

## CALCIUM BINDING TO CARDIAC TROPONIN AND THE EFFECT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE

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### 1. Introduction

The contractile activity of vertebrate striated muscle appears to be regulated by the troponin–tropomyosin complex located in the thin filaments of the myofibril [1]. In the absence of calcium, troponin inhibits the interaction of actin and myosin. Calcium binding to troponin reverses this inhibition and allows actin and myosin to form the crossbridges which initiate muscular contraction. Troponin consists of three subunits: TN-T, a tropomyosin binding protein, TN-I, a protein which inhibits the actomyosin ATPase, and TN-C which binds calcium [2]. While cardiac and skeletal muscle troponin are functionally similar, their three subunits differ in molecular weights, amino acid composition, and column elution patterns (2). Although much information is available on calcium binding to skeletal muscle troponin, little work has been done on cardiac troponin. Using skinned frog skeletal muscle fibers and mechanically disrupted rat ventricular muscle fibers, Kerrick and Donaldson [3] concluded that similar molecular mechanism were responsible for the  $\text{Ca}^{2+}$ -activation of the two muscle types. This was in contrast to the work of Ebashi et al. [4] who found that both the binding constant and capacity of cardiac troponin ( $3.4 \times 10^5 \text{ M}^{-1}$ , 1 mol  $\text{Ca}^{2+}/10^5 \text{ g protein}$ ) were lower than that of skeletal muscle troponin. Van Eerd has compared the amino acid sequences of cardiac and skeletal muscle purified TN-C [5] and

proposed that cardiac TN-C contains 3 calcium binding sites rather than the 4 sites assigned for skeletal TN-C. In view of the obvious differences between skeletal and cardiac troponins and the disagreement between experimental determinations of their calcium binding parameters, a more detailed analysis using purified troponin–tropomyosin was undertaken.

It had also been demonstrated that cyclic AMP-dependent protein kinase catalyzed the phosphorylation of TN-I in purified cardiac troponin [6,7] and that phosphorylation of TN-I was correlated directly with the increased contractility of rat hearts perfused with epinephrine [8]. Presumably stimulation of cyclic AMP formation by epinephrine resulted in activation of cyclic AMP-dependent protein kinase. Rubio et al. [9] suggested that phosphorylation of TN-I in the cardiac native actomyosin complex by the cyclic AMP-dependent protein kinase resulted in an increase in the calcium sensitivity of the actomyosin ATPase activity. This alteration of the actomyosin calcium sensitivity could arise from changes in the calcium binding parameters of troponin which regulate the ATPase activity. The purpose of this investigation was to characterize calcium binding to the cardiac troponin–tropomyosin complex and to determine if phosphorylation of the TN-I subunit in this complex altered the calcium binding parameters.

### 2. Experimental

Troponin–tropomyosin was purified from beef heart [6]. In order to determine the stoichiometry of bound calcium in addition to measuring the

**Abbreviations:** MOPS, morpholinopropane sulfonic acid; EDTA, [ethylene dinitrilo] tetra-acetic acid; EGTA, ethyleneglycol bis- $[\beta\text{-aminoethyl ether}] \text{ } N,N'$ -tetra-acetic acid.

affinity constants, it was essential to purify the troponin–tropomyosin as a complex which contained stoichiometric amounts of protein subunits. Quantitation of Coomassie Blue staining on SDS–polyacrylamide gels with corrections for differences in stain uptake by the individual purified protein components showed that one mole of each of the four proteins was present in the troponin–tropomyosin complex. Cardiac troponin–tropomyosin was phosphorylated by incubation with ATP,  $Mg^{2+}$  and cyclic AMP-dependent protein kinase as described [6] with incorporation of  $1.2 \pm 0.06$  moles of phosphate per mole of troponin–tropomyosin. The phosphate was incorporated specifically in the TN-I subunit in separate experiments using  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Total protein-bound phosphate in troponin–tropomyosin was measured before and after the phosphorylation reaction [6].

All solutions used for calcium binding were treated with Chelex-100 (Bio-Rad) to reduce contaminating calcium, then stored in plastic-ware. Less than  $1\ \mu\text{M}$  residual calcium was measured fluoro-

metrically in the buffers or protein solutions [10]. Proteins were dialyzed overnight against 250 mM KCl, 10 mM MOPS, 2 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.0, then dialyzed against three changes of 100 vol. 250 mM KCl, 30 mM MOPS, 2 mM  $MgCl_2$ , pH 7.0. Calcium binding to troponin–tropomyosin was measured by equilibrium dialysis with microcells [11] containing  $20\ \mu\text{l}$  of solution in each chamber. After mixing for 4 h,  $15\ \mu\text{l}$  was withdrawn from each chamber and the first  $10\ \mu\text{l}$  was discharged into a counting vial for measurements of radioactivity. No changes in protein concentration or protein-bound phosphate content were detected after dialysis. SDS–polyacrylamide gel electrophoresis indicated no significant proteolysis of troponin–tropomyosin after 4 h.

### 3. Results and discussion

A typical calcium binding curve for nonphosphorylated cardiac troponin–tropomyosin is shown in fig.1 along with skeletal muscle troponin–tropo-

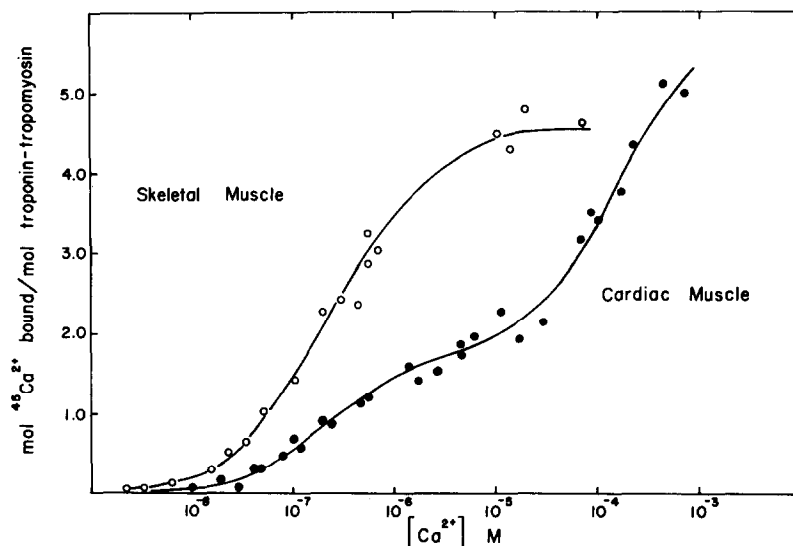


Fig.1. Effect of calcium concentration on calcium binding to cardiac and skeletal muscle troponin–tropomyosin. The total  $^{45}\text{CaCl}_2$  concentration was varied from  $2\ \mu\text{M}$  to  $1\ \text{mM}$  and aliquots were obtained after 4 h dialysis. The free calcium concentration was computed from a program using the ionic equilibria equations and binding constants of Donaldson and Kerrick [12]. The solid curves represent the best fit obtained by an iterative least squares procedure to the equation:

$$\text{Bound calcium} = \frac{n_1 K_1 [\text{Ca}^{2+}]}{1 + K_1 [\text{Ca}^{2+}]}$$

The values obtained for the cardiac proteins (●) were  $K_1 = 4.31 \times 10^6\ \text{M}^{-1}$ ,  $n_1 = 1.74\ \text{mol Ca}^{2+}\ \text{bound/mol protein}$  and  $K_2 = 6.4 \times 10^3\ \text{M}^{-1}$ ,  $n_2 = 4.2\ \text{mol Ca}^{2+}\ \text{bound/mol protein}$ . The values obtained for skeletal-muscle troponin–tropomyosin (○) are  $K_1 = 4.80 \times 10^6\ \text{M}^{-1}$  and  $n_1 = 4.37\ \text{mol Ca}^{2+}\ \text{bound/mol protein}$ .

Table 1  
Calcium-binding parameters of cardiac troponin-tropomyosin

Troponin-Tropomyosin	$K_1$ ( $M^{-1} \times 10^{-6}$ )	$n_1$ (mole/mole)	$K_2$ ( $M^{-1} \times 10^{-3}$ )	$n_2$ (mole/mole)
Nonphosphorylated (6)	$3.88 \pm 0.27$	$1.76 \pm 0.26$	$5.9 \pm 1.80$	$4.15 \pm 0.73$
Phosphorylated (5)	$4.16 \pm 0.55$	$1.60 \pm 0.13$	$5.4 \pm 1.68$	$4.22 \pm 0.75$

The values for  $K$  and  $n$  are averages  $\pm$  S.E.M. The number of determinations is shown in parentheses. The data from each experiment were analyzed for  $K$  and  $n$  values as described in fig.2. After incubation with cyclic AMP-dependent protein kinase  $1.2 \pm 0.06$  moles of phosphate/mole of troponin-tropomyosin were incorporated.

myosin. Analysis of these data by an iterative least squares procedure suggested the presence of a single class of binding sites for the skeletal muscle proteins with a maximum binding of 4 mol  $Ca^{2+}$ /mol protein. These results are essentially identical to the values reported for skeletal muscle troponin [13] in the presence of  $Mg^{2+}$ . In contrast the cardiac proteins appear to have 2 classes of binding sites. Calcium binding parameters of cardiac troponin-tropomyosin are summarized in table 1. The 2 classes of binding sites differed in their affinity constants by 3 orders of magnitude ( $K_1 = 3.9 \times 10^6 M^{-1}$ ,  $K_2 = 5.9 \times 10^3 M^{-1}$ ). Approximately 2 moles of  $Ca^{2+}$  were bound per mole of troponin-tropomyosin by the high

affinity sites. Thus at  $10 \mu M$   $Ca^{2+}$  cardiac regulatory proteins bind 2 moles of  $Ca^{2+}$  whereas skeletal muscle regulatory proteins bind 4 moles of  $Ca^{2+}$ . These results demonstrate another difference between the regulatory proteins from these two muscles.

Since  $Ca^{2+}$  binding to cardiac troponin-tropomyosin has been proposed to involve negative cooperativity [14], these data were further analyzed by a Hill plot (fig.2). The plot did not show a single line with a slope of less than unity which would be expected for negative cooperativity. Instead the curve was characteristic of 2 independent binding sites [15] with limiting slopes of 0.86 at each end. This value is slightly less than unity since the affinities for the

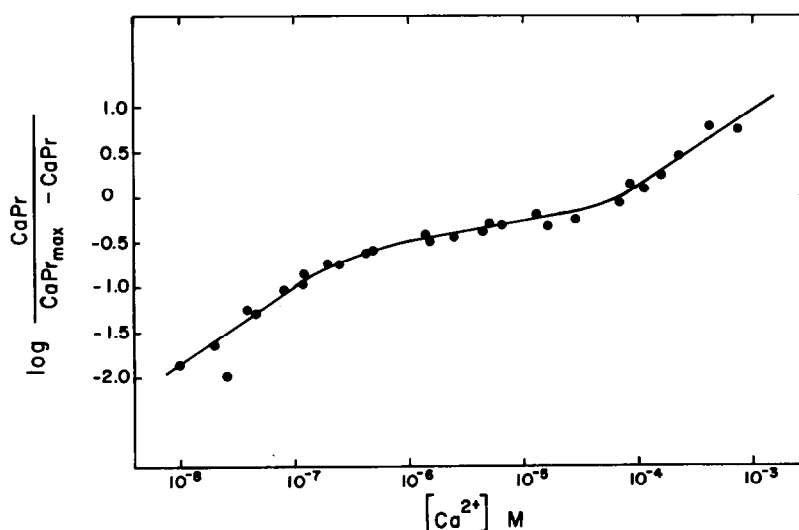


Fig.2. Hill plot of the calcium binding data of cardiac troponin-tropomyosin from fig.1. The solid curve represents the calculated isotherm obtained from the  $K_1$  and  $n_1$  values of fig.1.

2 classes are not separated by more than  $10^4$ . Also the slope in the middle of the curve is slightly greater than zero and reflects the fact that at these intermediate  $\text{Ca}^{2+}$  concentrations the amount bound is affected by the affinities for both classes of binding sites. The lack of negative cooperativity contrasts with the data of Reddy and Honig [14] and can probably be explained by the differences in  $\text{Ca}^{2+}$  concentrations used in the experiments. The Chelex method of binding is limited to  $\text{Ca}^{2+}$  concentrations from 10–90  $\mu\text{M}$  whereas with the use of an EGTA– $\text{Ca}^{2+}$  buffer system we were able to vary the free  $\text{Ca}^{2+}$  concentration from 0.004–1000  $\mu\text{M}$ .

From the amino acid sequence of the TN-C subunit of bovine cardiac troponin, van Eerd [5] has postulated that 3 calcium binding sites are present. However Solaro and Shiner [16] have estimated that only 2 calcium binding sites regulate cardiac actomyosin ATPase activity with an apparent affinity of approximately  $10^6 \text{ M}^{-1}$ . Therefore the high affinity calcium binding sites ( $K_1 = 3.9 \times 10^6 \text{ M}^{-1}$ ) in the troponin–tropomyosin complex which bind 2 moles of  $\text{Ca}^{2+}$  may regulate crossbridge formation. The importance of the low affinity sites which bind 4 moles of  $\text{Ca}^{2+}$  is not apparent at this time. Further work is obviously needed to identify the calcium-binding characteristics of the separated components of the troponin–tropomyosin complex.

Phosphorylation of cardiac troponin–tropomyosin by cyclic AMP-dependent protein kinase had no effect on either the affinity or the amount of calcium bound to the protein (table 1). Since the amount of endogenous phosphate in troponin–tropomyosin varied among different preparations, and was present in both the TN-I and TN-T subunits [6], the effect of this difference in phosphate content on calcium binding was also investigated. For two preparations of troponin–tropomyosin which contained 0.30 moles and 0.83 moles of phosphate per mole of protein respectively, the affinities and capacities of the protein for calcium were not significantly different from the values presented in table 1.

These results do not support the proposal [9] that phosphorylation of cardiac TN-I by cyclic AMP-dependent protein kinase would cause an increase in the calcium sensitivity of the cardiac actomyosin ATPase activity by increasing the affinity or capacity

for  $\text{Ca}^{2+}$  binding to troponin. Therefore a change in calcium-binding properties to cardiac troponin–tropomyosin is probably not the primary mechanism by which cyclic AMP may increase cardiac contractility. Other biochemical mechanisms related to the cyclic AMP-dependent phosphorylation of cardiac troponin must be explored.

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