Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 170-177

EVIDENCE FOR NOVEL 30,000-50,000M_r COFACTOR IN THE ACTIVATION OF MUSCLE

Jagdish GULATI and Árvind BABU

Departments of Medicine and Physiology & Biophysics
Albert Einstein College of Medicine
Bronx, New York 10461

Received January 20, 1988

ABSTRACT A new approach is described for reconstituting a fully desensitized skeletal muscle fiber to restore its contractility. These studies revealed a novel regulatory cofactor, 30-50,000Mr by filtration (26-55kDa by SDS PAGE). It was shown to be critical for the Ca^{2+}-activation in the physiological milieu. The cofactor was present in skeletal and cardiac muscles as well as in brain, but not in kidney and liver. The cofactor may be a second Ca^{2+} switch in a dual-regulation scheme for vertebrate muscle, or could provide an essential link in the cross-bridge cycle beyond activation.

Ca^{2+} ion triggers the contraction of vertebrate striated muscle by binding a specific subunit of troponin (TnC) in the regulatory complex(1). Classically, from x-ray diffraction studies on whole muscle, a steric blocking scheme was invoked where the Ca^{2+} binding initiates the cross-bridge cycle by shifting the tropomyosin position on the actin filament and allowing actin-myosin attachment(2,3). But in tests on isolated proteins, troponin-tropomyosin affected a step in the cycle beyond the actin-myosin attachment(4,5). Consistent with this, in low salt, weakly attached bridges are seen in skinned fibers in the absence of Ca^{2+}(6), even when three-fourths of the TnC was extracted(7). Meanwhile, advanced time-resolved x-ray diffraction patterns on whole muscle still support the steric mechanism(8). In skinned fibers too, the weak bridges are less abundant in high (physiological) salt(9). Reconstitution experiments on fibers with 100% TnC deletion are now presented, revealing an additional protein moiety of 30-50,000Mr, called cofactor B, that is critical for completing the Ca^{2+} regulation in the physiological milieu. As one of the possible mechanisms we suggest that this cofactor functions critically in the activation of muscle by blocking the (weak) bridge attachment, and that its effect is reversed under physiological conditions in the presence of Ca^{2+} or by low salt. Further, the presence of the regulatory cofactor, separate from troponin and tropomyosin, is a novel finding and provides major new insights into the contraction mechanism.

0006-291X/88 $1.50

METHODS

Skinned fibers from psoas (fast-twitch) muscle of hamsters were subjected to TnC-extraction treatment at 30°C either briefly (5-30min,-TnC) (10) or for 150-180min (OTnC) to obtain desensitized fibers. The extracting solution contained 5mM EDTA and 10mM imidazole (pH.7.2). Resting tension of the fiber was unchanged following extractions. The extracted fibers were transferred to a relaxing solution (80mM K-propionate, 5mM EGTA, 5.6mM $MgCl_2$, 20mM imidazole, 5mM ATP, 20mM phosphocreatine(CP) and 250units/ml creatine phosphokinase; pH 7.0). Activating solution was either the same (ionic strength 190mM, physiological, called HIS) except that CaEGTA was substituted for EGTA, or (LIS) containing 10mM imidazole, 5.6mM $MgCl_2$ (4mM free Mg^{2+}), 1mM Mg ATP (or 5mM MgATP and no CP), 1mM Ca EGTA, 5mM (CP) and 250 units/ml kinase and pCa was made 4.0. LIS force was independent of the Mg ATP level. For resensitization, the fibers were incubated in relaxing solution containing (0.4mg/ml) of either purified TnC or combined LC's (light chains 1,2, and 3) and TnC for up to 3 hours. Prolonging this incubation to 20 hours had no further effect. Myoplasmic extract, when used, was freshly prepared for each experiment using about a gram tissue in 1-2 ml extracting solution. The tissue was finely chopped and ultrasonicated and then extracted for 2-3 hrs at 30°C. The extract was centrifuged (1000G) and the supernatant reconstituted with added salts so that the final constituents were similar to the relaxing solution, except of course for EDTA. Additional 5mM $MgCl_2$ was added to swamp EDTA, and this reformulated solution is called the soup. 12-15 hr (overnight) incubation with the soup was used to regain full sensitization. The sarcomere length of 2.5um and the temperature during activation was 5°C. Fiber selection and transducers were as desribed(7,10,11).

RESULTS and DISCUSSION

Tension response on desensitized fibers. As shown in Fig. 1A, the force on Ca^{2+}-activation in physiological salt (HIS: 180mM) was 0-10% of the control (Po) on briefly treated fibers (-TnC; partial desensitization) and exactly zero after prolonged treatment (OTnC). The deletion of TnC was fairly selective in (-)TnC fibers (79% extraction) and reconstitution with purified rabbit TnC resulted in 80-100% recovery of force response. In contrast, (0)TnC fibers lost half of the LC2 (myosin light chain2) in addition to the loss of all of TnC as indicated by the gels (Fig. 2; also Table 1A). The force recovery of (0)TnC fibers was negligible (trace c in Fig. 1A) even after both TnC and LC2 were reloaded (lane c in Fig. 2; Table 1). This indicated that with the extended extraction treatment (i) some moiety (other than TnC) critical for Ca activation was leached out, or (ii) the contractile function of the fiber was permanently impaired.

Fiber response in low salt (LIS). To test whether the contractile function was wrecked, we compared the force of (0)TnC and (-)TnC fibers in 40mM salt. Fig. 1B shows that the restored (0)TnC fibers (+TnC + LCs) gave full force in low salt even though there was practically no force in physiological salt

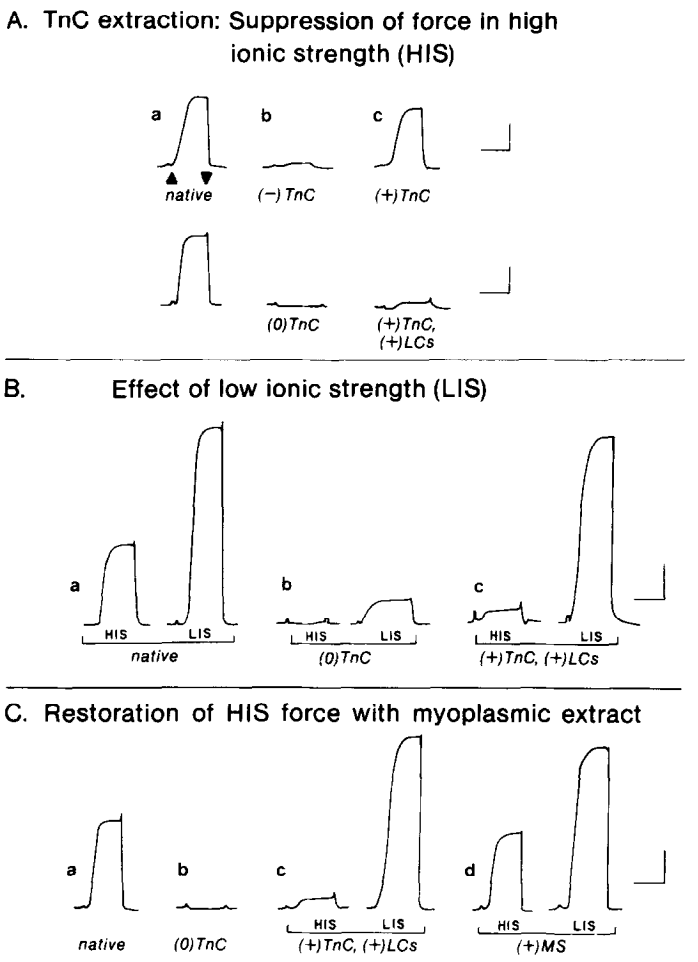

Fig. 1. Extraction of the regulatory cofactor from demembranated fibers. (A) Force responses in 180mM salt solution of a partially (-TnC) and a fully ((0)TnC) extracted fibers are compared. The residual TnC contents of such fibers are given in Table 1. To determine force, the relaxed fibers were transferred from solution with EGTA (free $Ca^{2+} < 10^{-8}$ M; ▼) to a high Ca^{2+} solution (10^{-4}M; ▲) (traces a). Both sets of fibers (-TnC and (0)TnC were nearly fully desensitized after the extraction treatments (traces b). On reconstitution with TnC, only the partially extracted fiber was found to be resensitized in physiological salt solution (HIS) (compare trace c on (-)TnC with that on (0)TnC). (B) shows the resensitization to Ca^{2+} in 40mM salt (LIS). In contrast to behavior in HIS, the (0)TnC fiber gives full force response in low salt. (C) Myoplasmic extract fully resensitizes the fiber in physiological milieu (HIS) (trace d). In each case, similar results were obtained on at least five fibers.

solution (force in low salt: $2.4P_o$, where P_o is force of the native fiber in 180mM salt solution). Force was negligible in low salt before the (0)TnC fiber was reconstituted with TnC and LCs (in Fig. 1B, the LIS force in trace b is 13% of that in a and c). There was no difference in the results by using LC2 instead of LC1+LC2+LC3 mixture. Of course, partially extracted fibers (-TnC) on reconstitution gave full recovery in both low and high salts (Fig. 1A; low salt force for this case not shown but was at the expected level of

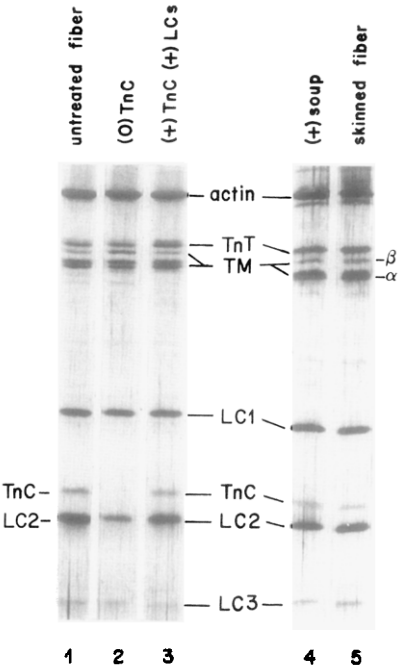

Fig. 2. SDS-PAGE runs on single fiber segments. Thin (0.75mm) gels were used and fixed with 5% gluteraldehyde prior to silver staining. (0)TnC (lane 2) indicates complete loss of TnC and 50% decrease in the LC2 band. The losses were recovered in lane 3. No difference could be ascertained between 3 and lane 4, suggesting that the regulatory cofactor missing in lane 3 is either below the sensitivity of the present gels and/or comigrates with another band. The examination of the bands above actin (not shown) also failed to show any prominent difference between 3 and 4. Lane 5 is another native fiber.

2.4Po). (-)TnC fiber also responded to Ca^{2+} activation in LIS even prior to reconstitution(7), but this force was between 0.5 - 1.0Po that is approximately 1/3 of the expected level $2.4P_o$. The low salt force in (-)TnC fibers could thus be explained by the residual TnC.

The present results showing full force recovery ($2.4P_o$) in low salt on (0)TnC fibers after TnC loading (Fig.1B) indicate that the intrinsic contractile machinery was preserved despite the prolonged extraction treatment. The persistent selective loss of force in high salt under these conditions then favors the idea that besides TnC another factor was essential to achieve Ca-activation in vertebrate fibers under physiological conditions and this cofactor was not conserved with extraction. An alternative possibility is that the restitution of LC2 and/or TnC in (0)TnC fibers is somehow imperfect, although quantitatively complete, and it is this imperfection that is corrected in low salt; but such a possibility appears unlikely.

Effect of myoplasmic soup If low salt had simply corrected an imperfection in the restoration of contractile machinery, it remained impermanent, because returning the fiber to high salt activating solution

Table 1A: RELATIVE AMOUNTS OF LIGHT CHAINS AND TnC[a]

	fiber treatment	TnC	LC3	LC2	LC1
1.	native(4)[b]	0.24±0.02	0.16±0.04	1.25±0.05	1.0
2.	-TnC(4)	0.05±0.01	0.16±0.02	1.06±0.07	1.0
3A.	0TnC(4)	0.00	0.15±0.02	0.59±0.13	1.0
B.	+TnC+LCs(4)	0.24±0.01	0.17±0.03	1.23±0.11	1.0
C.	+TnC+LCs+soup	0.30	0.14	1.05	1.0

Table 1B: RELATIVE TROPOMYOSIN (TM) AND TROPONIN-T (TnT)

		TM[c]		TnT	LC1
		α	β		
4.	native(8)	0.72±0.03	0.18±0.02	0.45±0.01	1.0
5.	0TnC(8)	0.70±0.06	0.19±0.02	0.47±0.04	1.0

(a) All amounts normalized to the LC1 bands.
(b) Data are on 4 or 8 fibers, except in 3C which are means of 2.
(c) α-TM was a doublet and the intensities were summed.

following the exposure to low salt still gave no force. On the other hand, replacement of the proposed cofactor should produce a permanent effect. To test this, myoplasmic soup was prepared with finely chopped (and ultrasonicated) rabbit psoas bundles in the EDTA solution. After 2-3 hr incubation in EDTA the concentrated myoplasmic extract was isolated. Following the incubation of (0)TnC fibers, already restored of LC's and TnC, in this extract, contractility in high salt was fully restored (trace d in Fig. 1C), indicating that the critical cofactor was present in the soup and could be reinstalled in the fiber.

Because TnT interaction with the remaining regulatory complex could be influenced by the salt concentration, it was necessary to check if TnT was the needed cofactor. TnT band is well separated in our gels (Fig. 2) and its intensity (relative to LC1) was found to be preserved in the (0)TnC fiber (Table 1B). Similarly, intensities of the tropomyosin bands were also conserved. Further, the (0)TnC fiber showed no recovery after treatment with purified TnT (200ug/ml) similar to that with the myoplasmic soup, suggesting that the new cofactor needed for Ca^{+} regulation is a separate moiety from the known regulatory system on the thin filament.

The cofactor in the soup could be inactivated by either (a) brief heating to 80°C, or (b) inclusion of trypsin. For trypsin treatment, 50ug/ml trypsin

Table 2: ACTIVITY IN DIFFERENT FRACTIONS OF THE SOUP

Fraction size (M.W.)	Tension recovery of 0TnC fiber in 180mM salt
<30kDa	-
<50kDa	+
>50kDa	+

Amicon mini-ultrafiltration cells were used with a series of membranes (YM30, XM50,YM100). Both top and bottom fractions were tested.- indicates less than 15% recovery and + 85 to 100% force recovery.

was added to the soup for 5 hrs at 20°C followed by the addition of 2mg/ml soybean trypsin inhibitor (Sigma). The soup fully retained its ability to regenerate the fiber when the trypsin inhibitor was added simultaneously with trypsin. This shows that the cofactor is a protein moiety.

Distribution of the cofactor amongst other tissues. The presence of the cofactor in a number of other tissues was evaluated by testing the activity of the appropriate cytoplasmic soup on psoas fibers. The cofactor was found in cardiac muscle as well as a slow-twitch muscle (soleus). The cofactor was not present in liver and kidney, but surprisingly the brain was positive. Because the brain is rich in calmodulin (10 to 20 fold over muscle, references 12 and 13), we tested for the ability of calmodulin to duplicate the cofactor-activity, and such a role was ruled out. Incubation of the fiber with purified CaM (bovine testes) was ineffective. Also, the addition to the soup of 1-10uM calmidazolium, a potent inhibitor of calmodulin in the phosphodiesterase system, was unable to block the cofactor activity. Similarly, the intrinsic calmodulin in the muscle also was not the cofactor, because the majority of calmodulin is in the particulate form (14), is not released on skinning and a loss of at most 20% was accompanied with prolonged TnC extraction(13). Similarly, the fact that the cofactor activity was found in soleus soup rules out parvalbumin because slow fibers are deficient of this calcium binding protein(15).

Molecular size. With Amicon ultrafiltration we found that the cofactor is between approximately 30,000 to 50,000Mr (Table 2). On SDS PAGE runs of the filtered soup, nine unidentified bands are found between 26 to 55kDa (Fig.3). Efforts are underway to isolate the particular band(s) amongst the nine with the major cofactor activity. Some of the bands are very likely extra since treatment of the tissue to obtain the soup was much more extreme than that during fiber extraction. For instance, a strong LC1 band is indicated in the

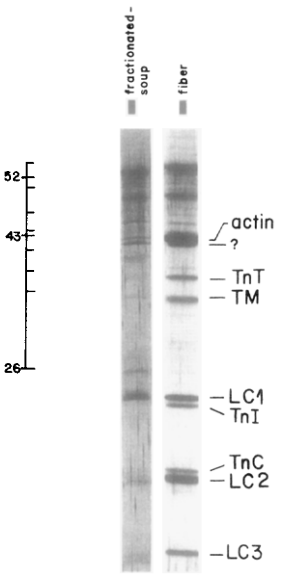

Fig. 3. SDS-PAGE run on ultra-fractionated soup from rabbit psoas muscle. The soup had been filtered successively through XM50 and YM30 Amicon membranes. The various bands in this fraction are compared with the psoas fiber, and 9 unidentified bands between 26-55kDa are noted. LC2 and TnC bands are weak in the soup lane because of the filtration through the YM30 membrane.

muscle soup (Fig.3) whereas the loss of LC1 from the fiber was minimal or not at all.and this discrepancy can easily be attributed to the different treatments.

Mechanisms for physiological action. One possibility for the mechanism of the action of the cofactor is that it is needed for the precise placement of LC2 on myosin heads. But this can not be a critical function of the cofactor because nearly half of the LC2 was undisplaced in (0)TnC fibers and these cross-bridges should have generated force on Ca-activation of the fiber. Another possible mode of action for the cofactor might be to regulate the attachment of weak cross-bridge attachments that is now considered to be one of the initial steps in the crossbridge cycle (16-18). Such a possibility would be attractive, for instance, if the co-factor bound Ca^{2+} in a separate step, and thus be a second switch in the cross-bridge mechanism(19). This could explain why force is made normally in the native fiber in physiological salt, even though the weak bridge attachments in the relaxed fiber were markedly limited (9) when Ca^{2+} was low. Alternatively, the putative cofactor-B could provide a needed link beyond the activation step: for instance, some critical transformation in the cross bridge cycle may be blocked in physiological salt in the absence of the cofactor. Additional studies in the future would be important to characterize the co-factor to fully understand the mechanism of muscular contraction.

ACKNOWLEDGEMENTS

We are grateful to Dr. John Kessler and Dr. Stan Scordilis for helpful discussions. We thank Dr. John Gergely for the generous gift of troponin T, and Dr. George Orr for Calmodulin. The grant support was NIH AR33736.

REFERENCES

(1). Ebashi, S. Proc R Soc London B 207, 259-86 (1980).
(2). Huxley,H.E. Cold Spring Harb Symp 37, 361-76 (1972).
(3). Wakabayashi,T., Huxley,H.E., Amos,L.A. & Klug,A. J Mol Biol 93, 477-97(1975).
(4). Chalovich,J. & Eisenberg,E. J Biol Chem 257, 2432-7 (1982).
(5). Rosenfeld,S. &and Taylor,E.W. J Biol Chem 262, 9984-93 (1987).
(6). Schoenberg et al Adv Exp Med Biol 170, 269-79 (1984).
(7). Babu,A., Pemrick,S. & Gulati,J. FEBS Letts 203, 20-24 (1986).
(8). Kress,M., Huxley,H.E., Faruqi,A. & Hendrex,J. J Molec Biol 188,325-42 (1982).
(9). Brenner,B., Yu,L. & Podolsky,R.J. Biophys J 46, 299-306 (1984).
(10). Babu,A., Scordilis,S., Sonnenblick,E. & Gulati,J.J Biol Chem 262, 5815-22 (1987).
(11). Gulati,J. & Babu,A. J Gen Physiol 86, 479-500 (1985).
(12). Klee,C.B. & Vanaman,T.C. Adv Prot Chem 35, 213-321 (1982).
(13). Orr,G., Babu,A., Voulalas,P. & Gulati,J. Biophys J 51, 324a (1987).
(14). Kalmut,H., Kotarba,J. & Strickland,K.Muscle & Nerve 10, 69-76 (1987).
(15). Heizmann,C.W., Berchtold,M.W. & Rowlerson,A.M. Proc Natl Acad. Sci 79, 7243-7247 (1982).
(16). Eisenberg,E. & Hill,T.L. Science 227, 999-1006 (1985).
(17). Huxley,H.E & Kress,M. J Muscle Res Cell Mol 6, 153-161 (1985).
(18). Huxley,A.F. Proc Roy Soc 183, 83-86 (1973).
(19). Babu,A. & Gulati,J. in Molecular Mechanisms of Muscle Contraction (Eds.H. Sugi and G. Pollack) Plenum, N.Y., in Press.