EFFECT OF THE TROPONIN C-LIKE PROTEIN FROM BOVINE BRAIN (BRAIN MODULATOR PROTEIN) ON THE Mg^{2+}-STIMULATED ATPase OF SKELETAL MUSCLE ACTOMYOSIN

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1. Introduction

Increasing evidence indicates that the divalent cation Ca^{2+} and the cyclic nucleotides act as central regulatory signals for numerous events in animal cells (for review, see [1–3]). In vertebrate muscle, Ca^{2+} regulates actomyosin activity primarily through its action on the calcium binding component of the troponin complex, troponin C. Recently, a ubiquitous troponin C-like protein with high affinity for Ca^{2+} has been shown to be present in relatively high levels as a soluble component in neurosecretory tissue of numerous vertebrate species [4] and in rapidly proliferating fibroblasts [5,6]. This protein, referred to as modulator protein*, is structurally similar to vertebrate muscle troponin C [7], and has previously been shown to possess two potential regulatory functions in animal cells. First, in the presence of calcium this protein will stimulate the activity of partially purified preparations of cyclic nucleotide phosphodiesterase as first shown by Cheung [8] and Kakiuchi [9]. Second, Cheung and co-workers [10,11] and others [12] have shown that modulator protein is also a calcium-dependent stimulator of partially purified preparations of Lubrol solubilized adenyl cyclase activity. We report here that modulator protein possesses troponin C-like activity in Ca^{2+}-regulated actomyosin systems from skeletal muscle. In the presence of troponin I and tropomyosin from skeleta muscle it is as effective in restoring Ca^{2+}-sensitivity to desensitized actomyosin as is the whole troponin complex and tropomyosin, both isolated from skeletal muscle.

*Because of its potential multiple regulatory functions, the protein has previously been termed a modulator protein [7].

2. Methods

2.1. *Muscle proteins*

Troponin from white skeletal muscle of the rabbit was prepared by the method of Ebashi et al. [13] and further purified by chromatography on DEAE-cellulose in 50 mM Tris–HCl, 15 mM 2-mercapto-ethanol, pH 8.0 [14]. Troponin C and troponin T were prepared by chromatography of troponin on DEAE-Cellulose in 8.0 M urea 50 mM Tris–HCl, 15 mM 2-mercaptoethanol, pH 8.0 [15]. Troponin I was purified from troponin B by chromatography on CM-Cellulose in 8.0 M urea, 50 mM sodium formate, 15 mM 2-mercaptoethanol, pH 4.0 [16]. Tropomyosin and desensitized actomyosin were prepared from white skeletal muscle of the rabbit as described by Cummins and Perry [17]. The purity of all protein preparations was checked before use by polyacrylamide gel electrophoresis in sodium dodecyl sulphate at pH 7.0 [18].

2.2. *Brain modulator protein*

Bovine brain modulator protein was prepared and tested for purity (greater than 95%) as described by Watterson et al. [7]. This preparation was identical in phosphodiesterase activator activity, physico-chemical properties, and in primary structure so far as

could be judged from tryptic peptide maps with the phosphodiesterase activator protein from bovine heart [19] prepared by a different procedure [20].

2.3. *Enzymic assays*

These were carried out in general as described by Schaub and Perry [21]. Specific details are described in the figure legends.

3. Results

Brain modulator protein behaved in a very similar manner to troponin C from white skeletal muscle in relieving the inhibition by white skeletal muscle troponin I of the Mg^{2+}-stimulated ATPase of desensitized actomyosin in the absence EGTA (fig.1). In both cases neutralisation of the inhibition was complete when the molar ratio of troponin C or modulator protein to troponin I was about 0.5 [22].

In the presence of EGTA, brain modulator protein differed quantitatively from troponin C from white skeletal muscle in that it was completely ineffective in relieving the inhibition of ATPase activity by troponin I, i.e., the mixture of muscle troponin I and brain modulator protein restored Ca^{2+}-sensitivity to the muscle actomyosin ATPase. At lower molar ratios of skeletal troponin C to troponin I, some Ca^{2+}-sensitivity of the ATPase was observed [22], although it was not complete and disappeared at higher ratios (fig.1a). Calcium sensitivity was restored to the system at all ratios of modulator protein to troponin I tested (fig.1b). Brain modulator protein alone or in the presence of troponin T had no effect on the Mg^{2+}-stimulated ATPase activity of desensitized actomyosin to which tropomyosin had been added, either in the presence of absence of 1 mM EGTA. No Ca^{2+}-sensitivity was obtained with any combination of the regulatory proteins in the absence of tropomyosin.

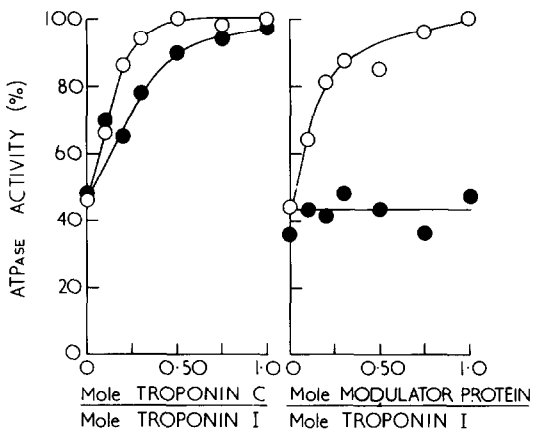

Fig.1. The effects of bovine brain modulator protein and rabbit skeletal muscle troponin C on the inhibition of the Mg^{2+}-stimulated ATPase of desensitized actomyosin by rabbit skeletal troponin I. ATPase assays carried out in 25 mM Tris–HCl, pH 7.6, 2.5 mM ATP 2.5 mM $MgCl_2$ with 450 μg desensitized actomyosin and 150 μg tropomyosin and in the presence or absence of 1 mM EGTA as indicated. Total volume 2 ml, incubated 5 min at 25°C. Activities expressed as percentage of that obtained in the absence of troponin I. (a) Effect of increasing amounts of troponin C from rabbit white skeletal muscle. (○) EGTA absent, (●) 1 mM EGTA. (b) Effect of increasing amounts of bovine brain modulator protein. (○) EGTA absent, (●) 1 mM EGTA.

When ATPase activities were compared at equal troponin I concentrations in the presence of tropomyosin, a mixture containing skeletal troponin I and brain modulator protein in the molar ratio of 1.0 : 0.5 was just as effective in restoring Ca^{2+}-sensitivity to the Mg^{2+}-stimulated ATPase as was the whole troponin complex from skeletal muscle containing troponin I, troponin C and troponin T in the molar ratio 1 : 1 : 1.

The above experiments would not reveal any differences in response at different Ca^{2+} concentrations as they were carried out in the presence of 1 mM EGTA which effectively reduced the Ca^{2+} concentration to less than 10^{-8} M. When the ATPase activity of the reconstituted actomyosin systems were assayed over a range of Ca^{2+} concentrations fixed by the use of a Ca buffer system [23] however, the troponin regulated system was 50% activated at a slightly lower Ca^{2+} concentration (about 0.5 pCa^{2+} unit) than that required to produce similar activation with the equimolar mixture of brain modulator protein and muscle troponin I (fig.2). As the Mg^{2+}-stimulated ATPase of desensitized actomyosin is very sensitive to ionic strength, enzymic assays were carried out at low ionic strength. Under these conditions maximal activation of myofibrillar and actomyosin ATPase occurs at pCa^{2+} values of about 7 [24].

A possible explanation of the difference in their abilities of modulator protein and troponin C to restore Ca^{2+}-sensitivity to the actomyosin ATPase was that the skeletal troponin C was slightly modified

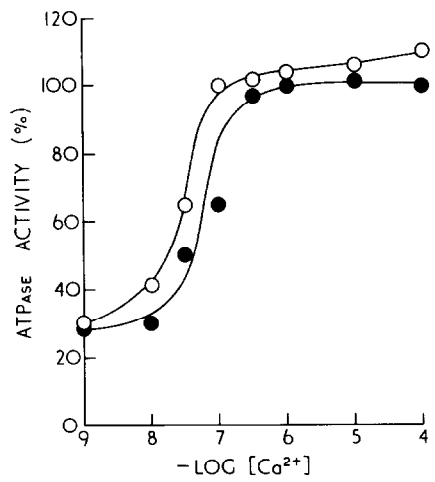

Fig.2. The effects of Ca^{2+} concentration on the ATPase activity of desensitized actomyosin in the presence of skeletal muscle troponin and an equimolar mixture of brain modulator protein and white skeletal muscle troponin I. Assay conditions generally as indicated for fig.1. Ca^{2+}–EGTA buffers were made as described by Charbarek and Martell [23]. (○) Troponin complex from white skeletal muscle of the rabbit, (●) equimolar mixture of bovine brain modulator protein and white skeletal troponin I.

by the procedures used in its preparation. This involves exposure to high concentrations of urea whereas brain modulator protein, which is obtained by very mild conditions, should have retained all the properties of the native protein. This was not the explanation, for after incubation for 12 h at 20°C in 8.0 M urea, 50 mM Tris–HCl, pH 7.6, and subsequent removal of the urea by dialysis against water, the ability of modulator protein to restore Ca^{2+}-sensitivity with skeletal troponin I was no different from that of untreated preparations of the protein.

All other samples of muscle troponin C tested, including those prepared from bovine heart and chicken breast, as well as the rabbit skeletal muscle protein modified by nitration [25] and carboxymethylation [26] were unable to restore complete Ca^{2+}-sensitivity to the actomyosin ATPase activity in the presence of added troponin I and tropomyosin.

On polyacrylamide gel electrophoresis at pH 8.6, brain modulator protein migrated rapidly to the anode with a mobility slightly less than that of troponin C from white skeletal muscle (fig.3a). In the presence of Ca^{2+}, a complex was formed with equimolar amounts of skeletal troponin I with a mobility of about 50% of that of the brain modulator protein. The complex was not formed in the absence of Ca^{2+}. It was similar to that formed by skeletal troponin C and troponin I [27] in its electrophoretic mobility and stability in 6.0 M urea in the presence of Ca^{2+}. With some preparations of skeletal troponin I, brain modulator protein formed several bands of complex (fig.3d). This phenomenon also is observed with skeletal troponin C although usually only a single additional band is seen when it is complexed with white skeletal troponin I.

In the presence of Ca^{2+} the electrophoretic mobility of modulator protein at pH 8.0 is slightly reduced as would be expected from the decrease in negative charge on the protein resulting from binding the cation. Skeletal muscle troponin C increases in mobility under similar conditions for the decrease in hydrated volume associated with the conformational change that occurs when calcium ions are bound more than compensates for the charge effect [27].

Evidence was also obtained from the electrophoretic studies for the formation of a brain modulator protein–skeletal troponin T complex (fig.3g) using conditions analogous to those under which the skeletal troponin C–skeletal troponin T complex can be demonstrated [28]. In the presence of EGTA the band corresponding to the complex disappeared completely (fig.3h). No evidence was obtained from the electrophoretic studies for direct interaction between brain modulator protein and tropomyosin. If however, skeletal troponin T was present interaction between tropomyosin and the brain modulator protein–skeletal troponin T complex could be demonstrated (fig.3k).

4. Discussion

The evidence from studies of the physical properties and amino acid sequence indicating that the brain modulator protein is similar to troponin C from skeletal muscle is further strengthened by the finding that it also possesses a number of the biological properties of muscle troponin C. These include the ability to neutralise the inhibitory activity of troponin I from skeletal muscle and to form Ca^{2+}-dependent complexes with troponin T and I, the latter being stable to high urea concentration. As troponin T

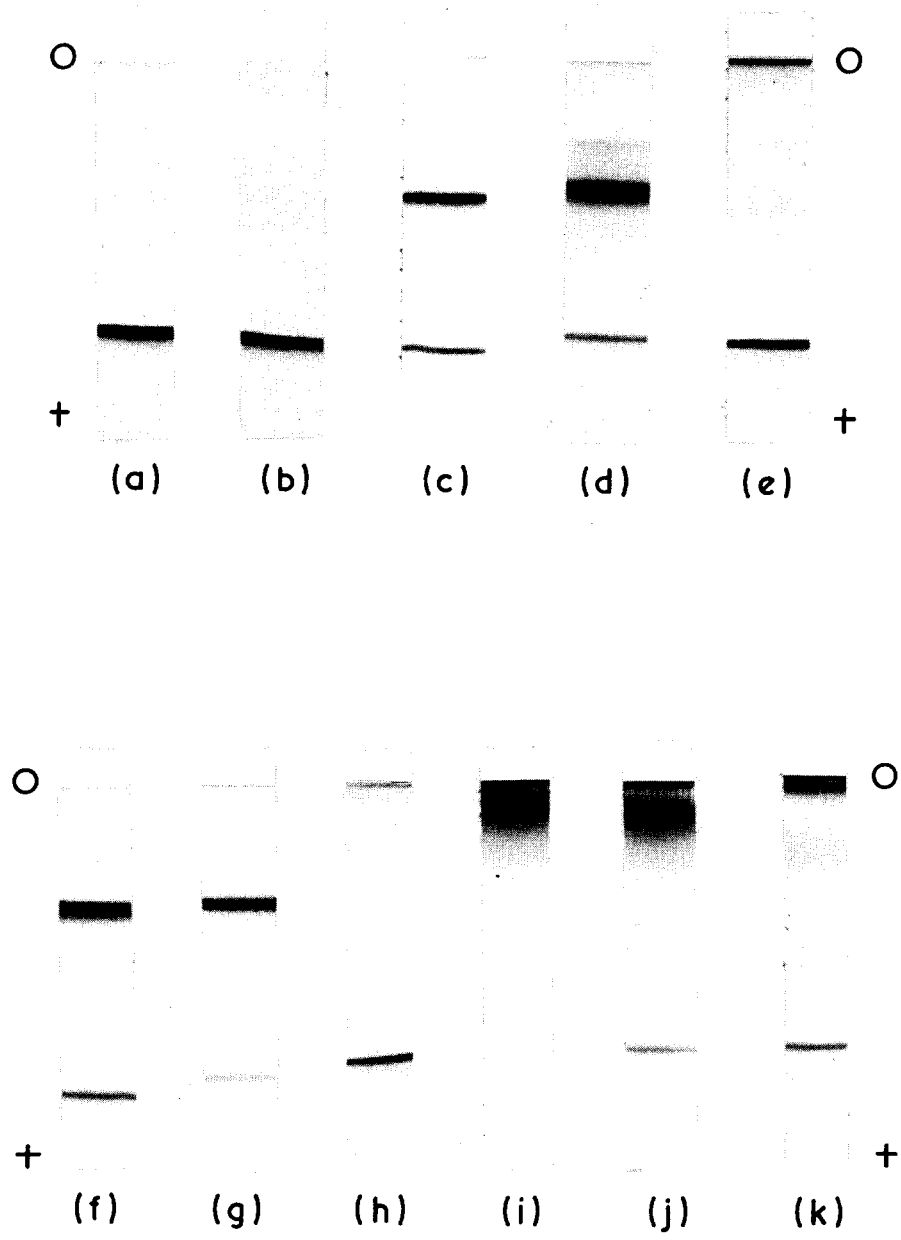

Fig.3. Electrophoresis of bovine brain modulator protein and the components of regulatory protein complex from rabbit skeletal muscle. Final conditions: 10% polyacrylamide, 25 mM Tris–80 mM glycine, pH 8.6 [18]. Gels stained in 0.25% Coomassie Brilliant Blue R in 50% (w/v) trichloroacetic acid and destained in methanol/acetic acid/water (5:1:14, by vol.) (o) Origin. (a) 15 μg brain modulator protein, (b) 15 μg skeletal muscle troponin C, (c) 10 μg skeletal troponin C + 5 μg skeletal troponin I, (d) 10 μg brain modulator protein + 5 μg skeletal troponin I, (e) 10 μg brain modulator protein + 5 μg skeletal troponin I + 2 mM EGTA, (f) 10 μg skeletal troponin C + 10 μg skeletal troponin T, (g) 10 μg brain modulator protein + 10 μg skeletal troponin T, (h) 10 μg brain modulator protein + 10 μg skeletal troponin T + 2 mM EGTA, (i) 40 μg skeletal tropomyosin, (j) 10 μg brain modulator protein + 10 μg skeletal tropomyosin (k) 10 μg brain modulator protein + 10 μg skeletal troponin T + 40 μg skeletal tropomyosin.

is considered to be the component that binds the troponin complex to specific sites on tropomyosin of the I filament of the muscle, the interaction between troponin T and troponin C is presumably the link involved in the process. Nevertheless, although the brain modulator protein possesses the property of interacting with skeletal troponin T, troponin T is not essential for the restoration of Ca^{2+}-sensitivity to the Mg^{2+}-stimulated ATPase of muscle actomyosin by brain modulator protein in the presence of muscle troponin I and tropomyosin.

The electrophoretic evidence suggests that neither troponin I nor brain modulator protein interact with tropomyosin. This implies that the troponin I–brain modulator protein complex need to be directly linked to tropomyosin for Ca^{2+}-regulation of the actomyosin ATPase in the system described. In the light of these facts and the observation that under certain conditions troponin T likewise is not required for Ca^{2+}-sensitivity of the ATPase skeletal actomyosin systems [22], the role of troponin T and of tropomyosin in the regulation of myofibrillar actomyosin ATPase activity requires re-examination. The results obtained with brain modulator protein are not easily reconciled with models involving troponin C linked to tropomyosin via troponin T and the blocking of sites on actin by the movement of the tropomyosin filament in the groove of the I filament [29–31]. Possibly troponin T has evolved as an additional regulatory protein for the highly developed fast-reacting control systems required for specialised muscle tissue.

The troponin C-like modulator protein is widely distributed in both vertebrate and invertebrate species [4,32,33]. It should be noted that only the bovine brain protein was tested in the present study. However, the physico-chemical properties, phosphodiesterase activator activities and tryptic peptide maps of the modulator proteins prepared from bovine, porcine, rabbit, rat and chicken brains are virtually identical [34]. These proteins would therefore, be expected to have very similar abilities to interact with the muscle actomyosin system used in the present study.

The relationship of the brain modulator protein to the troponin C-like protein isolated from brain by Fine et al. [35] is not clear. The two proteins show many similarities although, unlike the modulator protein, the troponin C-like protein of Fine et al. [35] apparently required troponin T for restoration of calcium sensitivity to skeletal actomyosin ATPase.

In addition to their highly conserved structural and functional properties, all modulator proteins tested to date contain a specifically trimethylated lysine residue. The role of this specific modification is unclear at present. However, rabbit skeletal muscle troponin C which lacks this methylated amino acid does not activate cyclic nucleotide phosphodiesterase [36] despite its structural similarity to modulator protein.

It can be concluded that troponin C-like modulator proteins present in many tissues may have a multi-functional role, controlling contractile activity as well as the metabolism of 3′,5′-cyclic adenylic acid. When tested with actomyosin and regulatory proteins derived from muscle, these troponin C-like proteins isolated from non-muscle tissue can provide valuable insight into the mechanism of action of the actomyosin regulatory protein system.

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