Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 558-565

CALMODULIN AS A MODEL FOR TROPONIN C

L. Castellani,[+] E.P. Morris[*] and E.J. O'Brien

Medical Research Council Cell Biophysics Unit
King's College
26-29 Drury Lane
London W.C. 2, England

Received August 8,1980

SUMMARY: We have investigated the ability of calmodulin to replace troponin-C in the troponin complex. Unlike troponin-C, calmodulin is able to confer Ca^{2+}-sensitivity on the actin-activated myosin subfragment-1 ATPase of filaments containing actin, tropomyosin and troponin-I in the absence of troponin-T. Gel electrophoresis of the supernatants and the sedimented filaments shows that the Ca^{2+}-sensitive reversible dissociation of calmodulin from the troponin-I causes this regulation. At high Ca^{2+} concentration troponin-I and calmodulin are associated, and they do not interact with filaments of actin and tropomyosin. At low Ca^{2+} concentration calmodulin dissociates from troponin-I, which is then able to bind to actin and tropomyosin filaments and inhibit the actin-activated myosin subfragment-1 ATPase. At no stage is calmodulin associated with the filaments. We conclude that although calmodulin seems to regulate the actin-activated myosin subfragment-1 ATPase similarly to the troponin-C plus troponin-T complex, it acts by a different mechanism. Our observations are consistent with a model in which there are two sites of interaction between troponin-I and troponin-C.

1. Introduction

Thin filament regulation in vertebrate skeletal muscle is mediated by the troponin complex which produces a Ca^{2+}-dependent change in the ability of the thin filament to activate myosin Mg^{2+}-ATPase (1).

Troponin is composed of three subunits, each of which has a distinct function. TnT[1] (30,503 M.W., ref. 2) binds strongly to tropomyosin (3). TnI (20,864 M.W., ref. 4) inhibits the activation of myosin ATPase by F-actin plus

---

[+]Present address: Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254.

[*]Present address: Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford Street, Boston, Massachusetts 02114.

[1]Abbreviations used in this paper are: TnC, troponin-C; TnI, troponin-I; TnT, troponin-T; SDS, sodium dodecyl sulphate; S-1, myosin subfragment-1.

0006-291X/80/180558-08$01.00/0

tropomyosin (5). TnC (17,868 M.W., ref. 6) relieves the inhibition brought about by TnI (7): in the troponin complex this occurs only when Ca^{2+} is bound to TnC.

Recently it has been shown that calmodulin, an ubiquitous Ca^{2+}-binding protein with a similar amino-acid sequence to TnC (8), is capable of functionally replacing TnC in the troponin complex (9). This protein has also been called Ca^{2+}-dependent regulator and modulator protein. It is involved in the regulation of phosphodiesterase (10,11) and a number of other Ca^{2+}-dependent cellular processes (12).

Amphlett et al. (9) found that calmodulin was not only able to replace TnC in the troponin complex, but it could also form a Ca^{2+}-sensitive regulatory complex with TnI, in the absence of TnT. This contrasts with the behaviour of TnC which, when TnT is not present, relieves the inhibition of TnI in a manner which is essentially unaffected by the Ca^{2+} concentration (7). Greaser and Gergely (7) also showed that full regulation is obtained only when TnT is present. Amphlett et al. (9), therefore, suggested that calmodulin was able to substitute for both TnC and TnT in the troponin complex.

We have studied the binding of calmodulin and troponin components to F-actin plus tropomyosin in order to investigate the mechanism by which calmodulin is able to replace TnT.

2. Materials and Methods

2.1 Protein preparation.

Actin from rabbit skeletal muscle was prepared by the method of Spudich and Watt (13). Tropomyosin was purified from a crude tropomyosin extract by hydroxyapatite chromatography (14). Troponin was prepared by the method of Ebashi et al. (15) and purified by that of van Eerd and Kawasaki (16). It was fractionated into components as described by Perry and Cole (17). TnT and TnC obtained by this method were quite pure. The TnI needed further purification as described by Wilkinson (18). The troponin components and the tropomyosin were stored freeze-dried at $-20^{o}C$. S-1 was prepared by the method of Weeds and Taylor (19) and stored in 50% glycerol at $-20^{o}C$. Calmodulin was purified from beef brain by a procedure similar to that of Watterson et al. (20), which involves ammonium sulphate fractionation, DEAE-cellulose chromatography and Sephadex G75 gel filtration (Alemá and Castellani, unpublished method).

2.2 ATPase assay.

ATPase measurements were made using a Radiometer pH-stat apparatus as described by Eisenberg and Moos (21). The reaction mixture contained 40 mM KCl, 2 mM $MgCl_2$ and 1 mM ATP. The assay was performed at pH 8.0 and the temperature was maintained at 25°C.

2.3 Binding experiments.

The binding experiments were performed in a solution containing 0.15M NaCl, 2 mM $MgCl_2$, 10 mM sodium phosphate, pH 7.0, and 1 mM ATP at 5°C. The final actin concentration was 1 mg/ml; the regulatory proteins were added in a 7:2:2:2:3 molar ratio of actin:tropomyosin:TnT:TnI:calmodulin. The regulatory components were added in excess to ensure maximal binding. The actin was polymerized in the presence of tropomyosin and added to a mixture of the other proteins. The Ca^{2+} concentration was adjusted to a very low level by adding EGTA to a final concentration of 0.3 mM. A high Ca^{2+} level was achieved by adding $CaCl_2$ to a final concentration of 0.1 mM. The samples were allowed to stand at 5°C for 1 hour and were then centrifuged at 90,000 xg for 1 hour to pellet the filaments.

The protein composition of both the pellets and the supernatants was analysed by SDS polyacrylamide gel electrophoresis by the method of Laemmli (22).

3. Results

3.1 ATPase assay.

Ca^{2+}-dependent regulation of the actin-activated S-1 ATPase was obtained with calmodulin both with and without TnT. Figure 1 shows the ATPase trace without TnT. The initial slope represents the actin-activated S-1 ATPase. The addition of tropomyosin reduces this rate (Fig. 1b), in agreement with the results of Eaton et al. (23). When both TnI and calmodulin are present, the high Ca^{2+} rate (Fig. 1d) is the same as that obtained with actin and tropomyosin, and the low Ca^{2+} rate (Fig. 1e) is close to that obtained with actin, tropomyosin and TnI. The presence of TnT did not affect the high Ca^{2+} rate, but increased the inhibition at low Ca^{2+} by about 10%.

3.2 Binding studies.

The mechanism of this regulation was investigated by a series of binding experiments of calmodulin and the troponin components to filaments of actin and tropomyosin. The composition of the filaments was examined by sedimentation and gel electrophoresis. The gels of the pellets and supernatants are shown in

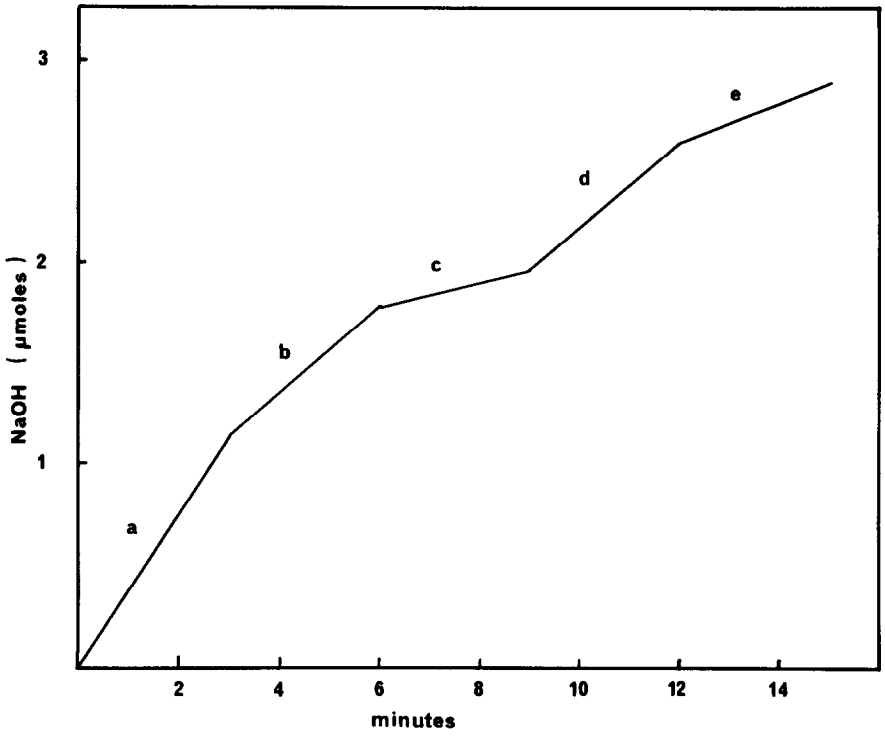

Figure 1. Schematic representation of the actin-activated S-1 ATPase measured by the rate of proton release. The intrinsic S-1 ATPase rate has been subtracted in each case. The proton release after the sequential addition of a) F-actin; b) tropomyosin (inhibition 43%); c) TnI (inhibition 84%); d) calmodulin (inhibition 43%) (trace amounts of Ca^{2+} in the assay mixture ensure that calmodulin is in its high Ca^{2+} form); e) EGTA added to a final concentration of 0.2 mM (inhibition 73%). The inhibition is evaluated so that it is 0% for the full actin-activated rate and 100% when the S-1 ATPase is not activated. The concentrations of proteins in the reaction mixture were: S-1, 0.2 mg/ml; actin, 0.2 mg/ml; tropomyosin, 0.1 mg/ml; TnI, 0.03 mg/ml; calmodulin, 0.06 mg/ml.

Figure 2. When the filaments are formed from actin, tropomyosin and TnI, the amount of TnI bound is not affected by changing the Ca^{2+} concentration. The pellets (Fig. 2a, lanes i and ii) contain all three proteins while the supernatants (Fig. 2b, lanes i and ii) contain mostly tropomyosin but very little TnI, both of which were added to excess. This observation is in agreement with the finding of Hitchcock (24) that the binding of TnI to F-actin plus tropomyosin does not saturate at a 1:7 molar ratio to the actin.

When calmodulin is present in addition to the previous combination, considerably more TnI is bound to the filament at low Ca^{2+} than at high Ca^{2+} and very little calmodulin is bound at either Ca^{2+} concentration.

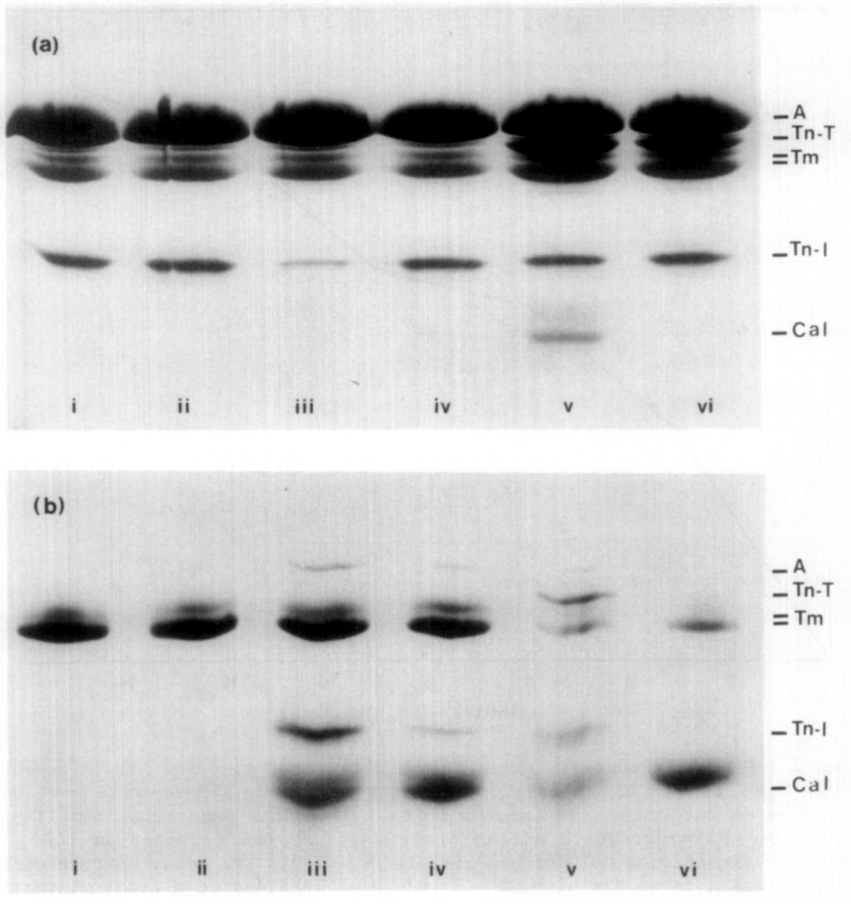

Figure 2. Fourteen per cent acrylamide SDS gels of (a): the pellets and (b): the supernatants obtained in the binding experiments. The combinations of proteins and Ca^{2+} concentrations used in forming the filaments were as follows: (i) and (ii) actin, tropomyosin and TnI; (iii) and (iv) actin, tropomyosin, TnI and calmodulin; (v) and (vi) actin, tropomyosin, TnT, TnI and calmodulin. In (i), (iii) and (v) the Ca^{2+} concentration was 0.1 mM. In (ii), (iv) and (vi) EGTA was included at a concentration of 0.3 mM.

The addition of TnT to the system causes most of the TnT, TnI and calmodulin to be bound to the filaments at high Ca^{2+} so that they are effectively reconstituted thin filaments in which calmodulin has replaced the TnC. At low Ca^{2+} calmodulin dissociates from the filaments and TnI and TnT remain. In fact comparison between the gels of the supernatants (Fig. 2b, lanes v and vi) shows that at low Ca^{2+} slightly more TnI and TnT are bound to the filaments. These gels also show that in the presence of TnT rather more

tropomyosin is pelleted. This is probably due to a direct precipitation of the tropomyosin by TnT as observed by Dabrowska et al. (25).

4. Discussion

Our ATPase measurements show that the calmodulin plus TnI complex, unlike TnC plus TnI, is capable of providing a Ca^{2+}-dependent regulation of the actin-activated S-1 ATPase. The degree of regulation is similar to that obtained by Amphlett et al. (9). The binding studies of calmodulin and TnI to the sedimented filaments show that this effect can be explained by the dissociation at low Ca^{2+} of calmodulin from TnI, which allows the TnI to bind to and inhibit F-actin plus tropomyosin. In contrast, TnI plus TnC bind to the filaments at low Ca^{2+} and dissociate as a complex at high Ca^{2+} (26), but at no stage inhibit (7). When the Ca^{2+} concentration is raised the binding between TnI and calmodulin is strengthened, causing the TnI to dissociate from the filament. This allows the actin-activated ATPase of the filament to return to the F-actin plus tropomyosin level. This interpretation is supported by the ATPase measurements, in which the high Ca^{2+} rate is similar to that with F-actin plus tropomyosin and the low Ca^{2+} rate approaches that obtained with TnI. Thus at no stage is calmodulin itself bound to the actin and tropomyosin filament. The regulation obtained derives from the reversible dissociation of TnI caused by calmodulin. This process can be represented schematically:

$$\underset{\text{inhibited}}{[\text{F-actin - Tm - TnI}]} + \text{Cal} \overset{Ca^{2+}}{\longleftrightarrow} \underset{\text{active}}{[\text{F-actin - Tm}]} + [\text{TnI - Cal:}Ca^{2+}]$$

The Ca^{2+} regulation of the activity of F-actin plus tropomyosin plus TnI by calmodulin is therefore achieved by a different mechanism from that of the TnC plus TnT complex. Our binding experiments to actin and tropomyosin filaments are consistent with the results obtained by native gel electrophoresis of the isolated proteins (9).

The major difference between calmodulin and TnC as regards their behaviour in the troponin complex is that the binding between TnI and calmodulin is weakened on the removal of Ca^{2+} to a much greater extent than is the binding between TnI and TnC. This suggests a model for the action of TnC in the troponin complex. In this model there are two sites on each molecule involved in the interaction between TnI and TnC. One pair of sites provides a link between the two subunits which is not affected by the binding of Ca^{2+}. The interaction between the second pair of sites only occurs when Ca^{2+} is bound to TnC. This second interaction prevents the binding of TnI to actin and thus relieves the inhibition. This would be the case if in TnI the second type of TnC binding site coincides with the actin binding site. According to this model calmodulin is essentially the same as TnC except that either it lacks the first type of binding site or the binding at this site is weaker.

It can be seen that this is an extension of the model proposed by Hitchcock et al. (26). The hypothesis that two different regions are involved in the interaction between TnI and TnC derives from the finding (27) that two fragments of TnI are capable of interacting with TnC but only one can inhibit the actin activation. Further evidence is the work of Leavis et al. (28) and of Weeks and Perry (29) with fragments of TnC which indicates that two distinct regions of the molecule are involved in the interaction with TnI.

It would seem, therefore, that TnC is a specialized version of calmodulin in which a permanent binding site for TnI has evolved. This would be an advantage in a system like skeletal muscle which responds rapidly to stimuli. If the TnC were dissociated from the regulatory complex in relaxed muscle, the delay in response would be increased by the time required for TnC to diffuse into the complex. It may be that this additional link is the cause of the reduced inhibitory effect of TnI in the TnI plus TnC complex, and the presence of TnT is required in whole troponin in order to restore full regulation.

ACKNOWLEDGMENTS: We are most grateful to Dr. S. Alemå, Mr. R. Bennett and Dr. D. Mercola for generous gifts of proteins. We wish to thank Mr. G. Johnson for valuable suggestions and Drs. P.M. Bennett, P. Leavis, S.S. Lehrer and G. Offer for their helpful criticism of this manuscript.

One of us (L.C.) was in receipt of a fellowship sponsored by the Accademia der Lincei and the Royal Society, under the European Exchange Program, and subsequently a fellowship from the European Molecular Biology Organisation.

REFERENCES

1. Ebashi, S. and Endo, H. (1968) Prog. Biophys. Mol. Biol. 18, 123-182.
2. Pearlstone, P.R., Carpenter, M.R., Johnson, P. and Smillie, L.B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1902-1906.
3. Greaser, M.L., Yamaguchi, M., Brekke, C., Potter, J. and Gergely, J. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 235-244.
4. Wilkinson, J.M. and Grand, R.J.A. (1975) Biochem. J. 149, 493-496.
5. Perry, S.V., Cole, H.A., Head, J.F. and Wilson, F.J. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 251-265.
6. Collins, J.H., Potter, J.D., Horn, M.J., Wilshire, G. and Jackman, N. (1973) FEBS Lett. 36, 268-278.
7. Greaser, M.L. and Gergely, J. (1971) J. Biol. Chem. 246, 4226-4233.
8. Vanaman, T.C., Sharief, F. and Waterson, D.M. (1977) in Calcium Binding Proteins and Calcium Function (R.H. Wasserman, R.A. Corradino, E. Carafoli, R.H. Krebs, D.H. MacLennan and F.L. Siegel, eds.) pp. 107-116. Elsevier-North Holland, New York.
9. Amphlett, G.W., Syska, H. and Perry, S.V. (1976) FEBS Lett. 72, 163-167.
10. Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 38, 533-538.
11. Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104-1110.
12. Cheung, W.Y. (1980) Science 207, 19-27.
13. Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866-4871.
14. Eisenberg, E. and Kielley, W.W. (1974) J. Biol. Chem. 249, 4742-4748.
15. Ebashi, S., Wakabayashi, T. and Ebashi, F. (1971) J. Biochem. 69, 441-445.
16. Van Eerd, J.P. and Kawasaki, Y. (1973) Biochemistry 12, 4972-4980.
17. Perry, S.V. and Cole, H.A. (1974) Biochem. J. 141, 733-743.
18. Wilkinson, J.M. (1974) Biochem. Biophys. Acta 359, 379-388.
19. Weeds, A.G. and Taylor, R.S. (1975) Nature 257, 54-56.
20. Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501-4513.
21. Eisenberg, E. and Moos, C. (1967) J. Biol. Chem. 242, 2945-2951.
22. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
23. Eaton, B.L., Kominz, D.R. and Eisenberg, E. (1975) Biochemistry 14, 2718-2724.
24. Hitchcock, S.E. (1975) Eur. J. Biochem. 52, 255-266.
25. Dabrowska, R.J., Polubnaya, Z., Nowak, E. and Drabikowski, W. (1976) J. Biochem. 80, 89-99.
26. Hitchcock, S.E., Huxley, H.E. and Szent-Györgyi, A.G. (1973) J. Mol. Biol. 80, 825-836.
27. Syska, H., Wilkinson, J.M., Grand, J.A. and Perry, S.V. (1976) Biochem. J. 153, 375-387.
28. Leavis, P.C., Rosenfeld, S.S., Gergely, J., Groback, E. and Drabikowski, W. (1978) J. Biol. Chem. 253, 5452-5459.
29. Weeks, R.A. and Perry, S.V. (1978) Biochem. J. 173, 449-457.