reaction of isoforms of TnC and TnI. On the basis of studies to date, it appears there are three domains of TnC and TnI that interact with each other. The first and most well characterized is a basic peptide of TnI containing the minimal sequence for inhibitory activity ($fsTnI_{104-115}$ and $cTnI_{137-148}$). This TnI peptide also reacts with actin and has been shown to bind to the NH_2-terminal acidic domains of fsTnC, specifically at $fsTnC_{50-60}$ and $fsTnC_{90-100}$. Primary sequence analysis of $fsTnI_{104-115}$ and $cTnI_{137-148}$ suggest relatively few specific sites of divergence. One specific site with potential functional significance, however, is at $fsTnI_{110}$. A proline residue at this position in the fast skeletal sequence is exchanged for a threonine residue at $cTnI_{143}$. Although both $fsTnI_{104-115}$ and $cTnI_{137-148}$ exhibit random coil structure in solution, their CD spectra are different, suggesting that the single residue substitution will alter conformational states of the inhibitory peptides in solution (Van Eyk et al., 1991). Additionally, an analogue of $fsTnI_{104-115}$ in which Pro_{110} has been exchanged for a glycine residue (G110-TnI) demonstrated that this substitution renders the peptide much more flexible (Campbell et al., 1992), potentially accounting for observed functional differences of $fsTnI_{104-115}$ and G110-TnI (Rüegg et al., 1989; Van Eyk & Hodges, 1988; Van Eyk et al., 1991). These structural data could therefore account for observed functional differences between the fsTnI and cTnI, in which it has been shown that fsTnI and $fsTnI_{104-115}$ are better inhibitors of both myofibrillar ATPase activity (Talbot & Hodges, 1981b) and skinned fiber force generation (Rüegg et al., 1989) than the corresponding cardiac variants. Whether this difference in inhibitory activity between the TnI isoforms is important in the differential response to acidic pH is not known. Inasmuch as the structure of this important region is different, independent of pH, it is reasonable to speculate that acidic pH will also affect the conformational states of the peptides differently.

The $fsTnI_{104-115}$ and $cTnI_{137-148}$ contain the minimal sequence for inhibitory activity, yet it is likely that the regions flanking these peptides are also of functional significance and may be important in the differential response to acidic pH. Although $fsTnI_{104-115}$ is the minimal sequence required for inhibition of actomyosin ATPase activity (Talbot & Hodges, 1981a,b), it is only about half as effective as native TnI in the same molar ratio at which fsTnI inhibits actomyosin ATPase activity by 50% (Talbot & Hodges, 1981a). Similarly, on a molar basis $fsTnI_{96-116}$ is only about 75% as effective as the entire fsTnI protein in its ability to inhibit ATPase activity (Syska et al., 1976). Interestingly, one of the regions flanking the inhibitory peptide has been shown to demonstrate a change in structure with acidic pH. Our laboratory has shown that when fsTnI was labeled with the fluorophore IAF at Cys_{133}, a decrease in pH resulted in a 30% drop in fluorescence (El-Saleh & Solaro, 1988). Within this region are three specific sites of divergence between fsTnI and cTnI with potential significance in the pH response: Val_{132} in fsTnI is exchanged for Glu_{165} in cTnI, His_{130} to Ala_{163}, and Asn_{139} to His_{172}.

Two other domains for TnC–TnI interactions are at $fsTnC_{126-136}$ and $fsTnI_{1-21}$ (Syska et al., 1976). Exact sites of interaction of these domains with fsTnI and fsTnC, respectively, have not been established, however. From indirect evidence, it is apparent that the N-terminal domain of fsTnI interacts with fsTnC in a metal-independent manner and thus may serve primarily a structural role (Sheng et al., 1992). The interaction of $fsTnI_{1-21}$ with fsTnC remains of interest, however, in that the N-terminus displays the greatest divergence between fsTnI and cTnI. The cTnI has an added N-terminal extension of 26–33 amino acids. Within this region are a number of proline residues which could allow for a folding of the peptide with potential interference of TnC binding by the N-terminal domain. We have shown, however, that this N-terminal extension does not play a significant role in the pH response, removal of the peptide having no effect on Ca^{2+} regulation or deactivation by acidic pH when reconstituted into cardiac myofibrillar preparations (Watanapermpool, 1994).

The issue of the role of TnC and TnC–TnI interactions in deactivation of myofilaments by acidosis has taken a new direction with the prospect of gene therapy or targeting of TnC for pharmacological interventions in myocardial dysfunction associated with ischemia. While our study was nearing completion, Metzger et al. (1993) published work suggesting an important role of TnC isoforms in differential responsiveness of striated myofilaments to decreased pH. Permeabilized single myocytes from transgenic mice overexpressing fsTnC in cardiac muscle showed a reduced sensitivity to deactivation by acidic pH. In light of the fact that pH sensitivity of cardiac myofilaments changes during postnatal development despite expression of a single isoform of TnC (Solaro et al., 1986), these data, as pointed out by Metzger et al. (1993), do not preclude a significant role of TnI isoforms in the pH response. Indeed, our results provide new insight into the crucial role of the TnC–TnI reaction in deactivation of striated myofilaments by acidic pH and how the isoform specificity of this interaction modulates the pH response.

ACKNOWLEDGMENT

We are grateful to Dr. John Mills for his thoughtful advice and to Dr. Anne Martin for her careful review and input during preparation of the manuscript.

REFERENCES

Blanchard, E. M., & Solaro, R. J. (1984) *Circ. Res. 55*, 382–391.
Blanchard, E. M., Pan, B.-S., & Solaro, R. J. (1984) *J. Biol. Chem. 259*, 3181–3186.
Brandt, P. W., Diamond, M. S., & Schachat, F. H. (1984) *J. Mol. Biol. 180*, 379–384.
Bremel, R. D., & Weber, A. (1972) *Nature New Biol. 238*, 97–101.
Campbell, A. P., Van Eyk, J. E., Hodges, R. S., & Sykes, B. D. (1992) *Biochim. Biophys. Acta 1160*, 35–54.
Carter, S. G., & Karl, D. W. (1982) *J. Biochem. Biophys. Methods 7*, 7–13.
Donaldson, S. K. B., & Hermansen, L. (1978) *Pflugers Arch. 376*, 55–65.
El-Saleh, S. C., & Solaro, R. J. (1988) *J. Biol. Chem. 263*, 3274–3278.
Fabiato, A. (1981) *J. Gen. Physiol. 78*, 457–497.
Guo, X., Wattanapermpool, J., Palmiter, K. A., Murphy, A. M. & Solaro, R. J. (1994) *J. Biol. Chem. 269*, 15210–15216.
Herzberg, O., Moult, J., & James, M. N. G. (1986) *J. Biol. Chem. 261*, 2638–2644.
Ingraham, R. H., & Swenson, C. A. (1983) *Eur. J. Biochem. 132*, 85–88.
Johnson, J. D., Collins, J. H., & Potter, J. D. (1978) *J. Biol. Chem. 253*, 6451–6458.
Johnson, J. D., Collins, J. H., Robertson, S. P., & Potter, J. D. (1980) *J. Biol. Chem. 255*, 9635–9640.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lehrer, S. S., & Leavis, P. C. (1974) *Biochem. Biophys. Res. Commun. 58*, 159–165.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 165–175.
Martin, A. F., Ball, K., Gao, L., Kumar, P., & Solaro, R. J. (1991) *Circ. Res. 69*, 1244–1252.
Metzger, J. M., & Moss, R. L. (1987) *J. Physiol. 393*, 727–742.
Metzger, J. M., & Moss, R. L. (1988) *Biophys. J. 54*, 1169–1173.
Metzger, J. M., & Moss, R. L. (1990) *J. Gen. Physiol. 428*, 737–750.
Metzger, J. M., Parmacek, M. S., Barr, E., Pasyk, K., Wan-In, L., Cochrane, K. L., Field, L. J., & Leiden, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9036–9040.
Moss, R. L. (1992) *Circ. Res. 70*, 865–884.
Moss, R. L., Lauer, M. R., Giulian, G. G., & Greaser, M. L. (1986) *J. Biol. Chem. 261*, 6096–6099
Moss, R. L., Nwoye, L. O., & Greaser, M. L. (1991) *J. Physiol. 440*, 273–289.
Pagani, E. D., & Solaro, R. J. (1984) in *Methods in Pharmacology* (Schwartz, A., Ed.) pp 44–61, Plenum Publishing Corp., New York.
Parmacek, M. S., & Leiden, J. M. (1991) *Circulation 84*, 991–1003.
Potter, J. D. (1982) *Methods Enzymol. 85*, 247–263.
Robertson, S. P., & Kerrick, G. L. (1979) *Pflugers Arch. 380*, 41–45.
Rüegg, J. C., Zeugner, C., Van Eyk, J. E., Kay, C. M., & Hodges, R. S. (1989) *Pflugers Arch. 414*, 430–436.
Sheng, Z., Pan, B.-S., Miller, T. E., & Potter, J. D. (1992) *J. Biol. Chem. 267*, 25407–25413.
Shiraishi, F., Kambara, M., & Ohtsuki, I. (1992) *J. Biochem. (Tokyo) 111*, 61–65.
Solaro, R. J., Kumar, P., Blanchard, E. M., & Martin, A. F. (1986) *Circ. Res. 58*, 721–729.
Solaro, R. J., Lee, J. A., Kentish, J. C., & Allen, D. G. (1988) *Circ. Res. 63*, 779–787.
Syska, H., Wilkinson, M., Grand, R. J. A., & Perry, S. V. (1976) *Biochem. J. 153*, 375–387.
Van Eyk, J. E., & Hodges, R. S. (1988) *J. Biol. Chem. 263*, 1726–1732.
Van Eyk, J. E., Kay, C. M., & Hodges, R. S. (1991) *Biochemistry 30*, 9974–9981.