Changes in Myofibrillar Activation and Troponin C Ca\(^{2+}\) Binding Associated With Troponin T Isoform Switching in Developing Rabbit Heart

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Postnatal development of the mammalian heart is associated with changes in the population of isoforms of the thin filament proteins. We correlated the change in thin filament proteins, which occur in rabbit hearts between 5 days and 22 days of age, with changes in Ca\(^{2+}\) dependence of myofibrillar ATPase activity, force generation, and troponin C Ca\(^{2+}\) binding. The preparations derived from the 5-day-old animals exhibited a high molecular weight isoform of troponin T not found in the hearts of the 22-day-old animals. Other troponin T isoforms were also found to be present in different relative amounts. No other major differences in thin filament protein composition could be identified. Compared with the 5-day-old rabbit heart preparations, the ATPase activity of myofibrils from 22-day-old rabbit hearts exhibited a reduced Ca\(^{2+}\) sensitivity. The pCa\(_{50}\) (negative log of the half-maximal-activity free Ca\(^{2+}\)) of the MgATPase activity was shifted by 0.15 pCa units with maturation. Maturation of the myofibrils was also associated with an increased effect of Mg\(^{2+}\) on pCa\(_{50}\). On increasing the Mg\(^{2+}\) from 2 to 10 mM at constant MgATP\(^{2-}\), the pCa\(_{50}\) of 5-day myofibrils was increased (shifted to the right) by 0.39 pCa units for 5-day-old rabbit hearts and 0.45 pCa units for 22-day-old rabbit hearts. Although similar changes in pCa\(_{50}\) of force developed by myofibrils were marginally significant, fibers from hearts of 5-day-old rabbits exhibited a greater Hill coefficient than hearts from 22-day-old rabbits (3.0 vs. 2.1). Despite the increased sensitivity of 5-day-old rabbit hearts to Ca\(^{2+}\), these hearts exhibited significantly less Ca\(^{2+}\) bound to myofibrillar troponin C than did the 22-day-old rabbit hearts. Moreover, the models that best described the Ca\(^{2+}\) binding data are different for the two age groups. Our data indicate that the Ca\(^{2+}\) activation and Ca\(^{2+}\) binding properties of myofibrillar troponin C are altered in developing cardiac myofibrils and that the changes in these properties may be influenced by changes in the troponin T isoforms present in the myofibril. (Circulation Research 1990;66:1204–1216)

Regulation of contractile activity of cardiac myofilaments by Ca\(^{2+}\) is dependent on the activity of a set of thin filament proteins consisting of the troponin complex (Tn) and troponomyosin (Tm)\(^{1}\). The Tn-Tm complex acts to inhibit the actin-myosin reaction at low intracellular free Ca\(^{2+}\), and with an increase of Ca\(^{2+}\) in the myofilament space, the inhibition is released. The Ca\(^{2+}\) signaling process is initiated by calcium binding to a single regulatory site on troponin C (TnC) and by tight binding of TnC and troponin I (TnI), the inhibitory unit of Tn, and is transmitted to the rest of the thin filament through troponin T (TnT) and Tm.\(^{1}\) Apart from the detailed mechanisms for this particular signal transduction process, an interesting feature of the regulatory scheme is that most of the thin filament proteins are now known to exist as populations of isoforms.\(^{2-12}\) This raises important questions concerning the functional significance of a particular isotype population as well as the regulation of the expression of the various isoforms.

With the exception of TnC, for which there is no evidence of isoforms, shifts in the isotype population of cardiac thin filament regulatory proteins have

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been documented best during postnatal development. α-Tm is predominant in the adult heart of small animal species, but in the neonate, there is a relative abundance of striated muscle β-Tm and both smooth and nonmuscle β-Tm.13 TnI and TnT have both been found to be present in an isotype population that differs with species and stage of development,2,5,6,7,12 and there is evidence that the changes in isotype population may be of functional significance.2,8,9 Solaro and colleagues2,14 presented evidence indicating the presence of isoforms of TnI in myofibrils from neonatal dog and rat hearts. In these species, the neonatal variant of TnI appears to confer a resistance to desensitization of myofibrillar calcium activation by acidosis. In rabbit, however, myofibrils isolated from newborn hearts exhibit the same sensitivity to acidic pH as the adult preparations.15 It is possible that this difference may be related to a lack of a shift in TnI isotype population in the postnatal developing rabbit heart. In the case of TnT, isoforms have been identified by various techniques and in various species. Adult beef heart has two distinct isoforms, which are easily identifiable by one-dimensional polyacrylamide gel electrophoresis (PAGE). These isoforms have been sequenced16 and shown to produce differences in the calcium activation of ATP hydrolysis by reconstituted thin filament myosin subfragment-1 preparations.11 In other species, multiple isoforms of TnT that change with age have been identified, but as yet the functional significance of these shifts has not been explored. Five isoforms of TnT have been identified in rabbit heart by Anderson et al.5 The proportion of each isoform was shown to vary with postnatal age. In the case of rat heart myofilaments,6,12,17 only two cardiac TnT isoforms have been identified, one embryonic and the other adult. The neonatal embryonic isoform has a higher molecular weight and is more acidic than the adult form.6,7

In the present paper, we have explored the functional significance of isotype shifts in thin filament proteins during development of the rabbit heart. We monitored thin filament activity from measurements of Ca2+ binding to myofilament TnC and activation of force and myofibrillar Mg2+ATPase activity in myofilaments prepared from adult and 22- and 4–5-day-old rabbit hearts. Our results indicate that the nature of Ca2+ regulation is different in the neonate than in the adult and that the difference may be attributable to shifts in the isotype population of TnT. Some of our results have been reported in abstract form.9

Materials and Methods

Preparations

New Zealand White rabbits were used for all preparations. Rabbits were anesthetized by intraperitoneal (for 5- and 22-day-old rabbits) or intravenous (for adult rabbits) injection of pentobarbital. The hearts were rapidly removed, and the myofilaments were prepared from left ventricular tissue pooled from hearts of 5-day-old, 22-day-old, and adult rabbits.

Myofibrils. Purified myofibrillar fractions were prepared essentially as described by Solaro et al,18 with Triton X-100 used to remove membranes. The hearts were homogenized in Teflon/glass tissue grinders at 4°C, and the proteolytic enzyme inhibitors pepstatin (0.5 μg/ml), leupeptin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (0.2 mM) were present during all steps of the isolation. All experiments with myofibrils were completed within 24 hours of preparation.

Skinned fibers. Detergent-extracted fiber preparations (skinned fibers) were prepared as described by Pan and Solaro.19 The fibers were peeled from left ventricular strips dissected from the hearts of 5- and 22-day-old rabbits. The strips were initially placed in an ice-cold “relaxing solution” containing 60 mM imidazole, 10 mM EGTA, 12 mM KCl, 2 mM free Mg2+, 5 mM MgATP, 1.2 mM creatine phosphate, and 10 units/ml creatine kinase at 175 mM ionic strength and pH 7.0. Fiber bundles less than 0.5 mm in diameter were teased and extracted in a “skinning solution” (relaxing solution plus 1% Triton X-100) overnight at 4°C with gentle agitation. The skinning solution was then replaced with a storage solution (relaxing solution containing 50% vol/vol glycerol). Fiber bundles were stored at −20°C and used within 2 weeks. The fibers from 5-day-old rabbit hearts ranged in diameter from 150 to 250 μm and had an average free length of 3.5–4 mm; the fibers from 22-day-old rabbit hearts ranged in diameter from 75 to 150 μm and had an average free length of 7 mm.

Measurement of Ca2+ Binding to Myofibrils

Ca2+ bound to myofibrils was measured with the aid of 45Ca and [3H]glucose as a solution space marker by using a modification of previously described procedures.19,20 Measurements were made at 1 mg/ml myofibrillar protein over a Ca range of 8–3 at 23±2°C and at 150 mM ionic strength. The binding solutions contained 60 mM imidazole, 1 mM glucose, 0.3 μCi/ml 45Ca, 0.3 μCi/ml 3H, 2 mM free Mg2+, 5 mM MgATP, 12 mM phosphocreatine, 1 unit/ml creatine kinase, and 50 μM CaEGTA. These conditions were achieved with total concentrations of MgCl2, CaCl2, KCl, EGTA, and ATP computed by a program that solves the multiequilibrium problem for all ionic species. To ensure equilibrium, the myofibrils were incubated in the binding solution for 45 minutes. After incubation, the myofibrils were centrifuged, and a sample of the supernatant fraction was assayed for protein according to the method of Lowry et al21 to correct for any unsedimented protein. Another sample of the supernatant was assayed for 45Ca and 3H activity in 10 ml Aquasol II (New England Nuclear, Boston, Massachusetts) with a scintillation counter (Beckman Instruments, Fullerton, California). 45Ca was extracted from the sedimented protein by suspension for 60 minutes in 1 ml “elution solution” containing (mM) CaCl2, 50, glucose 10, and KCl 100. The elution solution was then assayed for radioactivity in
Aquasol II. Bound Ca\(^{2+}\) was computed from the protein concentration, the total calcium in the binding solution, and the ratio of \(^{45}\)Ca and \(^3\)H counts in the supernatant and pellet, with correction for unsedimented protein.

**Measurement of Myofibrillar ATPase Activity**

Ca\(^{2+}\)-dependent MgATPase activity of myofibrils from 5- and 22-day-old hearts was measured by determination of release of P\(_i\) during a 10-minute incubation with (mM) free Mg\(^{2+}\) 2 or 10, imidazole 60, and ATP 5 at 30\(^\circ\)C, pH 7.00, and 150 mM ionic strength over a pCa range of 7.00–4.75. The total concentrations of calcium, EGTA, KCl, MgCl, and ATP were calculated as described above. The reaction was initiated with addition of ATP and terminated with 10% trichloroacetic acid. The precipitated protein was removed by centrifugation, and P\(_i\) was measured in the supernatant fraction as described by Carter and Karl.\(^{22}\) All experiments were performed in triplicate, and a minimum of two hearts from each age group was used for the myofibril preparations. A total of three sets of triplicate experiments were performed for each of the two groups.

**Force Measurements**

Force measurements were made by modification of previously described procedures.\(^{19}\) The detergent-extracted fibers were mounted between a fixed post and a force transducer element (AE810 Sensor Nor, Horton, Norway), and force was recorded on a strip chart recorder (Linear 1200, Thomas Scientific, Swedesboro, New Jersey). Two relaxing solutions were used: HR (with high [10 mM] EGTA concentration) and LR (with low [0.1 mM] EGTA concentration). Maximum force was determined using an activating solution (AS) at pCa 4.5. The maximum force was checked at the beginning and end of each series of pCa points. Results from preparations showing greater than a 10% decrease in maximum force were rejected.

Initial fiber length was set so that the sarcomere length was 2.2 \(\mu\)m; the laser diffraction pattern, which was obtained by shining a neon/helium laser beam through the midportion of the fiber, was used. Clear diffraction patterns were obtained for all experiments using fibers from 22-day-old hearts, whereas a clear diffraction pattern was not obtainable for the fibers from 5-day-old hearts. To approximate a resting sarcomere length of 2.2–2.3 \(\mu\)m, the micromanipulator was adjusted to give a fiber length that produced a minimal force in relaxing solution. The procedure was suggested by the data of Kentish et al\(^{23}\) and Hibberd and Jewell,\(^{24}\) which was obtained from skinned fibers of rat right ventricle. These authors noted that there was a nonzero initial force at low [Ca\(^{2+}\)] when the sarcomere length was 2.2–2.3 \(\mu\)m. A minimal resting tension was noted in the fibers from 22-day-old hearts in relaxing solution when the sarcomere length was 2.2 and 2.3 \(\mu\)m, as measured by laser diffraction. The sarcomere length was not controlled during the experiments once the initial fiber length was set, but it was rechecked with each reaplication of the HR solution. Results were rejected if the measured sarcomere length changed by 0.2 \(\mu\)m or more. The protocol for the sequence of solutions used for force measurement was HR, LR, AS, HR (until baseline was achieved), LR, pCa, HR (until baseline was achieved), LR, pCa, and so forth for the 22-day-old hearts. Two protocols were used to study the 5-day-old heart fibers in that repeated exposures to low pCa (<6.0) solutions resulted in extensive fiber damage. The first protocol for these fibers omitted the initial use of AS. The force generated by the fiber was measured by exposing the fiber to solutions having pCa 6.25, 6.125, 6.00, and 5.75 before the exposure to AS. The second protocol was the same as that used for the 22-day-old heart fibers. Data from the two protocols were combined for the 5-day-old fibers, since the pCa\(_{50}\) predicted by fitting the data to the Hill equation was the same regardless of the protocol used.

**Gel Electrophoresis and Immunoblots**

Myofibrillar preparations were analyzed by sodium dodecyl sulfate (SDS) PAGE essentially as described by Laemmli.\(^{25}\) Isoelectric and gradient gel systems were used to separate the myofibrillar proteins. Molecular weight markers were used to identify protein bands of known apparent molecular weight. Multiple samples of myofibrils from 5- and 22-day-old hearts were loaded onto each gel. The total protein loaded in each lane was identical as assessed by Lowry assay. Electrophoresis of myofibrillar proteins in 8% polyacrylamide gels, which were 0.75 mm thick, or 5–18% gradient polyacrylamide gels, which were 1.5 mm thick, was carried out at 200 V until the bromophenol dye front reached the bottom. Gels were stained with Coomassie brilliant blue (1.5 mm thick gels) or as described by Merrill et al.\(^{26}\)

The TnC content of myofibrillar preparations was quantitated by using alkaline urea PAGE in the absence of SDS as previously described.\(^{19}\) The TnC bands migrate well ahead of all other proteins in this system making accurate densitometry measurements possible. The density of TnC staining of known quantities of myofibrillar protein was compared with the density obtained with a series of TnC standards. The standards were chosen so that the amount on the gel was linear with density and bracketed the unknown amounts in the myofibrillar preparations.

Immunoblot analysis was carried out by the methods of Towbin et al.\(^{27}\) One-dimensional 8% SDS-PAGE gels were presoaked in buffer (25 mM Tris, 192 mM glycine, 20% vol/vol MeOH/H\(_2\)O, pH 8.3) for 15 minutes, sandwiched with nitrocellulose, and transferred electrophoretically by using the same buffer at a constant current of 200 mA for 1 hour. After rinsing with distilled water, the nitrocellulose was incubated in a solution containing 20 mM Tris buffer (pH 7.5), 500 mM NaCl, 0.05% Tween-20, and 1% bovine serum albumin. The primary antibody
(JLT-12) at a predetermined dilution was added with 1% bovine serum albumin in Tris-buffered saline. After 1 hour of incubation, the nitrocellulose was washed five times with distilled water and twice with Tris-buffered saline. Incubation with secondary antibody (goat anti-mouse immunoglobulin G horse-radish peroxide conjugate) was for 1 hour in Tris-buffered saline plus 1% bovine serum albumin. The nitrocellulose was washed as above, and the color reaction was developed with 4-chloro-1-naphthol as the substrate in the presence of H2O2.

Data Analysis

The Ca2+ binding data were fit to a model that provides for three classes of binding sites and allows the incorporation of cooperativity factors for the class I and class II sites. The mathematical form is given by

$$\text{bound Ca}^{2+} \text{ (nmol/mg protein)} = \frac{n_1 K_1^x [\text{Ca}^{2+}]^x}{1 + K_1^x [\text{Ca}^{2+}]^x} + \frac{n_2 K_2^y [\text{Ca}^{2+}]^y}{1 + K_2^y [\text{Ca}^{2+}]^y} + \frac{n_3 K_3 [\text{Ca}^{2+}]}{1 + K_3 [\text{Ca}^{2+}]^3}$$

where x is the cooperativity factor for class I sites, y is the cooperativity factor for class II sites, n1, n2, and n3 are the stoichiometric coefficients of class I, II, and III sites, respectively, and K1, K2, and K3 are the binding constants of class I, II, and III sites, respectively. The noncooperative form of this model (x = 1.0, y = 1.0) has been previously described in detail by Pan and Solaro. The model was initially run with the single constraint that the stoichiometric coefficient of the class I sites was twice the value of the coefficient of the class II sites (i.e., n1 = 2n2). A nonlinear least-squares regression curve fitting procedure (BMDP Statistical Software, Inc., Los Angeles, California) was used to fit the data to the model. Goodness of fit was determined by previously described statistical tests. The initial fits were nonweighted, but analysis of residual plots indicated that variance weighting was necessary. After the Ca2+ binding data and the ATPase activation data were combined and analyzed, it became clear that alternative models needed to be introduced, subjected to statistical analysis, and compared with the original noncooperative model in which n1 = 2n2 (this model is referred to as the H2L1 model hereafter). The Ca2+ binding data were fit to Equation 1 with various values for x and y and with either n1 = 2n2 or n1 = n3 for the ratios of the stoichiometric coefficients for the class I and class II binding sites. A more detailed description of the considerations and underlying assumptions of each model tested is given in “Appendix.”

To obtain values for [Ca2+]0 and nH, the pCa-ATPase activity and pCa-force data were fit to an empiric form of the Hill equation by using a nonlinear least-squares regression package (PCNONLIN; Statistical Consultants, Lexington, Kentucky):

$$\% \text{ activation} = \frac{100 [\text{Ca}^{2+}]^{nH}}{[\text{Ca}^{2+}]^{nH} + [\text{Ca}^{2+}]^{nH}}$$

Individual data points having standardized residuals with an absolute value greater than two were eliminated, and the regression was rerun. No pCa-force points were rejected, and at most, two of 13 points were eliminated from the ATPase data sets.

Regression parameters were taken as significant only if the 95% confidence limits did not include zero. Variance weighting was used when indicated by analysis of the residual distribution. If, after reduction of the number of parameters and proper weighting, the model still produced nonsignificant parameters, the model was judged inappropriate for the data. Models having different numbers of parameters were compared by the “extra sum of square principle” (F test) and the minimum Akaike information criteria estimation (MAICE) as previously described. Models with equal numbers of parameters but reflecting changes in binding stoichiometry were compared by the “run test,” the residual sum of squares, and mean-square error. Pairwise comparisons were made by the two-tailed t test when parametric analysis was appropriate. Multiple comparisons were made by analysis of variance. The use of parametric or nonparametric analysis was based on the results of the Wilk-Shapiro W test.

Results

SDS-PAGE and Immunoblot Analysis

Analysis of proteins of 5- and 22-day-old heart myofibrils by gradient PAGE is shown in Figure 1. Protein bands that could be identified on the basis of apparent molecular weight have been labeled in Figure 1. Within the limits of the one-dimensional gel system, the quantities of the thin filament proteins are identical for the two age groups. β-Tm (not labeled) was present in both preparations and migrated just ahead of TnT and behind TnI. Other protein bands indicated in Figure 1 were identified on the basis of apparent molecular weight, by comigration with pure standards, or by cross-reactivity with antibodies. A protein band subjacent to myosin light chain 1 that was present in 5-day-old but not in 22-day-old myofibrils was not identified in this way. In the region of the gel just below actin, there are two bands with a mobility corresponding to TnT. It is apparent that the faster migrating of these bands was present in reduced amounts in the 22-day-old myofibrils. However, to more clearly define these differences, we analyzed the myofibrillar preparations by using 8% PAGE for better separation and silver staining and immunoblotting for clearer detection. In the silver-stained gel (Figure 2A), the lane containing myofibrils from 5-day-old hearts exhibited four bands (TnT 2–5) with a mobility between actin and Tm. The same region of the gel with 22-day-old myofibrils also showed four bands. TnT 5, the fastest migrating band, is present in reduced amounts in the 22-
day-old myofibrils, and this may correspond to the reduction in the amount of the faster migrating TnT band in the gradient gel shown in Figure 1. An immunoblot of the 8% SDS gel is shown in Figure 2B. The primary antibody used to develop the blots was a monoclonal mouse anti-rabbit skeletal TnT antibody (JLT-12), which has been described by Jin and Lin. The bands that are labeled with the antibody are referred to as numbers one through four. Band number one is that with the highest apparent molecular weight (slowest mobility). Four bands appear in the lane containing the 5-day-old myofibrils: one, two, three, and four. The lane containing the 22-day-old myofibrils exhibits four bands as well: one (very faint), two, three, and four. Bands two and four are the most abundant in the 5-day-old myofibril lane; band four is clearly the dominant form in the 22-day-old myofibril lane. The highest molecular weight isoform of TnT (band one) almost completely disappears with development between 5 and 22 days of age. The next highest molecular weight form (band two) significantly decreases in abundance during the same period of development. TnT 3, which is not clearly apparent in the neonate, is somewhat more abundant at 22 days and is clearly evident at 3 months (data not shown). Except for TnT 5, which is not seen in the immunoblot and appears to decrease

**FIGURE 1.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation of myofibrillar proteins from 5- and 22-day-old rabbit ventricular muscle. Gradient gels were 5–18% polyacrylamide and were stained with Coomassie blue. MHC, myosin heavy chain; TNT, troponin T; TNI, troponin I; LC, myosin light chain. See text for description.

**FIGURE 2.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation of myofibrillar proteins from 5- and 22-day-old rabbit ventricular muscle. Panel A: Silver-stained gel showing multiple forms of troponin T (TNT). MHC, myosin heavy chain; TM, tropomyosin. Panel B: Results of immunoblot with monoclonal antibody to TNT. See text for description.
TABLE 1  Parameters of Ca²⁺ Activation of MgATPase Activity of 5- and 22-Day-Old Rabbit Postnatal Cardiac Myofibrils at 2 and 10 mM Free Mg²⁺

<table>
<thead>
<tr>
<th>Age</th>
<th>2 mM Mg²⁺</th>
<th>10 mM Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCa₅₀</td>
<td>nH</td>
</tr>
<tr>
<td>5 days</td>
<td>6.25±0.03*</td>
<td>1.66±0.16</td>
</tr>
<tr>
<td>22 days</td>
<td>5.88±0.06*</td>
<td>2.20±0.14</td>
</tr>
</tbody>
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*Statistical analysis by analysis of variance showed significant difference (p<0.05) for pCa₅₀ values between 2 and 10 mM Mg²⁺ and between values for 5- and 22-day-old rabbit myofibrils at both 2 and 10 mM Mg²⁺. Hill coefficients (nH) were not significantly different for 2 versus 10 mM Mg²⁺ data and for values of 5- versus 22-day-old rabbit myofibrils.

In abundance with development, there appears to be a shift from high molecular weight isoforms of TnT to lower molecular weight isoforms with postnatal development. This pattern is generally consistent with that found in rat⁶,¹²,¹⁷ and chicken⁷,¹²; the neonatal myocardium expresses the higher molecular weight isoform of TnT, whereas the lower molecular weight isoform is expressed in the adult myocardium. On the basis of the data from silver-stained gel and immunoblots, we conclude that in 5-day-old myofibrils, there are five bands; the first band is not observable on the silver-stained gel, whereas the fifth band is not seen in the immunoblot. This result confirms the report of Anderson et al.,⁵ who reported earlier that there are five isoforms of rabbit cardiac TnT and that the relative amount of these isoforms changes with development. Our results differ from those of Anderson et al in that we note a greater decrease in the abundance of TnT 2 between 5 and 22 days of age. The results shown in Figure 2B have been reproduced in our laboratory on multiple immunoblots (data not shown). The reasons for the differences between this report and that of Anderson et al may be related to methodology.

Activation of Mg²⁺-Dependent Ca²⁺-Activated ATPases

To assess functional changes in myofibrils that may be associated with changes in TnT isoforms, we measured Ca²⁺ activation of MgATPase activity of myofibrils from 5- and 22-day-old hearts (Table 1, Figure 3). The pCa₅₀ values are significantly different for the two groups at both 2 and 10 mM free Mg²⁺ (6.26±0.05 and 5.87±0.06 for 5-day-old myofibrils and 6.11±0.05 and 5.66±0.03 for 22-day-old myofibrils). The Hill coefficients (nH) were not significantly different between the two groups (1.7 and 2.20 for the 5-day-old myofibrils and 2.1 and 2.0 for the 22-day-old myofibrils for 2 and 10 mM Mg²⁺, respectively). Although the value of nH increased as free Mg²⁺ went from 2 to 10 mM for the 5-day-old myofibrils, the difference between the means was not significant.

Age-related differences in myofibrillar regulation were also reflected in the difference between the pCa₅₀ at 2 mM Mg²⁺ and at 10 mM Mg²⁺. This difference was significantly less for the 5-day-old myofibrils than for the 22-day-old myofibrils. Mg²⁺ has been found to bind to the low-affinity regulatory (class II) site of cardiac TnC but with an affinity constant about four orders of magnitude less than Ca²⁺.¹⁹ However, the Mg²⁺ concentration at pCa₅₀ is three to four orders of magnitude greater than that of Ca²⁺; therefore, the extent of binding of the two ligands to the regulatory sites is nearly identical. The difference in pCa₅₀ between the 5- and 22-day-old myofibrils with increasing Mg²⁺ concentration from 2 to 10 mM could be due to different class II site affinity constants for Ca²⁺ and Mg²⁺ or to a difference in the effect of Mg²⁺ binding to the class II sites of TnC of neonatal and adult myofibrils.

Force-pCa Relations

We also compared Ca²⁺-activated force of neonatal and mature myofibrils. The relation between normalized force and pCa for 5- and 22-day-old skinned fibers is shown in Figure 4. The skinned fibers from the 22-day-old hearts developed greater absolute force than the fibers from the 5-day-old hearts at full activation by a factor of 4 or 5. This result is consistent with earlier findings of Solaro et al.¹⁴ and with the observation that the neonatal myocardium has a lower density of myofibrils than mature myocardium.²⁹,³⁰ The pCa₅₀ values for force activation were not significantly different between
the two groups (5.92±0.06 for 5-day-old fibers vs. 5.81±0.03 for 22-day-old fibers). The Hill coefficients (3.1±0.35 for 5-day-old fibers and 2.1±0.1 for 22-day-old fibers) were significantly different at the 93% level. The marginally significant difference between the Hill coefficients is related to the large standard deviation for the value of nH in the 5-day-old fiber group. The values of pCa0.5 and nH for the 22-day-old fibers have smaller standard error values than those for the 5-day-old fibers. This is most likely the result of better control of initial sarcomere length and greater uniformity of fiber dimensions in the 22-day-old fiber experiments (see "Materials and Methods"). These findings are consistent with the results of Solaro et al.,14 who reported that the fibers of neonatal rat right ventricle had a higher pCa0.5 and nH than those from adult rat right ventricle. Differences resulting from variable expression of myosin heavy chain in the 5- and 22-day-old myocardium do not appear to be responsible for the differences in the ATPase activity—pCa and force—pCa relations. Shifts in the myosin heavy chain isoform population of adult rabbit heart myofibrils from one containing essentially 100% V1 to one containing 100% V3 did not significantly affect the pCa0.5 for activation of myofibrillar ATPase activity.31

Ca2+ Binding to Myofibrils

Differences in Ca2+ activation of myofibrils associated with TnT isoform switching may be related to changes in the Ca2+ binding properties of TnC. Therefore, we measured the relation between free Ca2+ and Ca2+ bound to myofibrils isolated from 5- and 22-day-old hearts (Figure 5). Previous studies have shown that, under the conditions of these measurements, the data represent Ca2+ bound exclusively to myofilament TnC for pCa values greater than or equal to 5.5. At lower pCa values, Ca2+ binding sites of myosin begin to titrate.19 Although not shown in Figure 5, Ca2+ binding measurements were made to pCa 3. As discussed in detail previously,19 full knowledge of the binding isotherm over this broad pCa range permits more accurate determination of binding parameters from nonlinear regression analysis. In fact, our experience with the present data indicates that without knowledge of the binding data up to pCa 3, significant binding parameters cannot be obtained.

The calcium binding data illustrated in Figure 5 for the two age groups were fit to a noncooperative form (x=y=1.0) of the three-parameter model described by Equation 1 in "Materials and Methods." Initial data analysis indicated that a constraint on the number of parameters and variance weighting were required. The constraint imposed was to set the number of class I sites equal to two times the number of class II sites. Despite the statistical manipulations, the value of K1 changed very little in any group from the original estimate produced by the nonconstrained, nonweighted regression (maximum change was 10%).

The results of the weighted constrained fits of the Ca2+ binding data to a noncooperative form of Equation 1 are given in Table 2. The binding constants (K1) for the class I sites were identical for the 22-day-old and adult myofibrils. The K1 of the 5-day-old myofibrils was less than the others by a factor of 3, and the difference was highly significant (p<0.01). The binding constants for the class II sites exhibited the same pattern; the values of K2 for the 22-day-old and adult myofibrils were not significantly different, whereas that of the 5-day-old myofibrils was less by a factor of 4–10. The K3 values for one data set of the 5-day-old group were marginally...
significant in that the 95% confidence intervals were within 10% of the parameter estimate of zero.

The values for the stoichiometric coefficients for the class III sites were not significantly different among the three groups. The value of \( K_1 \) for the 5-day-old myofibrils was significantly greater than that of the 22-day-old model myofibrils. The high stoichiometric coefficients and low binding constants of the class III sites are compatible with the assumption that these are the high-capacity low-affinity Ca\(^{2+}\) binding sites of myosin. If the \( n_i \) and \( K_i \) values predicted by the models are used to calculate a bound Ca\(^{2+}\) versus pCa curve, the result is an isotherm similar to that observed by Pan and Solaro\(^{19} \) for the binding of Ca\(^{2+}\) to myosin.

The class I and class II sites, analyzed with a noncooperative model, have binding constants similar to those expected for the high-affinity and low-affinity Ca\(^{2+}\) binding sites of cardiac TnC.\(^{19,32} \) The results of the regression analysis suggest that the high-affinity Ca\(^{2+}\) binding sites of myofibrillar TnC from hearts of 5-day-old rabbits have a threefold lower affinity for Ca\(^{2+}\) than the same sites of cardiac myofibrils from more mature animals (Table 2). The low-affinity site has a reduced Ca\(^{2+}\) binding affinity as well. As discussed below, this interpretation is model dependent, yet the apparent reduced affinity for Ca\(^{2+}\) is not due to a difference in available binding sites. Quantitation of myofibrillar TnC revealed no difference in the content of TnC or in the electrophoretic migration pattern of TnC in alkaline urea gels of myofibrils from 5- and 22-day-old hearts (Figure 6). The TnC content was 0.46 nmol/mg in the 5-day-old myofibrils and 0.44 nmol/mg protein for the 22-day-old myofibrils. The measured TnC content was close to the value of 0.5 nmol/mg protein previously demonstrated in dog heart myofibrils.\(^{19} \) This measured TnC content is in excellent agreement with that predicted by the regression of the Ca\(^{2+}\) binding data (0.5 nmol/mg protein). Therefore, differences in TnC content were not the cause for the differences in Ca\(^{2+}\) binding between the 5- and 22-day-old myofibrils.

### Relation Between TnC-Bound Calcium and Myofilament Activation

The observation that 5-day-old myofibrils bind less Ca\(^{2+}\) than 22-day-old myofibrils but that they are more sensitive to Ca\(^{2+}\) with regard to activation of the ATPase activity indicates that steady-state activation of the 5-day-old myofibrils occurs with substantially less Ca\(^{2+}\) bound to TnC than in the case of the 22-day-old myofibrils. To show this more clearly, we computed the relation between activation of ATPase activity and Ca\(^{2+}\) bound to the individual sites of TnC, using the binding constants from Table 2 and the values of [Ca\(^{2+}\)]\(_{50}\) and \( n_H \) for 2 mM Mg\(^{2+}\) from Table 1 for activation of ATPase activity. These relations (Figures 7A and 7B) represent the degree of saturation of the class I (high-affinity) sites of TnC and the class II (low-affinity) sites of TnC with Ca\(^{2+}\) at any level of ATPase activity at steady state. During the cardiac cycle, the class I sites are most likely always occupied with Ca\(^{2+}\) or Mg\(^{2+}\). The class II site exchanges Ca\(^{2+}\) during the cycle and is thus the “regulatory” site. The 22-day-old myofibrils exhibited a typical relation between occupancy of the low-affinity regulatory site and activation of the ATPase activity; at 90% of full activation, there was about 70% occupancy of the low-affinity site. This is identical to the relation between bound Ca\(^{2+}\) and activation of force in canine cardiac skinned fibers reported by Pan and Solaro.\(^{19} \) This is in striking agreement with the regulatory role of TnC in cardiac muscle.

### Table 2. Ca\(^{2+}\) Binding Properties of Rabbit Cardiac Myofibrils at Different Postnatal Ages

<table>
<thead>
<tr>
<th>Age</th>
<th>( K_1 )</th>
<th>( K_2 )</th>
<th>( n_3 )</th>
<th>( K_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days</td>
<td>( 4.1 \times 10^6 )</td>
<td>( 3.0 \times 10^6 )</td>
<td>72</td>
<td>532</td>
</tr>
<tr>
<td>22 days</td>
<td>( 1.2 \times 10^7 )</td>
<td>( 4.3 \times 10^5 )</td>
<td>123</td>
<td>286</td>
</tr>
<tr>
<td>Adult</td>
<td>( 1.2 \times 10^7 )</td>
<td>( 1.1 \times 10^5 )</td>
<td>63</td>
<td>361</td>
</tr>
</tbody>
</table>

\( K_1, K_2, K_3 \), and \( K_4 \) are the binding constants for the class I, II, and III sites, respectively, per Mol. \( n_1, n_2, \) and \( n_3 \) are the stoichiometric coefficients for the three classes of binding sites. The Ca\(^{2+}\) binding parameters were obtained from analysis of data shown in Figure 5 by least-squares curve fitting using a noncooperative model (HI-L model) with \( n_1 \) fixed at 1.0 and \( n_2 \) at 0.5 mol/mol myofilament troponin C as described in the text.

\( ^* \) p<0.05 vs. 22-day-old and adult rabbit.

\( ^\dagger \) p<0.05 vs. 22-day-old rabbit.
contrast to the predicted relations for 5-day-old myofibrils depicted in Figure 7A. In this case, the MgATPase activity is 90% of maximal activation with less than 10% of the low-affinity sites occupied.

This departure from the generally accepted idea that significant saturation of the class II regulatory site is required for activation of myofibrillar activity prompted us to search for an alternative to the H2L1 Ca2+ binding model for the 5-day-old myofibrils. The best model as judged by the criteria previously given is one in which the class I and class II sites have equal stoichiometric coefficients (n1=n2=0.5) and the cooperativity factor for the class II sites (γ) is 1.45.* We refer to this model as the C-H2L1 model. The binding constants from Table 3 for the C-H2L1 model and the pCa0 and Hill coefficient values for the myofibrils (Table 1) were used to generate Figure 7C. It can be seen that the activation of the myofibrillar ATPase activity proceeds as the saturation of the class II site increases and that the curve describing the saturation of the class II site exhibits cooperativity, as does the activation curve. This finding is consistent with the hypotheses and findings of Tobacman and Sawyer; Ca2+ binding to the low-affinity (regulatory) site may be cooperative, and the ATPase activity is an indirect measure of Ca2+ bound to that site.

The Ca2+ binding data from 22-day-old myofibrils were fit to a cooperative model as well, but the n1=2n2 constraint was maintained because an H2L1 model would not fit the data if the value of n1 (equivalent to n2) was constrained by the TnC content. The goodness of fit was only marginally improved by use of the cooperative model for the 22-day-old myofibrils (Table 3). The MAICE score was reduced only 3.5%, and the F test indicated that the noncooperative model should be retained as the better model. Therefore, the noncooperative model was retained and was used in the remainder of the analysis.

Therefore, it is apparent that the binding of Ca2+ to TnC in the cardiac myofibrils from 5-day-old animals proceeds by a different mechanism than the binding of Ca2+ to TnC of cardiac myofibrils from 22-day-old animals. This “mechanism” may be a functional “closure” of the second high-affinity site of TnC with binding of Ca2+ to one of the two high-affinity sites or with binding to the low-affinity site. The data analysis indicates that cooperativity does occur in the 5-day-old myofibrils during Ca2+ binding to the class II sites, but the analysis did not unequivocally support the assumption of cooperativity for the 22-day-old myofibrils.

**Discussion**

Regardless of which model is used to describe our results, it is important to emphasize that, in any case, the raw Ca2+ binding data indicate that the 5-day-old myofibrils are fully activated with about 2 mol total

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*Although the result of the curve fitting indicates that there is cooperativity among the class II sites, assignment of physical significance is a complex theoretical problem (see “Appendix”) because the cooperativity parameter is obtained empirically and not by rigorous modeling using the principles of statistical thermodynamics. The cooperativity factor does, however, put some constraints on the number of interacting sites, interaction energies, and the Hill coefficient (defined as four times the partial derivative of binding extent with respect to the natural log of concentration, with extent equal to one half of maximal).
Ca\(^{2+}\) bound per mole myofibrillar TnC, whereas the 22-day-old myofilaments achieve full activation at about 3 mol Ca\(^{2+}\) bound per mole TnC. Although the findings are not discussed here, we obtained similar results for the case of the relation between bound Ca\(^{2+}\) and force. Thus, the relation between bound Ca\(^{2+}\) and activation is clearly different between the two age groups.

An important question is whether the developmental differences in myofibrillar Ca\(^{2+}\) activation we show are due to changes in the TnT isoform population. The isoform distribution of TnT has been shown to change in concert with the isoforms of TnI during development in both the rat and chicken by using monoclonal antibodies against TnI and TnT.\(^{12}\) Yet the report of Nakanishi et al,\(^{13}\) which shows identical pH sensitivities for Ca\(^{2+}\) activation of newborn and adult rabbit myofilaments, suggests that there is no TnT isoform shift. We have not identified any major differences in the actin, TnC, TnI, or Tm protein isoforms present in the two groups of hearts studied by SDS-PAGE. This does not rule out the presence of isoforms having the same apparent molecular weight but different isolectric points. We also cannot rule out the presence of different isoforms of actin by the present analysis. The \(\alpha\)-skeletal actin is found in fetal, but not neonatal, rat heart.\(^{13}\) The cardiac and skeletal actin differs by only two amino acid residues at the N-terminus,\(^{3,6}\) and the two forms have been used interchangeably for study of reconstituted actin-myosin preparations with no differences in activity noted (R.J. Solaro, unpublished data). Schachat et al\(^{8}\) have shown that isoform populations of TnT and Tm are the major determinants of both Ca\(^{2+}\) sensitivity and cooperativity in fast skeletal muscle fibers. They found that the fibers expressing both TnT\(_2\) and an \(\alpha\)-\(\alpha\)-dimer of Tm had a higher pCa\(_{50}\) and a greater \(n_H\) than other fibers. Both Tm and TnT isoforms appeared to affect pCa\(_{50}\) and \(n_H\) independently in skeletal muscle fibers. In rats and other small animals, the \(\alpha\)-isoform of Tm is predominant in adult myocardial tissue.\(^{13}\) The \(\beta\)-isoform is found only in the fetal and newborn heart except in hypertrophy;\(^{13}\) skeletal muscle expresses \(\beta\)-Tm at all stages of development. If rabbit heart follows the same pattern as rat heart Tm isoform expression, the 5-day-old rabbit heart would be expected to contain some \(\alpha\)-\(\beta\)-heterodimer, whereas the 22-day-old hearts would be expected to contain predominantly \(\alpha\)-\(\alpha\)-homodimer Tm. If expression of the \(\alpha\)-homodimer Tm results in a higher pCa\(_{50}\) and \(n_H\) in heart, as in skeletal muscle, then the 22-day-old hearts should show greater cooperativity than the 5-day-old hearts. The fact that this was not observed suggests that the Tm isoform population is not very different at the two ages. This is supported by gels shown in Figures 1 and 2.

The major difference in thin filament composition between the two groups is in the population of isoforms of TnT present in the myofibril. This indicates that TnT isoype variations alone may be sufficient to produce changes in the Ca\(^{2+}\) binding and activation characteristics in cardiac muscle. The presence of multiple cardiac isoforms of TnT has been demonstrated in several species. In chicken,\(^{7,12}\) and rat,\(^{6,12,17}\) an isoform unique to the fetal and early postnatal animal has been identified. The sequence information for the chicken cardiac TnT and the two-dimensional PAGE analysis of the rat\(^{6}\) heart indicate that the fetal isoform is of a higher molecular weight and more acidic than the adult isoform of TnT. The fetal isoform of TnT is the product of alternative splicing, which includes an exon (number 5 in the chicken) or exons, not found in the messenger RNA coding for the adult TnT isoforms. Alternative splicing also takes place in skeletal muscle and produces a wide variety of TnT isoforms.\(^{3,4}\) Much of the variability occurs in the N-terminal region of the molecule.\(^{1,10,36}\) It is this portion of the TnT molecule

| Table 3. Summary of Binding Constants and Statistical Comparisons for Alternative Models for 5- and 22-Day-Old Rabbit Myofibril Ca\(^{2+}\) Binding Data |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Model             | Model rank      | SRS             | \(K_1\) (M\(^{-1}\)) | \(K_2\) (M\(^{-1}\)) | \(n_1\) | \(K_3\) (M\(^{-1}\)) |
| 5-day-old rabbit myofilaments | | | | | | |
| NC H\(_2\)L\(_1\) (Table 1) | 4 | 2.4 | 4.1 \times 10^6 | 3.0 \times 10^4 | 72 | 532 |
| C H\(_2\)L\(_1\)\(^\ast\) | 5 | 2.8 | 4.8 \times 10^6 | NS | 76 | 520 |
| NC H\(_2\)L\(_1\)† | 2 | 0.459 | 1.69 \times 10^7 | 1.04 \times 10^6 | 72 | 532 |
| C H\(_2\)L\(_1\)† | 1 | 0.209 | 2.01 \times 10^7 | 1.11 \times 10^6 | 70 | 542 |
| NC 2/3(H\(_2\)L\(_1\))† | 3 | 0.841 | 1.04 \times 10^7 | 5.9 \times 10^6 | 71 | 539 |
| 22-day-old rabbit myofilaments | | | | | | |
| NC H\(_2\)L\(_1\) | 1 | 2.537 | 1.2 \times 10^7 | 4.3 \times 10^5 | 123 | 286 |
| C H\(_3\)L\(_1\) | 1 | 2.129 | 1.3 \times 10^7 | 4.2 \times 10^5 | 120 | 294 |

SRS, sum of residuals squared from regression of data; \(K_1\), \(K_2\), and \(K_3\), binding constants of class I, class II, and class III sites, respectively; \(n_1\), \(n_2\), and \(n_3\), stoichiometric coefficients of class I, class II, and class III sites, respectively; NC, no cooperativity in model; H\(_2\)L\(_1\), model in which \(n_1=2n_2\); C, cooperativity of class II sites per Equation 1 (see text); H\(_2\)L\(_1\), model in which \(n_1=n_2\); NS, not significant. Best y value was 1.45 for H\(_2\)L\(_1\). Significant parameters for \(K_2\) could not be obtained using cooperative H\(_2\)L\(_1\). Best y value for 22-day-old C H\(_2\)L\(_1\) was 1.35. Models are ranked in order of goodness of fit (see text).

* All troponin C is assumed to be involved in Ca\(^{2+}\) binding.
† \(n_1=n_2=0.5\); all troponin C is assumed to be involved in Ca\(^{2+}\) binding.
‡ Approximately two thirds of troponin C available is assumed to be involved in Ca\(^{2+}\) binding.
that lies adjacent to the region in which nearest neighbor Tm molecules overlap\textsuperscript{30}; the C-terminal region has been found to interact with both TnC and TnI\textsuperscript{37,38}. The isoforms of bovine cardiac TnT have been found to confer different Ca\textsuperscript{2+} sensitivities in a system of reconstituted thin filament subfragment-1 preparations. In this system, the lower \textit{M}, TnT isoform was found to produce a greater Ca\textsuperscript{2+} sensitivity than the higher \textit{M}, form.\textsuperscript{11} Functional significance for the cardiac isoforms of TnT of other species has not been demonstrated to our knowledge.

The observed differences in activation of MgATPase activity and force generation with changing thin filament isoforms are not without precedent. However, the differences in the Ca\textsuperscript{2+} binding and the proposed binding models are, to our knowledge, unique. The present findings suggest that isoform shifts in cardiac TnT may influence other cardiac thin filament proteins by incompletely understood mechanisms. Studies of the interactions of the Tn subunits in skeletal muscle indicate that the conformational changes that occur in TnC on binding of Ca\textsuperscript{2+} to its high-affinity sites are modulated by either TnI or TnT, or both.\textsuperscript{39} TnT undergoes a conformational change on Ca\textsuperscript{2+} binding to the low-affinity sites of skeletal muscle TnC.\textsuperscript{40} Such interactions have not been conclusively demonstrated for cardiac Tn subunits, but our findings indicate that TnT influences cooperativity between thin filament units and that it also plays a role in determining the pattern of Ca\textsuperscript{2+} binding at equilibrium by native cardiac myofibrils.

Appendix

The noncooperative form of Equation 1 is the model to which the Ca\textsuperscript{2+} binding data were fit to develop Figures 7A and 7B. This model assumes that both high-affinity sites are nearly saturated before there is significant occupancy of the low-affinity site (H\textsubscript{1}L\textsubscript{1} model). The other implicit assumptions are that 1) all TnC molecules present in the myofibrils participate in Ca\textsuperscript{2+} binding, the binding is noncooperative, and the classes of sites are noninteracting (e.g., class I sites do not interact with class II sites); 2) the class I and class II sites do not interact with each other; and 3) there is no interaction among nearest neighbor sites of the same class. Alternative models describing the Ca\textsuperscript{2+} binding to 5-day-old myofibrils can be derived by altering the underlying assumptions. If the H\textsubscript{1}L\textsubscript{1} model is applied to the present data, the low-affinity site assumes a “regulatory” role in the activation of the MgATPase activity in the 22-day-old myofibrils (Figure 7B). This is not the case in the 5-day-old myofibrils (Figure 7A). The analysis shown in Figure 7A indicates that the activation of MgATPase activity is independent of the occupancy of the low-affinity site or that only a very low occupancy of this site is required for activation, since it is generally accepted that Ca\textsuperscript{2+} binding to the low-affinity site of cardiac TnC is necessary to remove the inhibitory effect of TnI on actin activation of myosin.\textsuperscript{1,33} In order for the ATPase activity to be fully activated with less than 10\% of class II sites occupied, a very high degree of cooperativity between Tn-Tm units is required. The Hill coefficient for the 5-day-old myofibril ATPase experiments does not support the assumption of such a high degree of cooperativity. Therefore, we considered alternative models that include a regulatory role to the class II sites without exceptionally high cooperativity.

The first alternative model was one in which only one class I site per TnC molecule is occupied and the other is effectively “closed.” Binding occurs at the class II (low-affinity) site (H\textsubscript{1}L\textsubscript{1} model) as the single class I site saturates, but there is no interaction between the class I and class II sites. The extent of Ca\textsuperscript{2+} binding for this model is correctly described by the sum of Langmuir isotherms (x=y=1.0) described by Equation 1 with n\textsubscript{1}=n\textsubscript{2}. If the Ca\textsuperscript{2+} binding versus pCa data for the 5-day-old myofibrils are fit to a model in which the stoichiometric coefficients of the high- and low-affinity sites are equal (n\textsubscript{1}=n\textsubscript{2}=0.5), the resulting affinity constants are K\textsubscript{1}=1.69 \times 10\textsuperscript{7} M\textsuperscript{-1} and K\textsubscript{2}=1.03 \times 10\textsuperscript{6} M\textsuperscript{-1} (Table 3). If these binding constants were used to generate a set of curves similar to those shown in Figures 7A or 7B, the low-affinity site would appear to be regulatory and exhibit the same relation between Ca\textsuperscript{2+} saturation of that site and percent maximal ATPase activity as seen in the case of the 22-day-old myofibrils (Figure 7B). Thus, the H\textsubscript{1}L\textsubscript{1} model is consistent with previously published work in that it maintains the essential role of Ca\textsuperscript{2+} binding to the low-affinity site of TnC for activation of ATPase (and force).

The H\textsubscript{1}L\textsubscript{1} model was also tested by using models incorporating cooperativity among class I sites or among class II sites but without interaction between the class I and class II sites. The exponents of \textit{x} and \textit{y} in Equation 1 were given values between 0 and 1.0 for negative cooperativity and greater than 1.0 for positive cooperativity. The class I sites did not exhibit any cooperativity as the goodness of fit declined significantly with the inclusion of a cooperativity factor for the class I sites. The class II sites did exhibit some cooperativity, and the goodness of fit improved as the value of \textit{y} increased from 1.0 to 1.45 depending on the model tested. Larger values resulted in a deterioration of the regression. The best fit of all models tested for the Ca\textsuperscript{2+} binding data from 5-day-old myofibrils was obtained by using the H\textsubscript{1}L\textsubscript{1} cooperative model with \textit{x}=1.0 and \textit{y}=1.45. The binding constants derived from this model were K\textsubscript{1}=2.01 \times 10\textsuperscript{7} M\textsuperscript{-1} for class I site and K\textsubscript{2}=1.11 \times 10\textsuperscript{6} M\textsuperscript{-1} for the class II sites.

It is important to note that Equation 1 is of the form of the Hill binding equation,\textsuperscript{41} which is purely valid only for a very limiting case; that case is one in which there is extreme cooperativity among either class I or class II sites. Extreme cooperativity implies that all subunits are in one or another conformational state and that the system is unstable otherwise.\textsuperscript{42} The formal derivation of the Hill binding equation requires that the values of \textit{x} (or \textit{y}) are
integer values, because they represent the number of interacting subunits. In the present context, if values of $x = y = 1.0$, the model is the sum of Langmuir isotherms for noninteracting sites and noninteracting classes of sites. For $x$ or $y$ different from 1.0, the physical significance of $x$ or $y$ in Equation 1 is difficult to interpret, because these parameters are obtained as the result of a curve-fitting routine. Despite this limitation, the cooperativity parameter $y$ from Equation 1 does place a limit on the number of interacting class II sites and places a limit on the energy of ligand plus site to ligand plus site interactions. The positive cooperativity implies that the interaction-free energies are negative, resulting in a thermodynamically favorable process.

In addition to the $H_1L_1$ model, a second model, which incorporated the apparent Ca$^{2+}$ binding stoichiometry for the 5-day-old myofibrils, was tested. The $H_1L_1$ and $H_2L_1$ models assume that all Tn subunits present in the myofibrils are involved in Ca$^{2+}$ binding. An alternative explanation for the data is that fewer Tn subunits are required to bind Ca$^{2+}$ to all three available sites in the 5-day-old hearts than in the 22-day-old hearts to achieve full activation. In this model all Tn subunits that bind Ca$^{2+}$ do so at all three sites (the two high-affinity and one low-affinity site), but not all Tn subunits are involved in Ca$^{2+}$ binding. This would imply that the binding of Ca$^{2+}$ to $X$ Tn subunits would make binding to the next $X/2$ Tn subunits unfavorable or that there are $X/2$ Tn subunits incapable of binding Ca$^{2+}$. Here the parameter $X$ is some arbitrary number, greater than 1, of Tn-Tm units. The value $X/2$ is empiric and based on the moles of Ca$^{2+}$ bound per mole of TnC for the 5-day-old myofibrils at the point where full ATPase and force activity are noted and where significant binding of Ca$^{2+}$ to myosin occurs. The lack of binding at some nearest neighbor Tn-Tm units implies either negative cooperativity or that some of the nonbinding units are noninteracting and do not take place in activation of ATPase activity or force. The latter assumption was incorporated into the binding model. The Ca$^{2+}$ binding model used to fit the data was similar to the $H_2L_1$ model in that the 2:1 stoichiometry between the high- and low-affinity sites was maintained, but the number of class I and class II sites available to bind Ca$^{2+}$ was restricted to two thirds of the total (2/3 $H_2L_1$ model). A model incorporating the assumptions of buried noninteracting sites with otherwise cooperatively interacting sites was not tested. The 2/3 $H_2L_1$ model fit the data better than the $H_2L_1$ model (Table 3) and yielded binding constants of $1.04 \times 10^7$ M$^{-1}$ for the class I sites and $5.9 \times 10^5$ M$^{-1}$ for the class II sites. This model also restores a "regulatory" role to the class II (low-affinity) site of TnC. However, the pCa-force data mitigate against acceptance of this model.

The results of fitting the 5- and 22-day-old myofibril Ca$^{2+}$ binding data to the different models are summarized in Table 3. The sum of residuals squared for one of the data sets is provided as an index of the goodness of fit for each model for the 5- and 22-day-old myofibril binding data sets. There appears to be cooperativity for binding of Ca$^{2+}$ to the 5-day-old myofibrils, but the data do not unequivocally support cooperativity in Ca$^{2+}$ binding to the 22-day-old myofibrils.

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