PHOSPHORYLATION OF THE INHIBITORY SUBUNIT OF TROPONIN AND ITS EFFECT ON THE CALCIUM DEPENDENCE OF CARDIAC MYOFIBRIL ADENOSINE TRIPHOSPHATASE

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1. Introduction

Although cyclic 3′,5′-AMP has been implicated in the positive inotropic response of cardiac muscle to catecholamines [1,2], the sequence of events leading to increased contractility is not understood. Many of the known actions of cyclic 3′,5′-AMP are mediated through protein phosphorylation [3], and it has been suggested [4] that changes in cardiac contractility could also involve this mechanism. Phosphorylation of proteins associated with cardiac sacroplasmic reticulum [5] and sarcolemma [6] by cyclic 3′,5′-AMP-dependent protein kinase has been reported, and also phosphorylation in vitro and in vivo of proteins of the contractile system [7–12]. In particular, phosphorylation of the inhibitory subunit of troponin (troponin-I) in perfused rat heart was correlated with increased force of contraction induced by catecholamines [9,12]. Phosphorylation of troponin-I in vitro was catalysed by cyclic 3′,5′-AMP-dependent protein kinase [8]. A hypothesis has been proposed in which phosphorylation of troponin-I in response to catecholamine stimulation of cardiac muscle could cause an increase in contraction by modifying the properties of troponin [13,14]. Support for this hypothesis was provided by the report of Rubio et al. [11], which showed that phosphorylation of guinea pig cardiac actomyosin by skeletal muscle cyclic 3′,5′-AMP-dependent protein kinase resulted in a decrease in the concentration of calcium required for activation of actomyosin adenosine triphosphatase (ATPase). Most of the phosphate was incorporated into a protein of mol. wt 28 000 which was probably troponin-I.

In this paper we report the finding that with contractile proteins from beef heart, phosphorylation of troponin-I caused an increase in the concentration of calcium required for activation of actomyosin ATPase. This result was obtained whether phosphorylation occurred with whole myofibrils, or with isolated native tropomyosin (tropomyosin + troponin). Additionally, treatment of the proteins with a phosphoprotein phosphatase with activity towards troponin-I caused a decrease in the calcium concentration required for activation. These results disagree with those of Rubio et al. [11], although slightly different preparations from different species were used.

2. Methods

2.1. *Preparation of proteins and enzymes*

Washed myofibrils were prepared from fresh beef heart muscle as described by Solero et al. [15]. This method incorporates two washes of the myofibrils with Triton X-100, which were essential for preparing myofibrils free of membrane contamination and proteolytic activity. The use of the detergent treatment did not result in changes in the calcium sensitivity of the actomyosin ATPase [15]. Desensitised myofibrils (myofibrils in which troponin had been removed) were prepared by repeated washing of myofibrils with 1 mM Tris–Cl, pH 8.5 [16]. Native

tropomyosin was prepared from myofibrils dried with ethanol by extraction with 1 M KCl, 25 mM Tris–Cl, 15 mM 2-mercaptoethanol, 0.1 mM $CaCl_2$, pH 8.0, at 20°C [17]. The extracted protein was dialysed against 5 mM Tris–Cl, pH 7.5, at 4°C and used without further treatment.

Cyclic 3′,5′-AMP-dependent protein kinase was prepared from beef heart essentially as described by Brostrom et al. [18], but omitting the calcium phosphate gel fractionation. The preparation was stimulated approx. 10-fold by cyclic 3′,5′-AMP. A crude phosphoprotein phosphatase preparation with activity towards phosphorylated troponin-I (as native tropomyosin) was prepared from rat heart. The method was as described for phosphatase from skeletal muscle [19] as far as the DEAE-Cellulose chromatography step. The preparation was also active towards phosphorylase *a*. Cyclic 3′,5′-AMP-dependent protein kinase inhibitor was prepared from rat skeletal muscle as described by Walsh et al. [20].

2.2. *Protein phosphorylation and desphosphorylation*

Cardiac myofibrils and native tropomyosin were phosphorylated in the presence of cyclic 3′,5′-AMP dependent protein kinase with the following conditions: 50 mM Tris–Cl, 5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM NaF, 15 mM 2-mercaptoethanol, 50 μM cyclic 3′,5′-AMP, 2 mM [γ-^{32}P]ATP (specific radioactivity approx. 20 μCi/μmol), pH 7.0. Native tropomyosin was added to concentration of 2 mg/ml, and myofibrils to 10 mg/ml. Protein kinase was used at concentrations of 0.1–0.4 mg/ml, and all incubations were at 30°C. [^{32}P]Phosphate incorporation during the reaction was followed by precipitation of the protein onto filter paper in 0.6 M trichloracetic acid [21]. The papers were extensively washed and radioactivity measured by liquid scintillation spectrometry. After phosphorylation, myofibrils were washed twice at 4°C with 60 mM KCl, 10 mM Tris–Cl, 15 mM 2-mercaptoethanol, 20 mM NaF, pH 7.5, before measurement of ATPase activity. Native tropomyosin was dialysed extensively at 4°C against 5 mM Tris–Cl, 20 mM NaF, 15 mM 2-mercaptoethanol, pH 7.5, after phosphorylation. Protein for total phosphate determinations or for analysis by polyacrylamide gel electrophoresis was precipitated in 0.6 M trichloracetic acid, resuspended in 0.1 M NaOH, and reprecipitated with 0.6 M trichloracetic acid. This was repeated until the supernatant after precipitation was free of radioactivity.

Dephosphorylation of cardiac myofibrils and native tropomyosin was carried out by incubation with rat heart phosphoprotein phosphatase. The incubations were carried out at 30°C in 50 mM Tris–Cl, 1 mM EDTA, 1.1 mM $MnCl_2$, 60 mM KCl, 15 mM 2-mercaptoethanol, pH 7.5, with 10 mg/ml myofibrils or 2 mg/ml native tropomyosin. The phosphatase was added to a concentration of 0.1 mg/ml, and activity measured by release of P_i [22]. After dephosphorylation, the myofibrils were washed as described above; the native tropomyosin was used without further treatment.

Total phosphate in the proteins was estimated after digestion in 2.5 N NaOH for 20 min at 100°C [23]. The P_i released was measured, after neutralisation, with ammonium molybdate/malachite green [22].

2.3. *Measurement of myofibril adenosine triphosphatase*

The ATPase activity of cardiac myofibrils was measured by the release of P_i from ATP. Myofibrils (native or reconstituted) were incubated at 30°C with 25 mM Tris–Cl, 30 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM ATP, 7.5 mM 2-mercaptoethanol, pH 7.0. Free Ca^{2+} was kept constant with 2 mM EGTA and the appropriate total Ca^{2+} concentrations [24,25]. The concentration of myofibrils (1 mg/ml) was adjusted such that the rate of ATP hydrolysis was linear for at least 10 min. When proteins which had previously been phosphorylated were present in the assay, 20 mM NaF was added to prevent dephosphorylation. At the end of the incubation period the reaction was stopped by the addition of trichloracetic acid, the protein was removed by precipitation, and P_i was measured [26] in the supernatant. Care was taken to ensure that all samples were incubated with the assay solutions for the same length of time to prevent differential non-enzymic ATP hydrolysis.

3. Results and discussion

3.1. *Phosphorylation and dephosphorylation of myofibrillar proteins*

When myofibrils were incubated with [γ-^{32}P]ATP, ^{32}P was incorporated into protein both in the absence

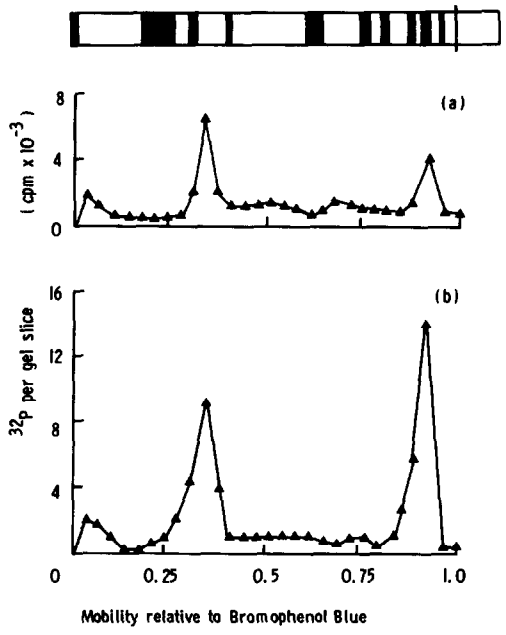

Fig.1. Phosphorylation of cardiac myofibril proteins following incubation with [γ-^{32}P]ATP and cyclic 3',5'-AMP-dependent protein kinase. Untreated (a) or phosphoprotein phosphatase treated (b) myofibrils were incubated with [γ-^{32}P]ATP and protein kinase as described in Materials and methods, precipitated with trichloracetic acid, and subjected to electrophoresis in 5% polyacrylamide in the presence of sodium dodecyl sulphate [27]. The gels were either stained for protein with Coomassie Brilliant Blue, or were sliced into 3 mm sections, digested in H_2O_2 [28], and ^{32}P measured by Cerenkov radiation.

and presence of added cyclic 3',5'-AMP-dependent protein kinase. In the absence of protein kinase there was a slow rate of incorporation which was only increased by 20% on addition of cyclic 3',5'-AMP. Addition of protein kinase and cyclic 3',5'-AMP gave a 8-fold stimulation of ^{32}P incorporation, which reached a plateau value of 0.4 nmol/mg myofibrillar protein in 5 min. Figure 1a shows that ^{32}P was incorporated into two regions of mol. wt 150 000 and 28 000. The region of mol. wt 28 000 was shown to be troponin-I by affinity chromatography of the myofibrils on troponin-C Sepharose in 8 M urea [29]. The band of mol. wt 150 000 was not identified, but a possibility was one of the proteins of the M-line, with mol. wt 155 000 [30].

As the ^{32}P incorporation with protein kinase was small, the total phosphate content of myofibrils was measured by alkaline hydrolysis, and gave a value of 9.4 ± 0.8 nmol/mg protein (mean±S.E.M.; n = 6). Treatment of myofibrils with phosphoprotein phosphatase for 1 h caused a reduction of protein bound phosphate to 7.0±0.6 nmol/mg protein (mean±S.E.M.; n = 4). Subsequent incubation of these myofibrils with protein kinase and [γ-^{32}P]ATP (+ NaF to inhibit phosphatase) gave a maximum ^{32}P incorporation of 1.2 nmol/mg protein. Figure 1b shows that after treatment with phosphatase ^{32}P was incorporated into the same molecular weight regions as before, with a larger incorporation into troponin-I.

Native tropomyosin contained four major proteins, troponin-T (mol. wt 41 000), tropomyosin (34 000), troponin-I (28 000) and troponin-C (19 000) with only minor amounts of contaminating proteins. This complex contained 10.3±0.8 nmol phosphate per mg protein (mean±S.E.M.; n = 4). If it was assumed that troponin-T contained 0.3 mol phosphate per mole [8], and the remaining phosphate was in troponin-I [8], the phosphate content of troponin-I was calculated to be 1.5±0.2 mol/mol (mean±S.E.M.; n = 4). This was similar to that reported for rabbit cardiac troponin-I [8]. Incubation of native tropomyosin with protein kinase and [γ-^{32}P]ATP resulted in the incorporation of ^{32}P specifically into troponin-I, and gave a total phosphate content of troponin-I of 1.8±0.1 mol/mol (mean±S.E.M.; n = 3). When native tropomyosin was incubated with phosphatase for 3 h, the total phosphate was reduced to 3.5±0.6 nmol/mg (mean±S.E.M.; n = 6). If it was assumed that phosphate was removed specifically from troponin-I, its phosphate content was 0.3±0.05 mol/mol (mean±S.E.M.; n = 6). Troponin-I isolated from perfused rat heart [9,12] contained 0.25 mol phosphate per mole in control perfusions and 1.5 mol/mol in perfusions with 0.1 μM isoprenaline. The phosphate content of troponin-I in the treated native tropomyosin were therefore very similar to those found in an intact tissue preparation.

3.2. *Effects of phosphorylation and dephosphorylation on myofibril adenosine triphosphatase*

Figure 2 shows the effect of phosphorylation and dephosphorylation on the Ca^{2+} dependence of cardiac native myofibril ATPase. There was an 8-fold increase in ATPase activity when the Ca^{2+} concentration was

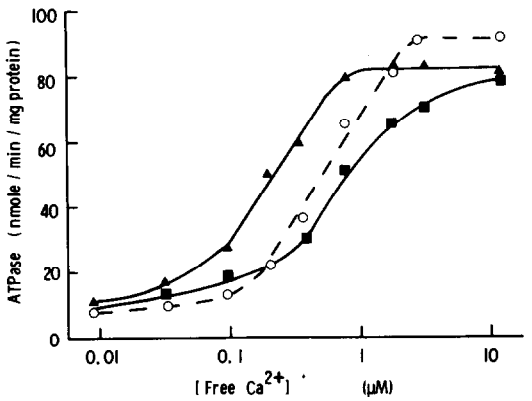

Fig.2. The effect of phosphorylation and dephosphorylation on the Ca^{2+} sensitivity of cardiac myofibril ATPase. Myofibrils were phosphorylated with protein kinase (■), dephosphorylated with phosphoprotein phosphatase (▲) or untreated (○- -○) as described in the text. Myofibril ATPase was measured in the presence of Ca/EGTA buffers at various free Ca^{2+} concentrations as described in Materials and methods.

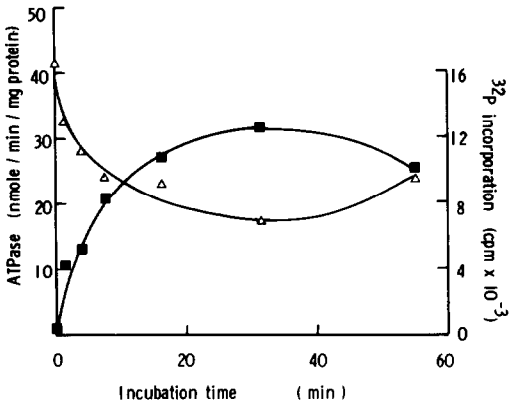

Fig.3. Time course of phosphorylation of cardiac myofibrils and loss of ATPase activity measured at 0.5 μM free Ca^{2+}. Myofibrils were incubated for the times shown with [γ-^{32}P] ATP and protein kinase as described in Materials and methods. The myofibrils were rapidly washed at 4°C in buffer containing 20 mM NaF, and ATPase (△) assayed with a Ca/EGTA buffer at 0.5 μM free Ca^{2+} as described in Materials and methods. ^{32}P incorporation (■) was measured by precipitation of the myofibrils on filter paper in 5% trichloracetic acid [21].

raised from 0.1 μM to 10 μM, and this was unaltered by changes in myofibril phosphate content. However, it was found that phosphorylation caused a slight increase in the concentration of Ca^{2+} required to activate the ATPase, and this was most noticeable at higher Ca^{2+} concentrations. Dephosphorylation of the myofibrils caused a large decrease in the concentration of Ca^{2+} required for activation, and this was observed at Ca^{2+} concentrations above 0.1 μM. The curves of fig.2 for the Ca^{2+} stimulated ATPase were fitted by non-linear least squares regression to the equation

$$v = V_{max}/(1 + K/[Ca^{2+}]^n) \quad (1)$$

where n is the Hill coefficient [31]. Note that K is not the concentration of Ca^{2+} where $v = 0.5\ V_{max}$. The value for n was not significantly different for each of the three curves, and was 1.76±0.11 (mean± S.E.). For the untreated myofibrils the K value was 0.38±0.05 μM (mean ± S.E.), for the protein kinase treated myofibrils 0.46±0.04 μM, and for the phosphatase treated, 0.058±0.015 μM. There was no significant difference in the K value between the untreated and protein kinase treated myofibrils (Student's t test, $P > 0.2$), but the difference between the untreated and phosphatase treated was highly significant ($P < 0.001$). As this effect of phosphorylation was the opposite of that found by Rubio et al. [11], various control experiments were performed. When untreated myofibrils were incubated with protein kinase as described in Materials and methods, but omitting [γ-^{32}P]ATP, or in the presence of protein kinase inhibitor protein, no phosphorylation of the myofibrils occurred and no changes in Ca^{2+}-dependent ATPase were seen. These experiments suggest that the effect seen on incubation with protein kinase was not caused by a different action to phosphorylation, such as proteolysis. In addition, when phosphatase treated myofibrils were subsequently rephosphorylated with protein kinase, the Ca^{2+} sensitivity of the ATPase was very similar to that of the untreated myofibrils (K value 0.41±0.09), showing the reversible nature of the changes. Figure 3 shows the parallel changes in phosphorylation and decrease in ATPase activity at 0.5 μM Ca^{2+} during incubation of myofibrils with protein kinase. This Ca^{2+} concentration was chosen from fig.2 as being the one which gave the largest difference in ATPase activity between phosphorylated and dephosphorylated myofibrils.

As two proteins, a 150 000 mol. wt component

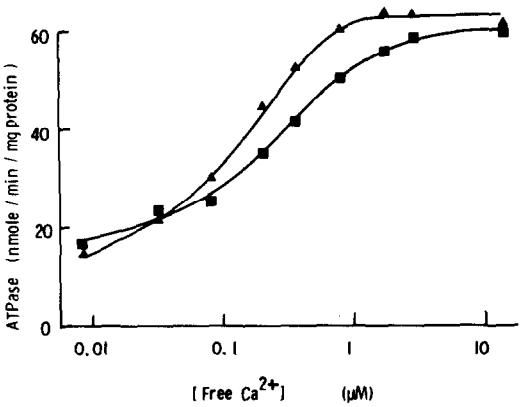

Fig.4. The effect of phosphorylation and dephosphorylation of cardiac native tropomyosin on the ATPase activity of reconstituted myofibrils. Native tropomyosin was phosphorylated with protein kinase (■) or dephosphorylated with phosphoprotein phosphatase (▲) as described in the text, and recombined with desensitized myofibrils. ATPase activity was assayed as described in Materials and methods in the presence of Ca/EGTA buffers.

and troponin-I, were phosphorylated in whole myofibrils, it was possible that some of the effects seen above were not caused exclusively by changes in troponin-I phosphorylation. Experiments were therefore performed with reconstituted myofibrils in which the phosphorylation of troponin-I only was altered. Native tropomyosin, which had been phosphorylated with protein kinase or dephosphorylated with phosphoprotein phosphatase, was added to desensitised myofibrils and the Ca^{2+} dependence of the ATPase measured (fig.4). There was an approx. three fold increase in ATPase activity when the Ca^{2+} concentration was raised from 0.1 μM to 10 μM, which was less than that seen with native myofibrils. This was probably owing to a slight loss of myofibrils integrity caused by the desensitization and reconstitution procedures. This was also reflected in a decrease in the n value (eq.1) (data of fig.4) to 1.4 ± 0.1 (mean±S.E.). As with the native myofibrils, phosphorylation of native tropomyosin (specifically troponin-I) caused an increase in the concentration of Ca^{2+} required to activate myofibril ATPase. The myofibrils reconstituted with phosphorylated native tropomyosin had a K value (eq.1) of 0.58±0.12 μM (mean±S.E.), while the value with dephosphorylated native tropomyosin was 0.22±0.05 μM (difference sig. at $p < 0.05$; Student's t test). These results show that phosphorylation of troponin-I alone was sufficient to increase the Ca^{2+} concentration required to activate myofibril ATPase.

3.3. *General discussion*

The results presented above differ from those of Rubio et al. [11], who found that phosphorylation of guinea pig native actomyosin caused a decrease in the Ca^{2+} concentration required for ATPase activation. This could have been caused by the use of cardiac muscle from different species, although it is unlikely that the same protein modification would cause opposite effects in guinea pig and beef heart. Also, experiments with rat heart myofibrils (not reported) showed similar effects to those from beef heart. Rubio et al. [11] used native actomyosin, which is a slightly different preparation to the washed myofibrils of the present study. It is possible that native actomyosin has a different protein composition to myofibrils which could account for the difference in the effects of phosphorylation, although the phosphorylation patterns ([11] and fig.1 above) were very similar. The use of skeletal muscle protein kinase by Rubio et al. [11] is also unlikely to be the reason for the difference, as the catalytic subunits of protein kinases from various tissues appear to be very similar in their substrate specificities [3]. As a possible explanation we wish to suggest that although Rubio et al. [11] observed an increase in ^{32}P incorporation on incubation with [γ-^{32}P]ATP and protein kinase, there was actually a loss of total protein-bound phosphate during this incubation. Myofibril preparations from beef and rat heart, and preparations of native tropomyosin, were contaminated with phosphoprotein phosphatase activity which cause dephosphorylation of troponin-I (and the 150 000 mol. wt component) unless inhibited by F^-. In all the incubations with protein kinase described above, NaF was present, and an increase in total phosphate was shown to have occurred. Rubio et al. [11] did not have F^- in their incubation, and it is therefore possible that dephosphorylation was occurring at the same time as ^{32}P was being incorporated.

These results throw into question the role of troponin-I phosphorylation in vivo as originally proposed [13,14]. It was suggested that phosphorylation could cause an increase in the interaction

between myosin and actin, either by increasing the maximum amount of interaction at saturating Ca^{2+} concentrations (equivalent to an increase in V_{max} of myofibril ATPase), or by decreasing the amount of Ca^{2+} required for activation. No evidence was provided in these studies for the former, and the change in calcium sensitivity that occurred was in the opposite direction. Troponin-I phosphorylation in perfused rat heart occurred at the same time as increases in contraction after stimulation by catecholamines [9,12]. If the decrease in calcium sensitivity of myofibril ATPase caused by phosphorylation of troponin-I in vitro reflects similar changes in vivo, the effect of catecholamines in the perfused heart must be at least two fold: to cause phosphorylation of troponin-I, and to increase the intercellular Ca^{2+} concentration during systole. Various mechanisms have been proposed for increasing cardiac cytoplasmic Ca^{2+} concentrations following catecholamine stimulation (reviewed in [5,6]) and it is probable that these or similar mechanisms are primarily responsible for the increase in contractility observed with positive inotropic agents.

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