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Biochemical and Biophysical Research Communications 369 (2008) 43–48
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In Memoriam

Background to the discovery of troponin and Setsuro Ebashi's contribution to our knowledge of the mechanism of relaxation in striated muscle

Abstract

The discovery of the actomyosin system provided for the first time a model system that enabled the study of the role of the muscle protein components in the contraction and relaxation cycle to be undertaken. It soon became apparent that ATP was essential for both processes but progress really began when it became clear that components both in the myofibrillar and sarcoplasmic fractions were involved in relaxation. After it was apparent that a trace of calcium was required for the activation of the MgATPase of the myofibrils it was shown that an active calcium pump was located in the sarcoplasmic reticulum. The report by Ebashi in 1963 that a new myofibrillar protein, troponin, was the target for calcium opened up the investigation of the calcium control of the MgATPase. Troponin was shown to be a complex of troponin C, I and T, each protein being under individual genetic control and existing in isoforms specific for the muscle type. The unique forms of troponin I and T in cardiac muscle make them the biomarkers of choice for cardiac injury.

Keywords: MgATPase; Actomyosin; Myofibril; Sarcoplasmic reticulum; Calcium; Troponin; Troponin C; Troponin I; Troponin T; Contraction; Relaxation; Biomarkers

One of the outstanding events that revolutionized the study of muscle was the discovery of actin and the actomyosin system by Szent-Gyorgyi and collaborators [1,2]. This discovery provided for the first time a protein model system that could be used to investigate the structural changes that occurred in muscle. At low ionic strength actomyosin is insoluble and in the presence of ATP undergoes a structural change, superprecipitation, comparable to the contractile process itself. Although the purified actomyosin complex did not relax it provided a model protein system in which both the contractile and relaxation processes could be studied.

By the early 1950s the evidence was strong that contraction in model systems such as actomyosin precipitates and fibres, myofibrils and glycerated fibres was accompanied by the hydrolysis of ATP. The substrate was MgATP which, unlike CaATP, is not an effective substrate for the ATPase of purified myosin. The remarkable effect of the interaction of actin with myosin is to enable the myofibril, composed largely of interacting actin and myosin filaments, to hydrolyse the MgATP in the soluble sarcoplasm with which it is bathed. A number of systems, some physiological and some not, that inhibited the actomyosin ATPase were invoked as relaxing factors (see Ref. [3] for review of this early work). It was becoming clear that ATP was essential both as a substrate for contraction and for relaxation. In the relaxed state of muscle in which the MgATPase is inhibited, the actomyosin complex is dissociated by the ATP present and the actin and myosin filaments do not interact. Thus modulation of the interaction of actin with myosin provides a mechanism for regulating the contractile and relaxing activity of muscle. Nevertheless at this time, although work with model systems in vitro was indicating the role of ATP, there was no detailed knowledge of the enzymic changes occurring on contraction in intact muscle. Some workers sought clear evidence that ATP hydrolysis was involved in contraction in situ [4]. Direct determinations on intact muscle by Davies and collaborators [5] confirmed that ATP hydrolysis was involved and justified the assumption that investigation of the mechanism for the regulation of actomyosin MgATPase activity would reveal the mechanism of relaxation.

Real progress began to be made in the study of the relaxing process with the report that a component in the supernatant of muscle homogenates, distinct from the myofibrillar fraction, was required to produce relaxation.

doi:10.1016/j.bbrc.2007.11.185

Bailey earlier had been struck by the observation that it was impossible to squeeze out sarcoplasm from freshly minced muscle but if it was allowed to stand at room temperature for half an hour or so this was readily achieved. The effect could be obtained when glycolysis was inhibited indicating that it was not the consequence of a pH change due to lactic acid production. Bailey set his research student Marsh to investigate the phenomenon [6–8]. Marsh was able to show that myofibrils in whole muscle homogenates shortened on standing but could be made to lengthen and increase their volume again by the addition of ATP. As this change could not be induced by ATP if the myofibrillar fraction was dispersed in fresh buffer he concluded that there was some relaxing factor that required ATP for activity was present in sarcoplasm; the Marsh factor. This factor also acted on model fibre preparations [9]. Just prior to this a lipoprotein microsome like fraction that possessed the Kielley-Myerhof MgATPase had been isolated from myofibril free homogenates of striated muscle [10]. It did not occur to me at the time, however, to test this fraction for relaxing faction activity. We now know that this was sarcoplasmic reticulum and indeed later it was used it as a relaxing factor preparation [11].

I first met Setsuro Ebashi under unusual circumstances. It so happened that in the late summer of 1953 I spent 6 weeks in Japan as manager of the Cambridge University rugby team that had been invited to tour the country. Probably, one of the first muscle scientists from the West to travel to Japan to since the war; I visited a number of departments interested in muscle research. It was apparent that Japanese scientists had suffered from deprivations during the war and even in 1953 they were still short of resources. Despite this their enthusiasm for muscle research was obvious. I was particularly impressed by the skinned muscle fibre preparation of Reiji Natori, then unknown in the West.

Ebashi was much interested in Marsh's findings and asked me to give a talk on the myofibrillar work. Like all of us he was fascinated by the fact that model systems such as isolated myofibrils shortened in the presence of ATP and did not lengthen after its removal. At that time he was working in the laboratory of Hiroshi Kumagi in the department of pharmacology and obviously as a young man of some promise he was held in high regard. Most investigators studying muscle relaxing factors were content to determine the effects on the MgATPase activity of their preparations with the conviction that contraction and relaxation involved stimulation and inhibition of the actomyosin enzymic activity. Ebashi, no doubt conditioned by his pharmacological background, consistently tested his preparations on the superprecipitation behaviour of actomyosin suspensions. He quite correctly considered that that this phenomenon was a more realistic model for the study of the contractile and relaxation processes.

Soon after Marsh's report Ebashi and collaborators [12], reported relaxing factor activity in two ammonium sulphate fractions of muscle extracts. One of these contained the Kielly-Meyerhof ATPase and was lipid rich, clearly identical with the microsome fraction that could be isolated by ultracentrifugation from muscle sarcoplasm from which myofibrils had been removed [10]. It was now becoming clear that both myofibrillar and sarcoplasmic components had special roles in the regulatory process. Bozler [13] also indicated the importance of a fraction other than that derived from the myofibrils by his report that ATP induced contraction and relaxation in fresh glycerated fibres but only contraction on washed fibres. He also demonstrated [13] that a divalent cation was involved for EDTA induced relaxation in glycerated fibres. At the time the evidence implied that the low rate of ATPase activity associated with relaxation might be caused by the binding or removal of magnesium from the system. Earlier evidence from direct intracellular injection of cations, however, had indicated that calcium was particularly effective in inducing contraction in the intact muscle cell [14,15].

Clear evidence that magnesium was not the cation involved was the demonstration that the myofibrillar MgATPase was strongly inhibited by EDTA concentrations less than one tenth of that of the magnesium present [16]. As EDTA has a slightly greater affinity for calcium than for magnesium [17] this observation implied that the former cation might be essential for activation of the MgATPase. At this stage we could not be certain that this was the case but a report by Raaflaub [18] drew our attention to another chelator that had been synthesized by Schwarzenbach, called 'glycolkomplexon' that was specific for calcium. Using 'glycolkomplexon' Raaflaub showed that removal of calcium inhibited the swelling of liver mitochondria. 'Glycolkomplexon', now known as EGTA, was not commercially available at that time but Schwarzenbach kindly sent me a sample. Low concentrations of this chelator strongly inhibited the myofibrillar MgATPase indicating that traces of calcium present in our preparations were essential for the myofibrillar MgATPase [19]. Careful work by Weber [20] indicated that increasing the calcium concentration from 10^{-6} to 10^{-5} M activated the myofibrillar MgATPase. We were struck and indeed puzzled by the fact the MgATPase of actomyosin prepared from purified actin and myosin did not require calcium [19]. Limited attempts to restore the calcium sensitivity to the synthetic actomyosin were unsuccessful and we concluded that on extraction some physical change that led to the loss of calcium sensitivity had occurred. It was left to Ebashi [21] to brilliantly demonstrate that there was another protein system, troponin, present in the myofibrils and crude preparations of actomyosin made from them, was essential for the calcium sensitivity.

In the 1950s there were a number of reports of protein fractions isolated from myofibrils that contained components that could not be identified as currently known myofibrillar components. Analysis of protein mixtures was

difficult at this time for protein gel electrophoresis had not been developed. Boundary electrophoresis by the Tiselius technique or ultracentrifugation were the methods then available. Both required relatively large amounts of protein and the resolution was not good (see [22]). Prolonged extraction of isolated myofibrils with a low ionic strength buffer at pH 7.0 yielded a viscous extract consisting mainly of two components that could distinguished by boundary electrophoresis. The minor component of this extract was tropomyosin whereas the other component, the so-called C-protein, which could not be identified with any then known myofibrillar component, had the properties of a pseudoglobulin [22–24]. We now know that this unidentified fraction clearly contained troponin as a major component for after Ebashi's discovery of troponin the low ionic extract of myofibrils was used as source of the regulatory complex e.g. [25,26].

The viscous complex extracted from myofibrils at low ionic strength contained tropomyosin and had properties very similar to Ebashi's 'native tropomyosin'. When the tropomyosin content of this fraction was estimated by the fall in viscosity on increasing the ionic strength [23] it gave a much higher value for the myofibrils than was accepted at the time. With hindsight one can conclude that the fall in viscosity was due to depolymerisation of the tropomyosin and dissociation of its complex with the troponin present in the extract. The 'extra protein' fraction obtained from high salt extracts of myofibrils [27] very probably also contained troponin.

Once the requirement of the myofibrillar MgATPase for a trace of calcium was established attention was directed to the role of the microsomal fraction, now identified as the sarcoplasmic reticulum. Work by Ebashi and others, for this now became a very active field, established that the sarcoplasmic reticulum was the site of a very powerful calcium pump that could reduce the calcium concentration in the sarcoplasm of resting muscle to below that required to activate the myofibrillar MgATPase [28–31]. ATP hydrolysis drives the pump leading to a store of calcium within the sarcoplasmic reticulum. On stimulation of the muscle the sarcoplasmic membrane becomes permeable to calcium resulting in an increase in concentration in the sarcoplasm thus activating the myofibrillar MgATPase and contraction, follows.

Thus although in the early 1960s the role of calcium and the sarcoplasmic reticulum in the relaxation process was clear, the mechanism by which low concentrations of calcium could modulate the myofibrillar MgATPase was not. Ebashi's report [21] that the fraction called 'native tropomyosin' isolated from muscle extracts was able to make the superprecipitation behaviour of desensitized actomyosin sensitive to EGTA was a massive step forward. 'Native tropomyosin' was subsequently shown to consist of tropomyosin associated with a new component that complexed with tropomyosin to increase the viscosity. This protein provisionally called the aggregation promoting factor was finally given the name troponin and shown to be the target protein for the calcium that activated the myofibrillar MgATPase [32–34].

Before it became clear that troponin was responsible for the sensitivity of the MgATPase of crude preparations of actomyosin to calcium the component responsible was provisionally referred to as the EGTA sensitising factor (ESF). In studies on the relation of tropomyosin to ESF it was observed that some preparations lost their factor activity and inhibited MgATPase activity in the absence of EGTA [35]. When purified this preparation was show to be specific for the actomyosin MgATPase. The inhibitory activity was unaffected by calcium and the preparation had no effect on the calcium activated ATPase of myosin [36,37]. Later to be called troponin I it was provisionally named the inhibitory protein and presumed to be derived from troponin or was a modified form of it. An important conclusion was that the inhibitory activity and the calcium requirement were independent properties of troponin and conceivably could be separated [38]. The implication that troponin was a complex of at least two components was shown to be correct by separation of the inhibitory and calcium binding activities on different proteins [39,40]. Intensive investigation of the complex nature of troponin by several workers lead to the isolation of a number of factors that were given different names by the groups involved. An additional protein, troponin T, of higher molecular weight than the inhibitory (TnI) and calcium binding (TnC) proteins was identified as the third component of the complex [26,41,42]. Possessing similar physical properties to TnI it forms a complex with tropomyosin, implying that that the latter protein has a functional role in the relaxation process. The field was much clarified when the nomenclature of TnC, TnI and TnT, suggested by Greaser and Gergely, was adopted at the Cold Spring Harbour Symposium on muscle in 1972.

Despite the detailed knowledge of the troponin components and their relationships that has been built up over the past 40 years it is not yet possible to describe in precise molecular terms how the MgATPase and hence contractile activity of the myofibril is controlled. It is widely accepted that the crucial event that controls the MgATPase of the myofibril and hence its functional state is the influence of the actin molecule on the enzymic centre of the myosin. Since the 1950s it has been presumed that tropomyosin was involved, possibly by blocking the interaction of actin with myosin, the so-called steric hypothesis. This was postulated on the basis of X-ray analysis that demonstrated the movement of the tropomyosin molecule on the thin filament of the myofibril during contraction. It was supported by some enzymic evidence that is not entirely convincing. Nevertheless with slight modifications this hypothesis still appears to be widely accepted.

It is somewhat surprising that in this theory tropomyosin is given the role of blocking the actomyosin interaction and hence inhibiting the ATPase rather than TnI, the inhibitory component of troponin that binds to actin and is specific for the actomyosin MgATPase of striated mus-

cle. Tropomyosin is also present in smooth muscle in which troponin is absent. It is not unique to contractile tissue for it is a widespread structural component in actin filaments in the cell. In striated muscle, however, it appears to have a functional relationship with TnI for it has the property of stimulating the inhibitory activity of this protein. In actomyosin systems free of tropomyosin maximal inhibitory activity is obtained with approaching one TnI molecule per actin monomer. In the presence of tropomyosin the maximum effect is obtained with one TnI molecule in the presence of up to seven actin monomers [43]. These in vitro results suggest that tropomyosin has the property of extending the inhibitory activity of TnI over a number of actin monomers and thereby compensating for the fact that in the filament there is only one troponin molecule for every seven actin monomers.

Another property of TnI that implies it has an important role in regulation is its ability to form a strong complex with TnC that is stable in 5 M urea [44]. This complex is calcium dependent and when the sarcoplasmic calcium concentration rises the TnI and TnC become strongly bound. In this condition the TnI is unable to inhibit the MgATPase of actomyosin and the myofibril contracts. When stimulation ceases the TnI TnC complex is much weakened and the former protein is able to exert its inhibitory activity on the actomyosin MgATPase, resulting in relaxation. A number of investigations indicate that in the presence of calcium structural changes occur in both proteins but much less is known of how the TnT component that interacts with tropomyosin, responds to the binding of calcium to the troponin complex. When the myofibril contracts tropomyosin moves its position in the actin duplex filament groove. The question still to be answered is, as was originally proposed, is this the intramolecular event that is responsible for controlling the interaction of actin and myosin filaments or is control exerted by a direct effect of TnI.

The steric hypothesis presents an elegant explanation of how the molecular changes initiated by the binding of calcium to troponin located close to every seventh actin monomer could influence the behaviour of the other six actin monomers in the repeat unit of the actin filament. In some way, possibly through TnT, the structural changes induced by the binding of calcium to TnC could cause the tropomyosin molecule, which extends along approximately seven actin monomers, to move its position in the groove. The long fibrous tropomyosin molecule is, however, rather flexible and it is difficult to envisage how the effect induced at one region of the molecule could lead to a lateral displacement of the whole molecule, 380 Å in length.

An alternative explanation postulates that the structural changes imposed on actin when TnI is bound are transmitted to the three actin monomers on either side of the actin monomer that binds the TnI in the troponin complex [45,46]. In the helical duplex of the F-actin filament there are a number of sites of interaction between monomers through which conformational changes in one actin monomer could be transmitted to adjacent interacting monomers. The role of the tropomyosin in this hypothesis is to stabilize the actin filament and thus facilitate the transmission of the conformational changes along the F-actin filament. The movement of tropomyosin on contraction can be considered to arise from the conformational change in the actin monomers that occurs when the interaction of actin with TnI is modified as a consequence of the binding calcium to TnC. The tropomyosin must adjust its position on the actin monomers to compensate for the conformational changes that have occurred.

If tropomyosin has a steric blocking role it would be expected to block the site or sites on myosin involved in the interaction with actin that enable it to hydrolyse MgATP. This condition would apply in resting muscle and be relieved on contraction. If this is the mode of action of tropomyosin it would be expected to inhibit the binding of actin to at least one of the sites on myosin considered to represent the regions to which actin binds. NMR studies with peptides of cardiac myosin considered to represent the binding sites for actin provide no evidence of inhibition by tropomyosin of actin binding to these sites [46].

A peptide region of TnI (residues 96–116 in rabbit fast muscle, [47]) possesses inhibitory activity that is stimulated by tropomyosin as is the case with the intact molecule. This peptide presumably binds to the major site of interaction of actin with myosin that is involved in regulating the enzymic properties. The binding of TnI inhibitory peptide to actin is slightly enhanced by tropomyosin. On balance the evidence that tropomyosin acts by directly blocking the action of actin on the myosin molecule is not yet convincing. The evidence available would suggest that its role is to stabilize the F-actin filament and facilitate the transmission to other actin monomers in the filament, the conformational change imposed by the TnI on every seventh actin monomer during the contraction relaxation cycle.

The discovery of troponin by Ebashi has stimulated the study of one of the most important complex protein interaction systems required for the function of higher animals. It has illuminated the role of calcium in this process and thrown important light on the role of this cation in other regulatory processes in cell metabolism. Complete understanding of the regulatory process in muscle will lead to a precise atomic description of the interaction of actin and myosin that is responsible for contraction itself, information which we do not yet have.

Quite apart from the light the discovery of troponin has thrown on the functional cycle in muscle and the stimulus it has given to many workers in the field it has provided a valuable clinical tool. The components of the complex exist in isoforms that are characteristic of the striated muscle type and which are under independent genetic control. Specific antibodies to the isoforms are readily prepared and can be used for typing muscle cells and detecting muscle

damage when they appear in the blood. The detection of TnT, and particularly TnI which is present in only one specific form in adult cardiac muscle, are the current methods of choice for the detection of cardiac muscle damage [48,49].

The discovery of troponin, for which we must thank Setsuro Ebashi, has had an enormous impact on muscle research and even over 40 years since its discovery hundreds of papers are appearing each year on the subject. Few scientists can claim that their work has such an influence.

I am grateful to Val Patchell for her help in the preparation of this article.

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Received 29 September 2007
Available online 26 December 2007

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