Mechano-chemical effects of Ca^{2+} in cross-linked troponin-C films

Anna E. Bukatina[a]*, Victor N. Morozov[b], Nikolai B. Gusev[c], Gary C. Sieck[a]

[a] *Departments of Anesthesiology, and Physiology and Biophysics, Mayo Foundation, 200 First Street SW, Rochester, MN 55905, USA*
[b] *Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia*
[c] *Department of Biochemistry, School of Biology, Lomonosov Moscow State University, Moscow 119899, Russia*

Received 23 May 2002; revised 18 June 2002; accepted 20 June 2002

First published online 1 July 2002

Edited by Amy M. McGough

Abstract Changes in troponin C (TnC) conformation upon Ca^{2+} binding forms the basis for regulatory and structural functions of TnC molecules. In the present study, Ca^{2+}-induced conformational changes in TnC were observed by mechanical measurements. TnC films were prepared by drying or electrospraying TnC solutions, cross-linked with glutaraldehyde, and isometric tension and stiffness measured as a function of pCa. An increase in Ca^{2+} from a pCa of 9 to 4 induced large-scale mechanical changes in the TnC films causing several percent shrinkage of the unloaded films. This shrinkage could be partially assigned to Ca^{2+} binding to the Ca^{2+}/Mg^{2+} sites of TnC.

Key words: Troponin C; Calcium; Calcium-binding protein; Conformational change; Cross-linking; Protein film

1. Introduction

Troponin C (TnC) is a Ca^{2+} receptor of the thin filament regulatory system in skeletal and cardiac muscles. Crystallographic studies of TnC revealed a dumbbell-shaped molecule, 75 Å long, consisting of C- and N-terminal lobes (C lobe and N lobe) of 25 Å in diameter connected by a nine-turn α-helix, three turns of which are fully exposed to solvent [1,2]. The C lobe contains two high-affinity Ca^{2+}-binding sites ($K_{Ca} \sim 10^7$ M^{-1}) which also bind Mg^{2+} ($K_{Mg} \sim 10^3$ M^{-1}). The N lobe incorporates two Ca^{2+}-specific binding sites with lower affinity ($K_{Ca} \sim 10^5$ M^{-1}) in skeletal TnC (sTnC) and one such site in cardiac TnC (cTnC) [3,4].

Binding of metal ions induces conformational transitions in TnC modifying its interaction with other muscle proteins (for review see [5,6]). Two Me^{2+} are always bound to the C lobe in situ and support a special protein conformation enabling permanent contact of TnC with TnI. The Ca^{2+} binding to the N lobe induces conformational changes in response to physiological changes in $[Ca^{2+}]$ switching muscle between relaxed and active states.

The purpose of the present study was to use mechanical measurements of cross-linked TnC films to directly observe conformational changes in TnC caused by Ca^{2+} binding. It was found that Ca^{2+} induced large changes in isometric force of the film, which partially reflect Ca^{2+} binding to the C lobe. These large-scale mechanical changes could be used as a sensitive probe to test binding of ligands to TnC.

2. Materials and methods

2.1. Proteins

The modified procedure described in [7,8] was used for preparation sTnC from rabbit skeletal muscles. Drs. H.M. Rarick and R.J. Solaro kindly provided us with a bovine cTnC preparation.

2.2. Preparation of protein films

Dry proteins were dissolved (10–20 mg/ml) in a buffer solution (0.5 mM KH_2PO_4, 0.1–0.2 mM DTT, pH 7.0). The protein solution (30–60 μl) was dialyzed overnight against 400 ml of the buffer. Before film formation, 45% sucrose and 10% glycerol (w/(w of dry protein)) were added to the dialyzed protein solution to protect protein molecules upon drying [9].

Dry protein films were formed either by fast drying of a droplet of the protein solution on a glass plate in a vacuum chamber [10] or by an electrospray deposition technique [9]. Dry protein films were then cross-linked in a vapor of 25% glutaraldehyde at 28°C [10]. Minimum time for the films to become insoluble was determined in preliminary experiments and subsequently used as the time for cross-linking (4 min for cTnC and 5 min for sTnC films). After cross-linking, dry films were kept in a freezer (up to several days) before use. Size of dry TnC samples used in experiments was 400–800 μm by 150–300 μm with a thickness of 2–5 μm.

2.3. Solutions

TnC films were tested in a buffer solution containing 25 mM imidazole, 150 mM KCl, and 1 mM NaN_3, pH = 7.00 at room temperature (19–21°C). To reach required pCa, 2 mM Ca^{2+}–EGTA buffers were added to this solution. Concentrations of free Ca^{2+} ions were calculated using equilibrium constants of Potter and Gergely [3] to enable a direct comparison of our data with those obtained on TnC solutions by these authors as well as by Holroyde et al. [4].

2.4. Mechanical measurements

The apparatus for measuring isometric force and Young's modulus was similar to that previously described [10] with some modifications in sample attachment. Briefly, the protein film in a buffer solution was attached between a force transducer and a piezoelectric bimorph actuator by pricking the film ends with two sharp tungsten tips. Small periodical changes in sample length (0.15 Hz with amplitude $< 1\%$) were applied to measure elastic modulus. Distance between the tips could also be adjusted with a micrometer screw to impose a constant strain. Usually the film ends were folded to reinforce the sites of piercing. Sometimes the ends were reinforced with microdroplets of a cyanoacrylate glue.

The attached protein film was placed in a flow chamber. After equilibration in the buffer solution with 2 mM EGTA, the film was slightly stretched. After that, isometric force and (occasionally) stiffness were measured in different solutions. To estimate the apparent affinity for Ca^{2+}, isometric force was recorded in solutions with different pCa. The apparent binding constants were estimated using Lineweaver–Burk double-reciprocal plot as shown in Figs. 2 and 3.

*Corresponding author. Fax: (1)-507-255 7300.
E-mail address: boukatina.anna@mayo.edu (A.E. Bukatina).

Abbreviations: TnC, troponin C

0014-5793/02/$22.00
PII: S0014-5793(02)03013-2

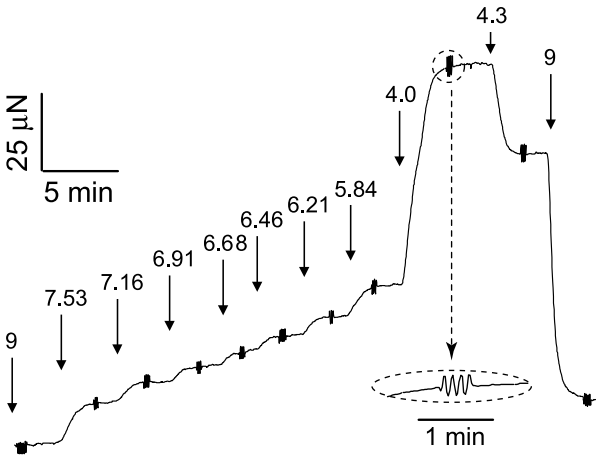

Fig. 1. Representative experiment demonstrating Ca^{2+} dependence of isometric force and stiffness of a sTnC film. Arrows indicate pCa of the solutions. The force changes induced by periodical changes in length (peak to peak amplitude, 0.9%, frequency, 0.15 Hz) were used to measure stiffness. In estimating stiffness, the amplitude of force changes was multiplied by a factor of 1.7, correcting for the frequency dependence of the measurement system.

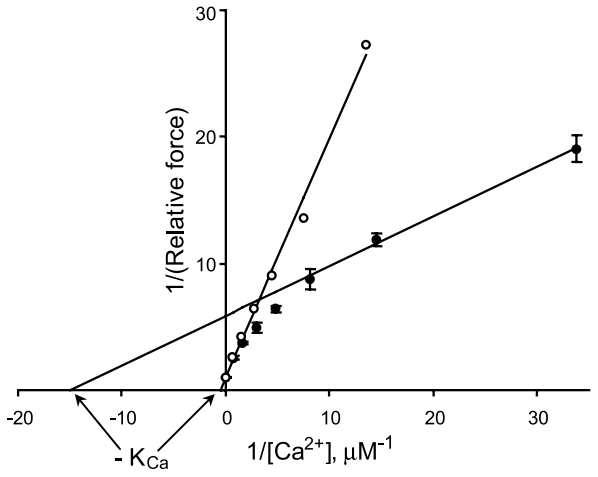

Fig. 2. Lineweaver–Burk double-reciprocal plots for force of cTnC film as a function of [Ca^{2+}]. Filled circles: without added Mg^{2+} ($n = 3$–4), open circles: with 2 mM $MgCl_2$ added to solutions (single experiment). Force in each curve was normalized to that at pCa 4. The data are fitted with linear regressions using the three lowest [Ca^{2+}] for the experiments without Mg^{2+}, and using all the data in the presence of $MgCl_2$. The best estimates for K_{Ca} are presented in Table 1.

2.5. Estimation of Ca^{2+}-induced changes in a length of unloaded TnC film

Assuming that the protein films both in free and ligand-bound states obey Hook's law, the strain was calculated according to the following formula:

$$(L_1-L_0)/L_0 = -((F_1/K_1)-(F_0/K_0))/L_0 \quad (1)$$

Here F_0, L_0, K_0 are the values of isometric force, unloaded length and stiffness in the buffer without Ca^{2+}. F_1, L_1, K_1 are the same parameters for the sample in the buffer with Ca^{2+}.

Data are presented as means ± S.E.M.

3. Results and discussion

3.1. General properties of cross-linked TnC films

Once placed in a water solution, dry TnC films swell increasing their volume approximately eight-fold. Such extensive swelling distinguishes TnC films from films of other globular proteins. For example, cross-linked amorphous lysozyme films swell only ~1.6–1.7-fold [11], which is typical for cross-linked amorphous films of globular proteins. The marked swelling of TnC films may be related to the presence of a large amount of COO^- groups and to the unusual structure of TnC, which combines fibrillar and globular domains. Straightening α-helices bent upon dehydration may be responsible for the observed high level of swelling.

Wet TnC films had an appearance of transparent gels. They were isotropic as judged from the absence of any birefringence. The films were highly elastic and could be reversibly stretched at least up to 50%.

Protein concentration in the films was estimated from the swelling ratio, taking into account the density of dry protein, 1300 g/l [12] and the molecular weight of TnC (18 000), and was found to decrease from 72 mM in dry film to 9 mM after swelling. Thus, wet TnC films contain about 16% protein.

The elastic modulus of TnC films (Table 1) was ~10^3 times smaller than that of protein crystals, 0.2 GPa [13]. Unlike protein crystals, where elastic modulus is mainly determined by direct physical contacts between neighboring protein molecules, the elastic modulus of amorphous protein films is notably smaller, being mostly determined by the elasticity of intermolecular links [14]. The difference between the modulus of crystals and amorphous films would be especially large for highly swollen and loosely packed films (e.g. compare protein concentration in TnC films, 9 mM, and in a lysozyme crystal, 55 mM [13]). Thus, changes in the elastic modulus of highly compliant TnC films should reveal changes in intermolecular contacts rather than changes in elasticity of protein molecules. On the other hand, changes in isometric force without changes in elastic modulus could reflect changes in size of protein molecules.

3.2. Mechanical effects of Ca^{2+}

TnC films responded to decrease in pCa from 9 to 4 by a

Table 1
Mechano-chemical properties of TnC films

Protein	Young's modulus[a] (kPa)		Shrinkage[a,b] by Ca^{2+} (%)	K_{Ca}[c] (M^{-1})	
	pCa 9	pCa 4		No Mg^{2+} added	2 mM $MgCl_2$
cTnC	550 ± 140 (4[d])	780 ± 130 (4)	2.5 ± 0.2 (4)	1.5×10^7	5.3×10^5
sTnC	120; 170	210; 310	8.4; 6	3.7×10^7	3.1×10^6

[a]No Mg^{2+} added.
[b]Relative changes in unloaded film length resulted from changes in pCa between 9 and 4 calculated by Eq. 1.
[c]If Ca^{2+}-binding curve has to be approximated by more than one binding constant only one of the highest affinity is presented.
[d]Figures in parentheses denote the number of samples used in measurements.

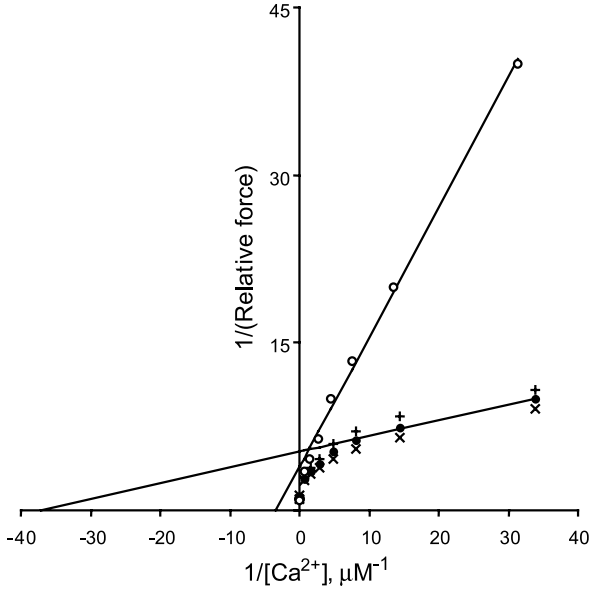

Fig. 3. Estimation of the apparent constants for Ca^{2+} binding with skeletal TnC. Filled circles: without added Mg^{2+} (average of two experiments with dried and electrospraying films denoted with '×' and '+' symbols, respectively), open circles: with 2 mM $MgCl_2$ added to solutions (single experiment). The data were normalized as indicated in the legend of Fig. 2. The lines are the best fits to the linear regressions calculated for the three lowest [Ca^{2+}] in the absence of Mg^{2+} and for the first seven lowest [Ca^{2+}] with $MgCl_2$.

large reversible increase in isometric force and a small increase in the Young's modulus (Fig. 1, Table 1). Corresponding to these mechanical changes, shrinkage of unloaded films (Table 1) was readily visible under a low-power microscope. Our data are consistent with a Ca^{2+}-induced shift in TnC conformation to a more compact structure [15], with an ~8% decrease in Stokes's radius [16]. Based on these results, we conclude that the observed mechanical effects are caused mainly by conformational changes in protein molecules rather than changes in intermolecular interaction.

In Fig. 1, it can be clearly seen that the mechanical properties of TnC films are sensitive to a very low level of free Ca^{2+} in solution (less than 10^{-7} M), which is indicative of a specific Ca^{2+} binding. Change in pCa from 9 to 5.8 caused an increase in force without considerable change in stiffness. Further increases in Ca^{2+} concentration up to pCa 4.0 resulted in additional increases in force accompanied by a notable raise of the stiffness.

The presence of notable deviation from the linear fitting of these data in the Lineweaver–Burk plot in the absence of Mg^{2+} (Figs. 2 and 3) indicates that several Ca^{2+}-binding processes with different affinities are responsible for the observed mechanical changes. To roughly estimate the highest apparent binding constants, we used data at the three lowest Ca^{2+} concentrations (Figs. 2 and 3). These estimates for both cTnC and sTnC (Table 1) are in a good agreement with the literature data for binding of Ca^{2+} to Ca^{2+}/Mg^{2+} sites of these proteins in solutions ($K_{Ca} = 1\times10^7$ M^{-1} for cTnC [4] and 2×10^7 M^{-1} for sTnC [3]).

The apparent binding constant for Ca^{2+} was reduced more than 10 times in the presence of Mg^{2+} (Table 1). Such a suppression of high-affinity Ca^{2+} binding by Mg^{2+} is in general agreement with the known competition of these two ions for the high-affinity sites [3,4]. Therefore, disappearance of high-affinity Ca^{2+} binding in the presence of Mg^{2+} provides additional support for our conclusion that the mechanical response of TnC films at low Ca^{2+} concentrations reflects Ca^{2+} binding to the high-affinity Ca^{2+}/Mg^{2+} sites.

Binding of Ca^{2+} to the high-affinity sites is known to be responsible for the majority of Ca^{2+}-induced conformational changes in TnC molecule [15]. To estimate the relative contributions of binding to the high-affinity sites to the total force changes, we used the intercepts of the fitting lines with the ordinates on Figs. 2 and 3. The intercepts give the inverse of force contribution due to binding with the specific sites. When related to the total force effect of pCa change from 9 to 4, the contribution of high-affinity binding was estimated to be 17% for cTnC and 19% for sTnC. Percentage of film deformation corresponding to such force changes (Eq. 1) is larger since there was a virtual absence of stiffness changes at low Ca^{2+} concentrations (Fig. 1). Thus, even in cross-linked protein films, TnC molecules display large conformational changes in response to Ca^{2+} binding with high-affinity sites.

Binding of Ca^{2+} to the regulatory sites on TnC could be expected to contribute to mechanical effects at pCa between 6 and 4. A significant stiffness increase in this pCa range (Fig. 1) suggests, as it was discussed above, changes in interaction between molecules. These changes could be attributed to several processes, e.g. to formation of Ca^{2+}-dependent hydrophobic intermolecular contacts [17] or to formation of Ca^{2+} bridges arising from non-specific Ca^{2+} binding, similar to that known in mechano-chemistry of polyelectrolyte fibers [18]. Thus, processes of different origin are likely responsible for the mechanical changes between pCa 6 and 4, but given the lack of data in this pCa range (Fig. 1) we cannot draw detailed conclusions.

This study demonstrates large-scale mechanical changes of TnC films in response to Ca^{2+}, which likely reflects changes in TnC ligand-binding states. Such a technique might be useful in primary screening of target proteins for drugs affecting muscle contraction. This technique is not limited to TnC, as indicated by preliminary results showing that the method can also be used to study the troponin complex (TnC, troponin T, troponin I). For the skeletal troponin complex film we found the apparent $K_{Ca} = 7\times10^6$ M^{-1} in the presence of 2 mM $MgCl_2$, which is very close to $K_{Ca} = 5\times10^6$ M^{-1} obtained in solutions by directly measuring Ca^{2+} binding [3].

Acknowledgements: This study is supported by NIH grants HL 34817 and HL37680.

References

[1] Herzberg, O. and James, M.N.J. (1985) Nature 313, 653–659.
[2] Satyshur, K.A., Rao, T.S., Pyzalska, D., Drendel, W., Greaser, M. and Sundaralingam, M. (1988) J. Biol. Chem. 263, 1628–1647.
[3] Potter, J.D. and Gergely, J. (1975) J. Biol. Chem. 250, 4628–4633.
[4] Holroyde, M.J., Robertson, S.P., Johnson, J.D., Solaro, R.J. and Potter, J.D. (1980) J. Biol. Chem. 255, 11688–11693.
[5] Filatov, V.L., Katrukha, A.G., Bulargina, T.V. and Gusev, N.V. (1999) Biokimiya 64, 969–985.
[6] Gordon, A.M., Homsher, E. and Regnier, M. (2000) Physiol. Rev. 80, 853–924.
[7] Greaser, M.L. and Gergely, J. (1971) J. Biol. Chem. 246, 4226–4233.
[8] Potter, J.D. (1982) Methods Enzymol. 85, 241–263.

[9] Morozov, V.N. and Morozova, T.Ya. (1999) Anal. Chem. 71, 1415–1420.
[10] Morozov, V.N. and Morozova, T.Ya. (1992) Anal. Biochem. 201, 68–79.
[11] Morozov, V.N., Morozova, T.Ya., Kachalova, G.S. and Myachin, E.T. (1988) Int. J. Biol. Macromol. 10, 329–336.
[12] Klapper, M.H. (1971) Biochim. Biophys. Acta 229, 557–566.
[13] Morozov, V.N. and Morozova, T.Ya. (1993) J. Biomol. Struct. Dyn. 11, 459–481.
[14] Morozov, V.N. and Morozova, T.Ya. (1981) Biopolymers 20, 451–467.
[15] Leavis, P.C. and Gergely, J. (1984) CRC Crit. Rev. Biochem. 16, 235–305.
[16] Bayers, D.M. and Kay, C.M. (1982) Biochemistry 21, 229–233.
[17] Blechner, S.L., Olah, G.A., Strynadka, N.C., Hodges, R.S. and Trewhella, J. (1992) Biochemistry 31, 11326–11334.
[18] Katchalsky, A and Zwick, M. (1955) J. Polymer Sci. 16, 221–234.