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The Phosphorylation of Troponin I from Cardiac Muscle

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1. Troponin I isolated from fresh cardiac muscle by affinity chromatography contains about 1.9mol of covalently bound phosphate/mol. Similar preparations of white-skeletal-muscle troponin I contain about 0.5mol of phosphate/mol. 2. A 3′:5′-cyclic AMP-dependent protein kinase and a protein phosphatase are associated with troponin isolated from cardiac muscle. 3. Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase catalyses the phosphorylation of cardiac troponin I 30 times faster than white-skeletal-muscle troponin I. 4. Troponin I is the only component of cardiac troponin phosphorylated at a significant rate by the endogenous or a bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase. 5. Phosphorylase kinase catalyses the phosphorylation of cardiac troponin I at similar or slightly faster rates than white-skeletal-muscle troponin I. 6. Troponin C inhibits the phosphorylation of cardiac and skeletal troponin I catalysed by phosphorylase kinase and the phosphorylation of white skeletal troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase; the phosphorylation of cardiac troponin I catalysed by the latter enzyme is not inhibited.

It is now well established that three proteins of the myofibril, namely myosin, troponin I and troponin T, undergo phosphorylation and dephosphorylation reactions (see Perry *et al.*, 1975, for review), although as yet no clear experimental evidence has been presented for the biological role of these processes. Most of the phosphorylation studies on troponin have been carried out on the complex isolated from fast skeletal muscle (Bailey & Villar-Palasi, 1971; Stull *et al.*, 1972; Pratje & Heilmeyer, 1972; Perry & Cole, 1973, 1974*a*,*b*). Our earlier investigations indicated that phosphorylase kinase would catalyse the phosphorylation of troponin T and troponin I when these proteins were incubated separately with the enzyme. With the troponin complex, however, practically all of the phosphorylation occurred on troponin T, for in the complex the main phosphorylation site on troponin I, threonine-11 (Moir *et al.*, 1974; Huang *et al.*, 1974), is masked by troponin C. On the other hand, phosphorylation catalysed by a 3′:5′-cyclic AMP-dependent protein kinase from bovine cardiac muscle was restricted to troponin I when either the troponin complex or the isolated components were incubated with the enzyme (Perry & Cole, 1974*b*). The rates of phosphorylation of the troponin components isolated from fast skeletal muscle were relatively slow with purified preparations of phosphorylase kinase and 3′:5′-cyclic AMP-dependent protein kinase. If these are the only kinases available *in vivo* to phosphorylate troponin in fast skeletal muscle, some question must arise as to their ability to modify or regulate the rapid sequence of events occurring during contraction in this type of muscle.

Following the report of Reddy *et al.* (1973) that cardiac troponin was phosphorylated by an endogenous 3′:5′-cyclic AMP-dependent protein kinase, we have shown that the cardiac troponin I component of molecular weight 28000 is the main site of phosphorylation by the endogenous protein kinase (Perry & Cole, 1974*b*). On the other hand, Reddy & Schwartz (1974) report that a protein of 23000 daltons is the main component to be phosphorylated. In our preliminary experiments the phosphorylation of troponin I by a 3′:5′-cyclic AMP-dependent protein kinase present in the heart was 5–10 times more rapid with the cardiac form than with the skeletal form (Perry & Cole, 1974*b*), suggesting that phosphorylation of this protein might be significant in regulating cardiac contractile activity. Such a process under the control of 3′:5′-cyclic AMP could be one of the molecular mechanisms responsible for the effects of the catecholamines on the contractile activity of the heart (see Robison *et al.*, 1971, for review).

The present paper is a study of the phosphorylation of troponin I from cardiac troponin with emphasis on those features that differ from skeletal muscle and which may be of significance for the function of cardiac muscle. Some preliminary aspects of the work have been briefly reported elsewhere (Perry *et al.*, 1975).

Methods and Materials

Troponin and components

Troponin was prepared from skeletal and cardiac muscles by the methods of Ebashi *et al.* (1971) and

Tsukui & Ebashi (1973) respectively. Both preparations were fractionated into troponin I, troponin C and troponin T as described elsewhere (Perry & Cole, 1974*b*).

Troponin I was also isolated directly from whole fresh skeletal and cardiac muscle by the affinity-chromatographic method of Syska *et al.* (1974) with rabbit white-skeletal-muscle troponin C linked to Sepharose 4B. Hearts that had been removed immediately after death were used fresh or after storage at −20°C. They were homogenized in 10vol. of 9M-urea, 75mM-Tris–HCl, pH8.0, 1mM-$CaCl_2$, 15mM-2-mercaptoethanol (final pH adjusted to 8.0 at room temperature with 1M-HCl), equilibrated against the buffer and applied to an affinity column (1g wet wt. of troponin C–Sepharose per g of muscle). In some cases, particularly with ox heart or small-scale rabbit heart preparations, obvious vascular tissue was dissected out to avoid contamination with troponin I-like components, of different electrophoretic mobility to cardiac troponin I, which can be isolated from vertebrate smooth muscle (H. Syska & S. V. Perry, unpublished work). With larger affinity columns containing 100g of Sepharose–troponin C up to 40mg of troponin I could be isolated from 120g of cardiac tissue. This procedure was also used for the isolation of homogeneous troponin I from troponin. Troponin I made by affinity chromatography was at least 95% pure as judged by polyacrylamide-gel electrophoresis of 100μg in 0.1% sodium dodecyl sulphate, 0.1M-sodium phosphate buffer, pH7.0, as described (Weber & Osborn, 1969; Perry & Cole, 1974*b*).

Phosphorylase kinase experiments

Phosphorylase kinase free of 3′:5′-cyclic AMP-dependent protein kinase and myosin light-chain kinase activities was prepared from white skeletal muscle of the rabbit (Cohen, 1973). Preparations (1mg/ml) after Sepharose 4B chromatography were stored at −20°C in 10% (w/v) sucrose solution. Unless otherwise stated in the text, incubations with phosphorylase kinase were carried out under the following conditions. The protein substrate (0.1–1.0mg/ml) was incubated at 30°C with phosphorylase kinase (0.01–0.1mg/ml) in 50mM-Tris–50mM-α-glycerophosphate (diluted fivefold from a stock buffer adjusted to pH8.6 at room temperature with 1M-HCl), 5mM-magnesium acetate, 0.5mM-$CaCl_2$, 25mM-NaF, 1mM-dithiothreitol and ATP (1–2mM), which usually contained [γ-^{32}P]ATP (1–100μCi/ml) in a final volume of 1ml. The reaction was stopped with 0.5vol. of 15% (w/v) trichloroacetic acid, and, if less than 0.5mg of protein was present, bovine serum albumin was then added (0.1ml of a 1%, w/v, solution). The precipitate was washed, dissolved and assayed for radioactivity as described (Perry & Cole, 1974*b*).

Bovine cardiac protein kinase

Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase (0.01–0.1mg/ml) was incubated with substrates, unless otherwise indicated in the text, at 30°C in 50mM-Tris–50mM-α-glycerophosphate (diluted fivefold from a stock buffer adjusted to pH7.2 at room temperature with 1M-HCl), 5mM-magnesium acetate, 0.1mM-3′:5′-cyclic AMP, 25mM-NaF, 1mM-dithiothreitol, ATP (1–2mM), which usually contained [γ-^{32}P]ATP (1–100μCi/ml) and, if troponin C was present, 0.5mM-$CaCl_2$, all in a final volume of 1ml. Incorporation of ^{32}P into the protein was estimated as for phosphorylase kinase.

Determination of phosphate

Phosphate was determined as described (Perry & Cole, 1974*b*) by direct estimation using the micro method of Bartlett (1959). Radiochemically it was determined by the Čerenkov method in a Philips scintillation counter in which d.p.m. were estimated by the channels-ratio method.

Determination of protein

Proteins were usually determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, unless the molar ratio of troponin components was required, when their initial concentrations after dialysis against 20mM-KCl were determined by a micro Nesslerization procedure (Strauch, 1965) assuming the protein contained 16% N.

Materials

Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase, bovine serum albumin and lysine-rich calf thymus histone were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. [γ-^{32}P]ATP was purchased from The Radiochemical Centre Ltd., Amersham, Bucks., U.K., as the freeze-dried NH_4^+ salt. Frozen rabbit hearts were obtained from The Buxted Rabbit Co., Great Totease Farm, Buxted, Sussex, U.K. Protein kinase inhibitor from rabbit skeletal muscle was kindly donated by Dr. P. Cohen. Sepharose 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Results

Characterization of cardiac troponin and isolation of components

Troponin prepared from frozen rabbit hearts by the method of Tsukui & Ebashi (1973) migrated as three major bands corresponding to troponin I, troponin C and troponin T on gel electrophoresis in sodium dodecyl sulphate. The preparations were less satisfactory than those obtained from rabbit white skeletal muscle, for they often contained smaller amounts of other proteins migrating with different

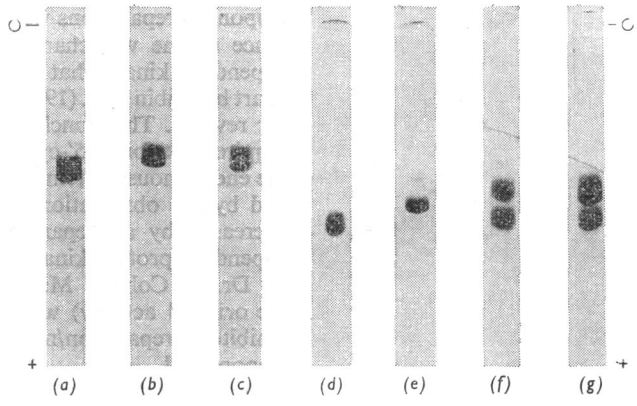

Fig. 1. *Polyacrylamide-gel electrophoresis of components of the troponin complex from cardiac and white skeletal muscle*

Electrophoresis conditions: 10% (w/v) acrylamide, 0.1% (w/v) sodium dodecyl sulphate, 100mM-sodium phosphate buffer, pH7.0 (Weber & Osborn, 1969). O, Origin. All preparations of troponin I were prepared by affinity chromatography. (*a*) 20μg of rabbit skeletal troponin T; (*b*) 10μg of rabbit cardiac troponin T; (*c*) 5μg of rabbit skeletal troponin T+5μg of rabbit cardiac troponin T; (*d*) 10μg of rabbit white skeletal troponin I; (*e*) 10μg of rabbit cardiac troponin I; (*f*) 10μg of rabbit skeletal troponin I+20μg of rabbit cardiac troponin I; (*g*) 10μg of rabbit skeletal troponin I+20μg of bovine cardiac troponin I.

mobilities. The reason for this was not clear and preparations were not improved significantly by increasing the ATP concentration in the initial extraction buffer to 2mM. These contaminants may have been due to partial proteolytic degradation during storage of the rabbit hearts, which were supplied frozen in bulk. Troponin prepared from fresh bovine hearts was more satisfactory but still contained other proteins, mostly of apparent molecular weight greater than 41000 but with a persistent contaminant of about 23000. Despite the presence of contaminants, rabbit cardiac troponin prepared from frozen hearts was satisfactorily fractionated into troponin I, troponin T and troponin C by the methods described (Perry & Cole, 1974*b*). The best preparations of troponin I as judged by homogeneity on electrophoresis were obtained by affinity chromatography directly from whole muscle or from troponin preparations (Fig. 1).

Troponin I and troponin T from cardiac muscle migrated on electrophoresis in sodium dodecyl sulphate with mobilities corresponding to molecular weights of 28000 and 41000, compared with values of 23000 and 37000 for the equivalent skeletal proteins (Fig. 1). On electrophoresis in 6M-urea, pH3.2 (Panyim & Chalkley, 1969) troponin I from skeletal and cardiac muscle migrated as a closely spaced doublet. The appearance of this doublet did not correlate with the extent of phosphorylation of the preparation.

Phosphorylation of cardiac troponin

Most preparations of troponin obtained from rabbit or bovine hearts had appreciable amounts of a 3′:5′-cyclic AMP-dependent protein kinase associated with them, whereas similar endogenous enzymic activity was absent from skeletal-muscle troponin preparations (see Perry *et al.*, 1975). The cardiac troponin preparations had significant protein kinase activity in the absence of 3′:5′-cyclic AMP, but activity was enhanced up to 10-fold by the addition of the nucleotide to the assay medium. On aging, the activity of the endogenous protein kinase decreased, but was significant after storing freeze-dried troponin preparations at −20°C for several months.

On the addition of bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase the rate of phosphorylation of the cardiac troponin often did not increase. With troponin preparations of lower endogenous kinase activity an increase in rate was obtained. The maximum rates of phosphorylation obtained with cardiac troponin in the presence or the absence of added enzyme were usually 5–15 times greater than those obtained with white-skeletal-muscle troponin and added enzyme.

Phosphorylation of cardiac troponin by endogenous or added bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase was restricted to the 28000-mol.-wt. component over the early stages of incubation. On prolonged incubation, significant although low amounts of phosphorylation occurred in the 41000-mol.-wt. band (Fig. 2). A minor component of bovine cardiac troponin of 23000 daltons was not phosphorylated. When about 10mg of troponin that had been phosphorylated with endogenous kinase and [γ-^{32}P]ATP was applied to a 50g column of troponin C–Sepharose in 9M-urea, 75mM-Tris–HCl, pH8.0, 15mM-2-mercapthoethanol, 1mM-$CaCl_2$ (final

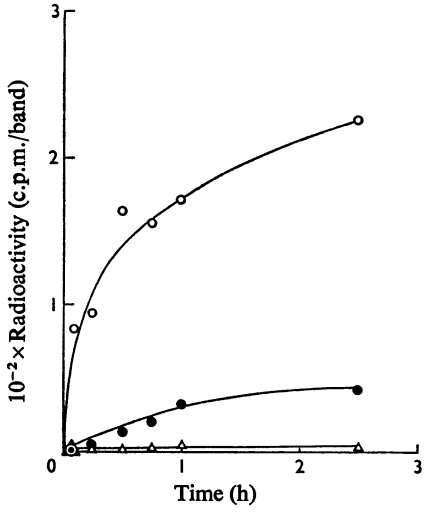

Fig. 2. *Phosphorylation of the components of cardiac troponin by the endogenous protein kinase*

Rabbit cardiac troponin (0.5mg/ml) was incubated without added enzyme under the standard conditions used for assaying 3′:5′-cyclic AMP-dependent protein kinase (see the Methods and Materials section). Total volume was 6ml. Portions (1ml) were removed at the times indicated, pipetted into 0.5ml of 15% (w/v) trichloroacetic acid, the precipitate was dissolved and dialysed at 37°C overnight against 4% (w/v) sodium dodecyl sulphate, 5M-urea, 1% (v/v) 2-mercaptoethanol, 10mM-sodium phosphate buffer, pH7.0. Electrophoresis was then carried out on 10% (w/v) polyacrylamide gels in 0.1% (w/v) sodium dodecyl sulphate, 0.04M-monobasic sodium phosphate, 0.06M-dibasic sodium phosphate buffer, pH7.0 (Weber & Osborn, 1969). The radioactivity of the separated components was determined as described (Perry & Cole, 1974*b*). ○, Troponin I; ●, troponin T; △, troponin C.

pH adjusted to 8.0 at room temperature with 1M-HCl), practically all of the radioactivity was held on the column. On application of the same urea buffer to which 10mM-EGTA [ethanedioxybis(ethylamine)-tetra-acetic acid] had been added, all the radioactivity was eluted associated with a single component migrating on electrophoresis in sodium dodecyl sulphate with a mobility corresponding to 28000 daltons. Thus the 28000-mol.-wt. component complexes with troponin C in the presence of Ca^{2+} and high urea concentrations after phosphorylation by endogenous kinase. This also confirms that the component of cardiac troponin phosphorylated by the endogenous kinase is troponin I, for the latter is the only protein in muscle that binds to a troponin C–Sepharose column under these conditions.

Properties of the endogenous protein kinase of cardiac troponin

The endogenous protein kinase activity of cardiac troponin preparations was probably due to the presence of the well-characterized 3′:5′-cyclic AMP-dependent kinase that has been isolated from the heart by Rubin *et al.* (1972) (see Walsh & Krebs, 1973, for review). This conclusion was supported by the requirement for 3′:5′-cyclic AMP for full activity of the endogenous protein kinase (see previous section) and by the observation that its activity was much decreased by a preparation of 3′:5′-cyclic AMP-dependent protein kinase inhibitor (kindly donated by Dr. P. Cohen). Maximum inhibition (30% of the original activity) was obtained when 0.5mg of inhibitor preparation/ml was used with 0.5mg of troponin/ml.

Further evidence for the identity of the endogenous enzyme with the 3′:5′-cyclic AMP-dependent protein kinase was suggested by the effects of bivalent cations on its activity. Mg^{2+} was the best activator; Co^{2+} was nearly as effective and Mn^{2+} gave some activity. Ca^{2+} was completely ineffective and also inhibited the activity with Mg^{2+}, whereas EGTA had no effect (Fig. 3). The enzyme activity was also decreased to 50% of its original value by increasing concentrations of KCl over the range 0–0.5M.

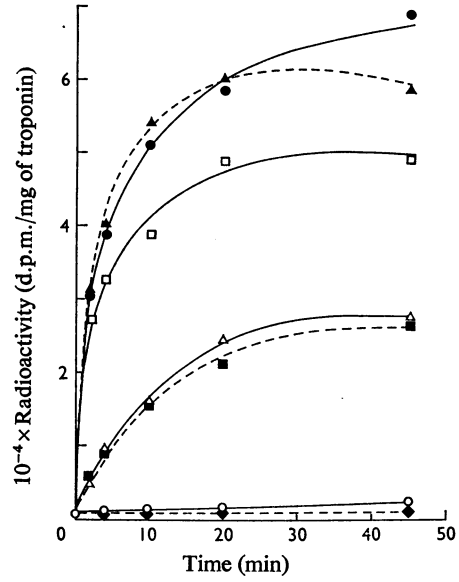

Fig. 3. *Effect of bivalent metal ions on the phosphorylation of cardiac troponin catalysed by endogenous 3′:5′-cyclic AMP-dependent protein kinase*

Bovine cardiac troponin (0.8mg/ml) was incubated under standard conditions (see the Methods and Materials section) with the omission of dithiothreitol and NaF, in the presence of the metal ions indicated (all 5mM) and [γ-^{32}P]ATP (1.25mM; 2.5μCi/ml). ○, No added metal ions; ●, $MgCl_2$; △, $MgCl_2$+$CaCl_2$; ▲, $MgCl_2$+1.25mM-EGTA; ♦, $CaCl_2$; ■, $MnCl_2$; □, $CoCl_2$.

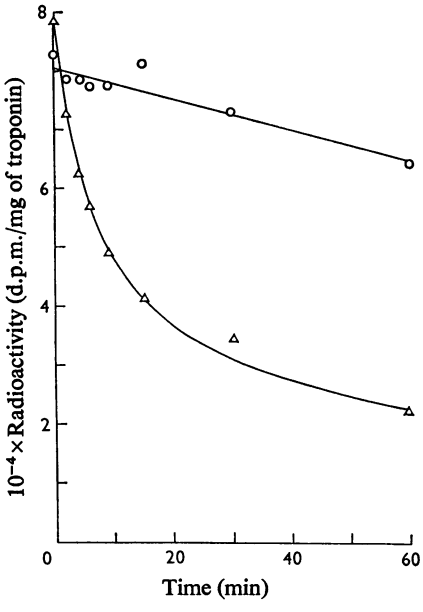

Fig. 4. *Effect of bivalent metal ions on the activity of the endogenous phosphatase of cardiac troponin preparations*

Cardiac troponin (12mg/ml) was incubated with [γ-^{32}P]-ATP (4mM; 100μCi/ml) for 1h at 30°C without added enzyme under standard conditions (see the Methods and Materials section) for 3′:5′-cyclic AMP-dependent protein kinase-catalysed phosphorylation. The reaction mixture (1.25ml) was desalted by application to a column (11.5cm ×2cm) of Sephadex G-25 equilibrated with 20mM-Tris–16mM-HCl, pH7.5, 5mM-dithiothreitol. Fractions containing the main protein peak corresponding to troponin were pooled and stored frozen before use. The phosphorylated troponin (0.2mg/ml) was incubated at 30°C in 50mM-Tris–45mM-acetic acid, pH7.4, 5mM-dithiothreitol, containing either 10mM-magnesium acetate or 1mM-$MnCl_2$. Samples (1ml) were taken at the times indicated and 0.1ml of 1% (w/v) bovine serum albumin was added to each sample after the addition of 5% (w/v) trichloroacetic acid. After centrifugation for 20min at 2000rev./min the precipitates were assayed for radioactivity. ○, Magnesium acetate; △, $MnCl_2$.

With some preparations, the amount of ^{32}P incorporation catalysed by the endogenous enzyme rose to a maximum value and began to fall after prolonged incubation. The presence of a protein phosphatase in the troponin preparations was confirmed by incubation of troponin with [γ-^{32}P]ATP (25μCi/ml; 2.5mM) under the standard conditions for phosphorylation by the endogenous enzyme and then removing excess of ATP by gel filtration on Sephadex G-25. When the ^{32}P-labelled troponin was subsequently incubated at pH7.4 without additional enzyme, a fairly rapid release of ^{32}P was obtained in the presence of Mn^{2+}. Mg^{2+} was less effective as activator of the endogenous phosphatase (Fig. 4), although the requirement for this cation varied with different preparations of troponin.

Phosphate content of troponin preparations

Perry & Cole (1974*b*) reported that whereas troponin from white skeletal muscle usually contained about 1mol of covalently bound P/80000g, more variable amounts, from 0.5 to 2mol of P/85000g, were present in cardiac troponin. Only troponin T contained significant amounts of P after fractionation of skeletal troponin (Perry & Cole, 1974*b*), but both troponin I and troponin T isolated from cardiac troponin contained significant amounts of P (Table 1). When isolated directly from whole fresh muscle by the affinity-chromatographic method (Syska *et al.*, 1974) troponin I from both white skeletal and cardiac muscles was phosphorylated (Table 1). The amount of P in cardiac troponin I isolated in this way was on average about 2mol/28000g. This value was consistently higher than that of troponin I isolated from cardiac troponin by the standard methods (0.5mol/28000g). The P content was also higher than that of troponin I isolated directly from skeletal muscle by affinity chromatography. If the intact heart was kept 2h after death at room temperature before isolation of troponin I the amount of P bound to troponin I was still high. Covalently bound phosphate was slowly lost from troponin I maintained in solution in 10mM-HCl.

Phosphorylation of cardiac troponin I

Initial rates of phosphorylation by 3′:5′-cyclic AMP-dependent protein kinase of cardiac troponin I

Table 1. *Phosphate content of troponin and its components*

Phosphate contents are average values determined as described by Perry & Cole (1974*b*). Troponin I was isolated by affinity chromatography, except for data from Perry & Cole (1974*b*). Values in parentheses indicate number of preparations (underlined) and the range of the values obtained. Apparent molecular weights used were as follows. Skeletal muscle: troponin, 80000; troponin I, 23000; troponin T, 37000. Cardiac muscle: troponin, 87000; troponin I, 28000; troponin T, 40000.

		Phosphate content (mol of P/mol)		
Muscle	Species	Troponin	Troponin I	Troponin T
Cardiac	Rabbit	1.1 (8; 0.7–2.3)	1.9 (5; 1.2–3.1)	0.37 (3; 0.3–0.5)
	Cow	0.88 (4; 0.5–1.7)		
Skeletal	Rabbit	1.0*	0.1*	0.7*
	Rabbit		0.53 (4; 0.4–0.9)	

* Data of Perry & Cole (1974*b*).

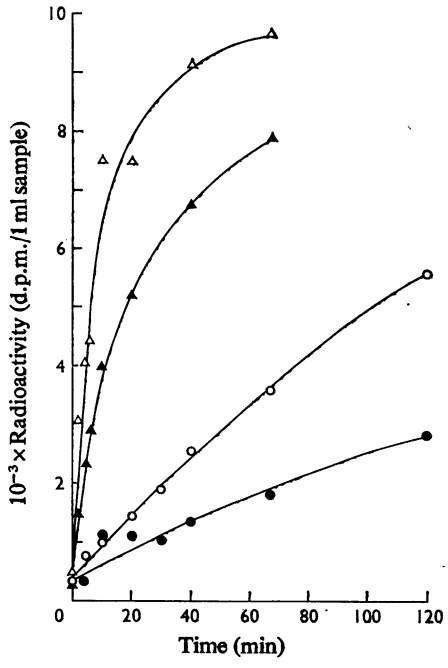

Fig. 5. *Comparison of the rates of phosphorylation of cardiac and skeletal troponin I catalysed by bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase*

Approximately equimolar solutions of rabbit cardiac troponin I (0.135mg/ml) and rabbit skeletal troponin I (0.11mg/ml) of known phosphate content, previously dialysed against 20mM-KCl, were incubated at 30°C with bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase (0.02mg/ml) and [γ-^{32}P]ATP (1mM; 2μCi/ml) under standard conditions (see the Methods and Materials section), with the addition of 4mM-KCl. Samples (1ml) were precipitated with 0.5ml of 15% (w/v) trichloroacetic acid, and 0.1ml of 1% (w/v) bovine serum albumin was added before the precipitates were washed and assayed for radioactivity by the usual procedure. △, Cardiac troponin I (0.3mol of P/mol); ▲, cardiac troponin I (1.0 mol of P/mol); ○, skeletal troponin I (0.0mol of P/mol); ●, skeletal troponin I (0.4mol of P/mol).

of low or zero P content were 30 times greater than with the skeletal protein (Fig. 5). With both forms of troponin I the initial rates were lower the higher the P content of the preparation. The maximum amounts incorporated varied between 0.3 and 1.0mol of P/mol of cardiac troponin I (10 experiments). The lowest amounts of phosphate incorporated on incubation with bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase were obtained with the troponin I preparations of highest phosphate content.

With phosphorylase kinase cardiac troponin I was phosphorylated not more than twice as fast and occasionally more slowly than the troponin I from skeletal muscle (Fig. 6). With phosphorylase kinase significantly more phosphate was transferred to cardiac troponin I than was the case with the protein kinase. Up to 2.5mol/mol of protein was incorporated on prolonged incubation with the enzyme.

If cardiac troponin was phosphorylated with the protein kinase system until phosphate incorporation had almost ceased, rapid phosphorylation again occurred on subsequent addition of phosphorylase kinase, and vice versa. After phosphorylation by both enzymes in this way the total phosphate content was at least 3mol/mol of cardiac troponin I.

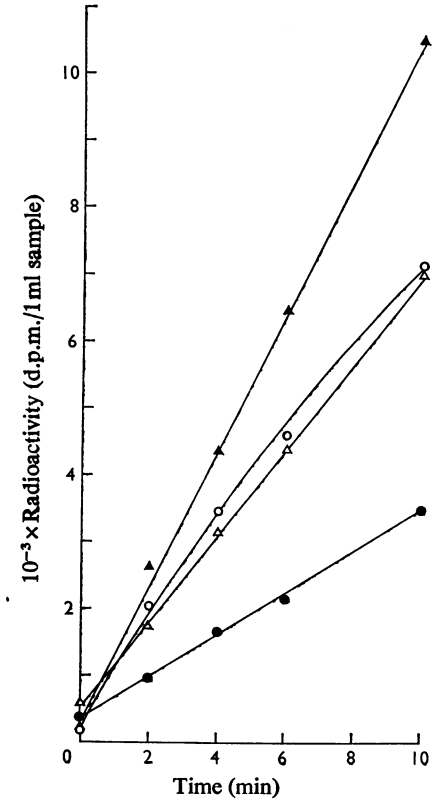

Fig. 6. *Comparison of the rates of phosphorylation of cardiac and skeletal troponin I catalysed by phosphorylase kinase*

Cardiac and skeletal troponin I at the same concentrations as used for the experiment illustrated in Fig. 5 were incubated with phosphorylase kinase (0.02mg/ml) and [γ-^{32}P]-ATP (1mM; 1.5μCi/ml) under standard conditions (see the Methods and Materials section). Otherwise procedures were as for Fig. 5. The radioactivity incorporated in the phosphorylase kinase (less than 10% of total) has been deducted from values shown. △, Cardiac troponin I (0.3mol of P/mol); ▲, cardiac troponin I (1.0mol of P/mol); ○, skeletal troponin I (0.0mol of P/mol); ●, skeletal troponin I (0.4mol of P/mol).

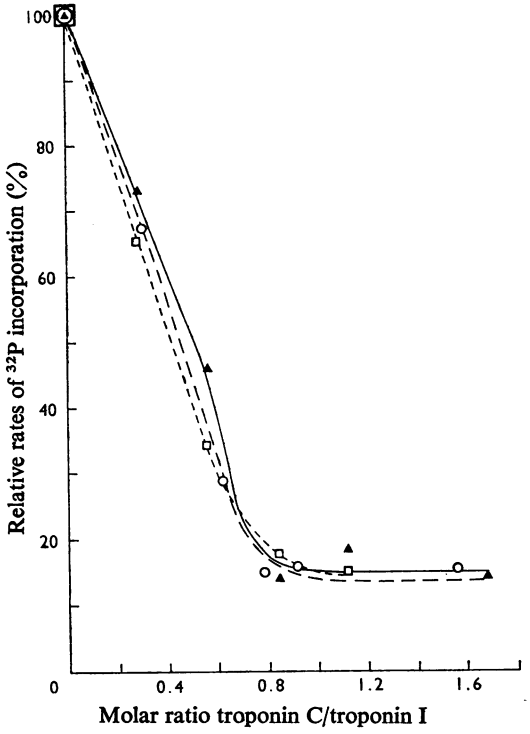

Fig. 7. *Effect of skeletal and cardiac troponin C on phosphorylation of cardiac troponin I catalysed by skeletal phosphorylase kinase*

Rabbit cardiac troponin I (0.1 mg/ml) was incubated with phosphorylase kinase (0.025 mg/ml) and [γ-^{32}P]ATP (1 mM; 1 μCi/ml) and troponin C from rabbit mixed skeletal and rabbit and bovine cardiac muscles at the molar ratios shown, for 15 min under standard conditions (see the Methods and Materials section) with the addition of 20 mM-KCl where necessary to bring the final concentration to 6.5 mM throughout. The incorporation of phosphate into troponin I in the absence of troponin C (about 0.9 mol of P/mol) was taken to be 100%. The blank due to the incorporation of phosphate into phosphorylase kinase (less than 10%) has not been deducted. The molecular weights of cardiac troponin I and all preparations of troponin C were taken to be 28000 and 18000 respectively. ○, Rabbit mixed skeletal troponin C; ▲, rabbit cardiac troponin C; □, bovine cardiac troponin C.

Effects of interaction of troponin C on the phosphorylation of cardiac troponin I

The phosphorylation of rabbit cardiac troponin I by phosphorylase kinase was markedly inhibited by troponin C in a manner similar to that reported for troponin I from fast skeletal muscle (Perry & Cole, 1974*b*). The effect was obtained with troponin I from rabbit cardiac muscle and cardiac troponin C from the same or a different species (Fig. 7). With increasing relative amounts of troponin C, inhibition increased in a linear manner and the effect was complete when troponin I and troponin C were present in equimolar ratio. In similar incubation conditions, phosphorylation of phosphorylase *b* by phosphorylase kinase was not inhibited significantly with molar ratios of troponin C/substrate of 5:1.

The effect of troponin C on phosphorylation by bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase differed markedly with cardiac or fast skeletal muscle troponin I as substrates. With fast skeletal troponin I, troponin C either from cardiac or fast skeletal muscle inhibited phosphorylation with a similar stoichiometry to that observed with phosphorylase kinase. The phosphorylation of cardiac

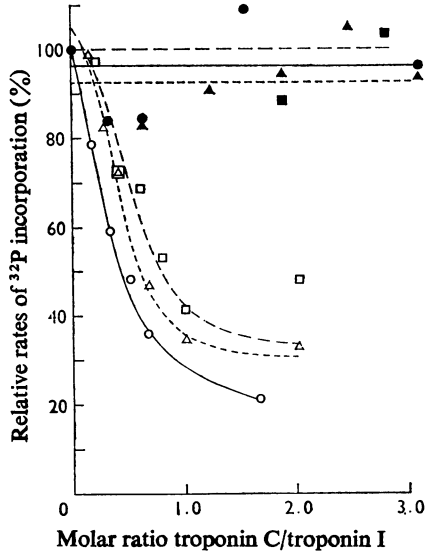

Fig. 8. *Effect of skeletal and cardiac troponin C on the phosphorylation of skeletal and cardiac troponin I catalysed by a bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase*

Rabbit skeletal troponin I (0.15 mg/ml) was incubated with protein kinase (0.025 mg/ml) for 45 min with [γ-^{32}P]ATP (1 mM; 1.6 μCi/ml) under standard conditions (see the Methods and Materials) with the addition of 12 mM-KCl. Rabbit cardiac troponin I (0.1 mg/ml) was incubated with the same protein kinase (0.01 mg/ml) for 15 min with [γ-^{32}P]ATP (1.25 mM; 2 μCi/ml) in similar conditions with 15 mM-KCl. In each case troponin C preparations were added in the molar ratios shown. The molecular weights of cardiac and skeletal troponin I were taken as 28000 and 21000 (Wilkinson & Grand, 1975) respectively; the molecular weight of troponin C was taken as 18000. The maximum phosphate incorporation was about 0.15 mol/mol for skeletal troponin I and about 0.2 mol/mol for cardiac troponin I. ○, △, □, Skeletal troponin I; ●, ▲, ■, cardiac troponin I. ○, ●, Rabbit skeletal troponin C; △, ▲, rabbit cardiac troponin C; □, ■, bovine cardiac troponin C.

troponin I by bovine cardiac cyclic AMP-dependent protein kinase, however, was not inhibited by either skeletal or cardiac troponin C (Fig. 8).

The significance of the results with protein kinase was evaluated by studying the effect of troponin C on phosphorylation of lysine-rich histone catalysed by bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase. Inhibition of phosphorylation of this substrate was obtained as the molar ratio of troponin C increased. In this case about 2 mol of troponin C/mol of histone (estimated mean molecular weight 15000) was required for 60% inhibition.

Discussion

The general features of the phosphorylation of cardiac troponin by phosphorylase kinase and by a 3′:5′-cyclic AMP-dependent protein kinase of cardiac muscle are similar to those reported for troponin from fast skeletal muscle. There are, however, quantitative and qualitative differences between the two systems which imply that phosphorylation of troponin I may be of special significance for the metabolism of cardiac muscle. These are as follows.

1. The phosphate contents of troponin, and particularly of troponin I, isolated from cardiac muscle are significantly higher than is the case with skeletal troponin. (The variable values obtained with cardiac troponin I may be due to the active kinase and phosphatase enzymes present in the preparation.)

2. The much more rapid phosphorylation of cardiac troponin I catalysed by a cardiac 3′:5′-cyclic AMP-dependent protein kinase.

3. Phosphorylation of cardiac troponin I by a 3′:5′-cyclic AMP-dependent protein kinase is not blocked by troponin C, as is the case with troponin I from fast skeletal muscle.

The amino acid sequence of rabbit white-skeletal troponin I has been determined (Wilkinson & Grand, 1975) and the main site of phosphorylation by a bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase shown to be serine-118 (Moir *et al.*, 1974; Huang *et al.*, 1974). This residue is immediately adjacent to the basic peptide (residues 96–117) that has inhibitory activity on the Mg^{2+}-stimulated adenosine triphosphatase of actomyosin and which interacts with actin (H. Syska, J. M. Wilkinson, R. J. A. Grand & S. V. Perry, unpublished work). Interaction between troponin I and other components of the myofibril may be, in part at least, electrostatic in nature. In that case the basic actin-binding region of troponin would be expected to interact with the acidic region close to the *N*-terminal of the actin molecule (Elzinga *et al.*, 1973). The introduction of a negatively charged phosphate group close to the basic region of troponin I would then modify the strength of binding to actin. Thus phosphorylation could regulate the inhibitory action of troponin I on the actomyosin adenosine triphosphatase. Although this hypothesis has been developed from studies on the skeletal system, there is strong evidence for the conservation of the primary structure of actin, and so the interaction sites on actin and troponin I from cardiac muscle are likely to be similar. A decrease in the binding constant of the troponin I–actin interaction could either increase the adenosine triphosphatase activity or change the time-sequence of activation of the adenosine triphosphatase. Either or both of these possibilities may help to explain the mechanism of the pharmacological effects of the catecholamines on cardiac muscle (see Robison *et al.*, 1971). The positive chronotrophic effect of catecholamines is restricted to a relatively small number of myocardial cells and has received little biochemical study. The increase in force of contraction, the inotrophic effect, has been widely studied and much evidence exists that this effect is mediated by 3′:5′-cyclic AMP (Robison *et al.*, 1971). Troponin I is the only myofibrillar protein that has so far been shown to be phosphorylated by 3′:5′-cyclic AMP-dependent protein kinase and therefore provides a direct link between the catecholamines and the contracto-regulatory system. Cardiac troponin I is much more rapidly phosphorylated than its skeletal counterpart, which implies that this process may have a special significance for the cardiac muscle, on which catecholamines have much more marked effects. The report by England (1975) that a close correlation exists between the increases in force of contraction and extent of phosphorylation of troponin I from rat heart in response to adrenaline supports this view.

Less can be postulated about the functional significance of phosphorylation of cardiac troponin I catalysed by phosphorylase kinase. From analogy with the skeletal system the main sites phosphorylated by each enzyme would be expected to be different, although the possibility of a common site cannot be excluded at present. Troponin C inhibits phosphorylation of troponin I by phosphorylase kinase, so this phosphorylation will not occur when troponin I and troponin C interact strongly. This occurs in the presence of Ca^{2+}, which is also required to activate phosphorylase kinase. Although cyclical phosphorylation–dephosphorylation could occur in phase with the contractile cycle of the myocardium, these studies *in vitro* suggest that the sites on troponin I are blocked when phosphorylase kinase is most active.

Despite the obvious similarities between the properties of troponin from cardiac and skeletal muscles, the functional difference between these two types of muscle may be reflected in the interaction of cardiac troponin I and troponin C. In skeletal muscle, troponin C blocks the sites on troponin I phosphorylated by phosphorylase kinase and by a 3′:5′-cyclic AMP-dependent protein kinase. The phosphorylation of cardiac troponin I catalysed by the latter enzyme is unaffected by troponin C. Thus this protein

either interacts with cardiac troponin I in a different way or the site phosphorylated by bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase is in a different position in relation to that phosphorylated by phosphorylase kinase. This might not be unexpected because, whereas the cardiac troponin I molecule has an apparent molecular weight of 28000, that of troponin I from fast skeletal muscle is 23000. It does suggest, however, that in the cardiac system, the sites on troponin I specific for the 3′:5′-cyclic AMP-dependent protein kinase are available for phosphorylation throughout the contractile cycle, irrespective of whether troponin I is complexed with troponin C.

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