The Site of Phosphorylation of Troponin I in the Perfused Rabbit Heart

THE EFFECT OF ADRENALINE

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1. On treatment of the perfused rabbit heart with adrenaline, the total covalently bound phosphate of troponin I increased from 1.14 mol of phosphate/mol to 1.86 mol of phosphate/mol. 2. Covalently bound phosphate could be identified only in the region of the molecule of cardiac troponin I consisting of residues 1–48. 3. When ^{32}P -labelled orthophosphate was present in the perfusion medium the phosphate at serine-20 became radioactively labelled. This residue was the only significant site of phosphorylation that could be identified. 4. The addition of adrenaline caused a 4–5-fold increase in covalently bound [^{32}P]phosphate. Virtually all of the ^{32}P was located at serine-20. 5. It was concluded from these studies that the extent of phosphorylation of serine-20 of cardiac troponin I increased from 30–40% in the control perfused heart to about 100% in the presence of adrenaline.

Troponin I from fast skeletal and cardiac muscles can be phosphorylated at specific sites by 3':5'-cyclic AMP-dependent protein kinase and phosphorylase kinase (for review see Perry, 1979). In the case of troponin I from fast skeletal muscle the role of the process in functioning tissue is unknown. Some phosphorylation presumably occurs *in vivo*, for, when isolated by affinity chromatography under conditions in which enzymic activity is not expected to occur, troponin I from skeletal muscle contains 0.4–0.5 mol of phosphate/mol (Cole & Perry, 1975). The covalent bound phosphate of troponin I in skeletal muscle, however, does not apparently exchange readily with the intracellular pools of ATP, for Ribolow *et al.* (1977) could find no incorporation of phosphate into this protein during tetanus in frog gastrocnemius and semitendinosus muscles. From studies *in vitro* (Perry & Cole, 1974; Cole & Perry, 1975) the phosphorylation sites of skeletal-muscle troponin I would be expected to be blocked in the complex due to the presence of troponin C.

The situation with the phosphorylation of cardiac troponin I is clearly different. When isolated from rabbit hearts by affinity chromatography it contains much more covalently bound phosphate than troponin I isolated from fast skeletal muscle by the same procedure, usually ranging in amount from 1 to 2 mol of phosphate/mol (Cole & Perry, 1975; Moir & Perry, 1977). Likewise, the cardiac-muscle protein is much more rapidly phosphorylated by

3':5'-cyclic AMP-dependent protein kinase, a process that is not significantly blocked by troponin C (Cole & Perry, 1975; Moir & Perry, 1977). Rabbit cardiac-muscle troponin I contains an *N*-terminal sequence of 26 residues that is not present in the skeletal-muscle protein (Wilkinson & Grand, 1978) and which contains a serine residue at position 20 that is readily phosphorylated by 3':5'-cyclic AMP-dependent protein kinase *in vitro* (Moir & Perry, 1977). Rapid phosphorylation of this residue is responsible for the much higher rate of phosphorylation catalysed by the 3':5'-cyclic AMP-dependent protein kinase of cardiac-muscle troponin I compared with the skeletal-muscle protein. Phosphorylation at serine-146 of cardiac-muscle troponin I is also catalysed by the 3':5'-cyclic AMP-dependent protein kinase. This would be expected, for the residue occurs in an analogous position in the molecule to serine-117 of troponin I from fast skeletal muscle that is the preferred site of phosphorylation by this enzyme (Moir & Perry, 1977).

The evidence that the extent of phosphorylation of troponin I increased when the perfused mammalian heart was treated with adrenaline (England, 1975; Solaro *et al.*, 1976) strongly suggested that the phosphorylation of troponin I had a specific role in intact cardiac muscle. We have therefore investigated the sites of phosphorylation of troponin I in the rabbit heart perfused by the Langendorff procedure to compare them with those identified by

studies *in vitro* (Moir & Perry, 1977). It has been shown that serine-20 is partially phosphorylated in the normal beating rabbit heart and that the phosphate moiety attached to this serine residue is virtually the only phosphate group that exchanges with the intracellular phosphate pool. On intervention with adrenaline all of the increase in the covalent phosphate content of cardiac-muscle troponin I is accounted for by further phosphorylation of serine-20.

Materials and Methods

Sephadex G-25, G-50 and G-75 and Sepharose 4B were obtained from Pharmacia, London W.5, U.K. Sodium [^{32}P]orthophosphate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Trypsin (treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one) and pepsin were purchased from Cambrian Chemicals, Croydon CR0 4XB, U.K. Thermolysin (thermophilic-bacterial proteinase, type X), adrenaline (as L-adrenaline bis-tartrate), O-phosphoserine and O-phosphothreonine were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Blue Brand Medical X-ray Film (BB 54), DX80 Developer and FX40 Fixer were obtained from Kodak, Birmingham 2, U.K.

Perfusion of rabbit hearts

Hearts were removed rapidly from stunned rabbits and after washing for 15–30 s in ice-cold saline (0.9% NaCl) were perfused by the Langendorff procedure (Morgan *et al.*, 1961) at 37°C with a modified Krebs–Henseleit buffer containing 118 mM-NaCl/4.7 mM-KCl/1.2 mM-MgSO₄/0.2 mM-KH₂PO₄/25 mM-NaHCO₃/2.5 mM-CaCl₂/0.5 mM-disodium EDTA/5 mM-glucose (Krebs & Henseleit, 1932). Any alterations to this medium were made by the addition of stock solutions and resulted in dilution of the perfusate by less than 1.0%. The force and rate of contraction were recorded on a polygraph (George Washington, Sheerness, Kent, U.K.) with a force transducer [UFI Dynamometer; range 2 oz (56.7 g)] attached to a stainless-steel hook inserted into the apex of the heart. After an initial period (2–5 min) the force and rate became regular and remained virtually constant for up to 100 min. Hearts were usually perfused after the initial period for a further 15 min for equilibration before additions were made to the perfusate.

For experiments on the incorporation of ^{32}P the initial 15 min equilibration was performed in the normal buffer and buffer containing ^{32}P (10 $\mu\text{Ci/ml}$) as sodium orthophosphate was then introduced into the apparatus. Perfusion was usually carried out for a further 15 min before the isolation of ^{32}P -labelled troponin I both in control experiments and after adrenaline treatment.

When required, adrenaline was then added to the perfusion medium to a final concentration of 4 μM and perfusion was continued until the increase in the force of contraction was maximal (approx. 30 s after addition of adrenaline). Hearts perfused with adrenaline were cut down at the peak of the force change and homogenized within 10–15 s in a small Waring blender-type homogenizer with 40–50 ml of solution containing 9 M-urea/75 mM-Tris/15 mM-mercaptoethanol/1 mM-CaCl₂, brought to pH 8.0 with 11.4 M-HCl. Control hearts were treated in the same manner except that the short perfusion in the presence of adrenaline was omitted.

Isolation of troponin I

Troponin I was isolated immediately from the urea extracts of perfused hearts by affinity chromatography using rabbit skeletal-muscle troponin C bound to Sepharose 4B (Syska *et al.*, 1974). The urea concentration was maintained at 9 M throughout the isolation procedure to ensure that enzymes capable of the phosphorylation or dephosphorylation of troponin I were inactivated. Urea was removed from the purified troponin I by dialysis against 1 mM-NH₄HCO₃ (3 $\times$ 200 vol.) or by gel filtration on columns of Sephadex G-25 or G-50 equilibrated and eluted with 0.01 M-HCl. Purity was determined by electrophoresis in 0.1% sodium dodecyl sulphate/0.01 M-sodium phosphate buffer (pH 7.0) (Weber & Osborn, 1969).

Determination of phosphate and nitrogen contents of troponin I samples

Protein was estimated by Nesslerization (Strauch, 1965) assuming a mol.wt. of 23 500 derived from the amino acid sequence of rabbit cardiac-muscle troponin I (Grand *et al.*, 1976). Phosphate was determined by the method of Bartlett (1959) with 10 M-H₂SO₄ (0.5 ml) for digestion; after reaction of the acid hydrolysate with aminonaphthol sulphonc acid reducer and ammonium molybdate, the final volume was made up to 5.0 ml.

Identification of the phosphorylated residues of cardiac-muscle troponin I

Cardiac-muscle troponin I (100–300 μg) isolated from control and adrenaline-stimulated hearts that had been perfused in the presence of ^{32}P were hydrolysed *in vacuo* at 110°C in 5.7 M-HCl (0.5 ml) for 1.5–3 h. After drying *in vacuo* the hydrolysates were applied to Whatman no. 1 paper (57 cm $\times$ 20 cm) and subjected to electrophoresis at 3 kV for 2–3 h at pH 2.0 (Wilkinson, 1974) in the presence of authentic samples of phosphoserine, phosphothreonine and [^{32}P]P_i. The sheets were dried, stained with ninhydrin and ^{32}P -containing material was located by radioautography.

Identification of phosphorylated peptides

Cardiac-muscle troponin I (5–25 mg) labelled with ^{32}P after perfusion under the appropriate conditions was digested with trypsin, thermolysin, pepsin or CNBr, the latter being performed in 0.1 M-HCl to minimize hydrolysis of phosphopeptides. Peptides containing ^{32}P were purified by gel filtration, paper electrophoresis and paper chromatography and were detected by radioautography. The methods employed have been described in detail elsewhere (Moir & Perry, 1977; Moir *et al.*, 1977).

Amino acid analysis

^{32}P -containing peptides were hydrolysed *in vacuo* for 20 h in 5.7 M-HCl at 110°C. Amino acid analysis was performed on a Locarte amino acid analyser (Wilkinson *et al.*, 1972). No corrections were made for the loss of serine.

Results

Phosphate content of troponin I isolated from perfused rabbit hearts

The phosphate content of troponin I prepared by affinity chromatography from rabbit hearts perfused for 15 min with modified Krebs–Henseleit buffer was 1.14 ± 0.20 mol of phosphate/mol (five preparations). If adrenaline ($4 \mu\text{M}$) was included in the perfusion medium, the phosphate content of the troponin I at the peak of the force response increased to 1.86 ± 0.50 mol/mol (five preparations). These values are consistent with those routinely obtained in this laboratory for rabbit hearts perfused in the presence or absence of adrenaline. The values for the phosphate content of cardiac-muscle troponin I reported in the present study are significantly lower than those reported previously from this laboratory (Solaro *et al.*, 1976). In the previous study the phosphate values were consistently higher because of the slight differences in the H_2SO_4 concentration in the solution used for colour development for experimental determinations and for calibration of the colour response. This was due to significant loss of

H_2SO_4 during the wet ashing procedure in the presence of relatively large amounts of organic material. The method as described in the Materials and Methods section is not subject to this error (C. M. Jones, R. J. Solaro & S. V. Perry, unpublished work). When ^{32}P -labelled sodium orthophosphate was included in the perfusion medium, radioactivity was incorporated into the troponin I (Table 1), indicating that the protein-bound phosphate was exchanging with the intracellular phosphate pool.

If hearts perfused with ^{32}P -labelled phosphate were subsequently treated with adrenaline ($4 \mu\text{M}$) and the troponin I isolated at the peak of the resulting force increase, the amount of covalently bound ^{32}P increased 4–5 times compared with the value in control hearts that had not been treated with adrenaline (Table 1).

When troponin I was isolated from control and adrenaline-treated hearts perfused with ^{32}P -labelled phosphate and subsequently hydrolysed in 5.7 M-HCl for approx. 2 h, radioactivity was found to be associated with phosphoserine separated by electrophoresis at pH 2.0 (see the Materials and Methods section). No detectable amounts of radioactivity were associated with phosphothreonine in these digests and no phosphothreonine could be observed by staining with ninhydrin (Fig. 1).

Distribution of phosphate in the CNBr peptides of troponin I from perfused hearts

Previous studies (Grand *et al.*, 1976; Moir & Perry, 1977) have shown that when rabbit cardiac-muscle troponin I is completely digested with CNBr, free homoserine and four peptides designated peptide CCN3 (residues 1–48), peptide CCN2 (residues 49–150), peptide CCN4 (residues 152–197) and peptide CCN5 (residues 198–206) are obtained. These peptides are arranged in the troponin I molecule as illustrated in Fig. 2.

The gel-filtration elution pattern of a CNBr digest of troponin I from a control heart applied to a column of Sephadex G-75 equilibrated and eluted with 0.01 M-HCl is illustrated in Fig. 3(a). A similar elu-

Table 1. Incorporation of ^{32}P into the troponin I of rabbit hearts perfused in the absence and presence of adrenaline. Hearts were homogenized after perfusion with ^{32}P -labelled orthophosphate and troponin I isolated by affinity chromatography as described in the Materials and Methods section. Radioactivity was estimated in whole proteins. Values in parentheses indicate the numbers of hearts used in each experiment.

Experiment	Radioactivity incorporated (c.p.m./heart)		Ratio of radioactivity in adrenaline/control
	Control	Adrenaline	
JS6	695 (1)	3297 (1)	4.7
JS9	929 (1)	5240 (2)	5.6
JS89	2425 (2)	11720 (2)	4.8
JS233	2850 (2)	12459 (2)	4.4
H351	6079 (2)	17826 (2)	2.9

Fig. 1. Radioautography of acid hydrolysates of ^{32}P -labelled rabbit cardiac-muscle troponin I

Troponin I (100–300 μg) isolated from 9 M-urea extracts of control and adrenaline-treated hearts was incubated at 110°C for approx. 2 h *in vacuo* in 5.7 M-HCl (approx. 0.5 ml) and the hydrolysates were examined by high-voltage electrophoresis at pH 2.0 (see the Materials and Methods section). (a) (i) Shows phosphoserine (ringed), ninhydrin stained; (ii) shows a radioautogram of an acid hydrolysate of rabbit cardiac-muscle troponin I perfused under control conditions; (iii) shows a radioautogram of ^{32}P -labelled orthophosphate; (iv) shows phosphothreonine (ringed), ninhydrin stained. (b) (i) shows phosphothreonine (ringed), ninhydrin stained; (ii) shows a radioautogram of an acid hydrolysate of rabbit cardiac-muscle troponin I from adrenaline-stimulated hearts; (iii) shows phosphoserine (ringed), ninhydrin stained. Abbreviation: O, origin.

tion pattern was obtained in three different experiments.

Some phosphate was found in a fraction containing the larger partial-digestion products that were eluted in a peak that was virtually unretarded on gel filtration and which was somewhat variable in amount between digests (cf. Figs. 3a and 3b). Most of the phosphate was present in peak A, which consisted of a partial-digestion product represented by a peptide that contained residues 1–150 and in peak C1, which contained the *N*-terminal peptide CCN3. Little phosphate was found in peptides containing residues 49–150 (peak B) or residues 152–197 (peak C2). A more complete separation of the two peptides present in peak C could be achieved by re-chromatography of this material on Sephadex G-50 (Moir & Perry, 1977); this confirmed that phosphate was bound to the fragment containing residues 1–48 rather than the other component in peak C, the

peptide containing residues 152–197. A similar distribution of phosphate was obtained if the CNBr peptides from troponin I isolated from adrenaline-treated hearts were fractionated in the same manner. The distribution illustrated in Fig. 3(b) is typical of the five different experiments that were carried out. Although the same peptide fractions contained phosphate as in the control experiments, the amount present on a molar basis was greater in the adrenaline-treated hearts.

If ^{32}P -labelled phosphate was included in the perfusion buffer the distribution of ^{32}P in the CNBr peptides from control hearts was exactly the same as was the phosphate in the unlabelled cardiac troponin I. Fig. 4(a) is typical of the results obtained in four different experiments. A similar distribution was obtained when the troponin I was prepared from muscle at the peak of response to adrenaline (Fig. 4b). In the latter case, however, the amount of ^{32}P in-

Fig. 2. Representation of the primary sequence of rabbit cardiac-muscle troponin I indicating the relative position of the CNBr peptides CCN2, CCN3, CCN4 and CCN5 (Grand *et al.*, 1976) and the phosphorylation sites specific for 3':5'-cyclic AMP-dependent protein kinase

Numbers are residue positions in the sequence. Abbreviations used: N, N-terminus; C, C-terminus (from Moir & Perry, 1977).

incorporated into peptide CCN3 in three different experiments ranged from five to seven times greater per mole than was the case with the control muscles.

Identification of amino acid residues that are phosphorylated in the perfused heart

^{32}P -labelled troponin I isolated from rabbit hearts perfused with Krebs-Henseleit buffer containing ^{32}P was digested by using a mixture of trypsin and thermolysin (1:1, by weight) in 50 mM- NH_4HCO_3 and the digest was fractionated by gel filtration on a column (2.5 cm $\times$ 110 cm) of Sephadex G-25, equilibrated and eluted with 50 mM- NH_4HCO_3 . All of the radioactivity was located in a single peak (Fig. 5) and the ^{32}P -labelled peptides were separated by electrophoresis at pH 6.5 of the fraction containing radioactive material followed by descending chromatography in n-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) or by electrophoresis at pH 2.0 and 3.5. Four radioactive peptides were separated by electrophoresis at pH 6.5 (Fig. 6a), two of which, peptides 2C and 3C, contained 85% of the recovered radioactivity (35% of that applied to the gel-filtration column). The two other ^{32}P -containing peptides, peptides 1C and 4C, were obtained in low yields and could not be characterized. The amino acid composition of peptides 2C and 3C differed by the presence of a second arginine residue in peptide 2C (Table 2).

In a previous study from this laboratory similar peptides were isolated after tryptic digestion of rabbit cardiac-muscle troponin I that had been incubated with [γ - ^{32}P]ATP and 3':5'-cyclic AMP-

dependent protein kinase (Moir & Perry, 1977). These peptides and those obtained in the present study were derived from the sequence Arg-Ser-Asp-Arg-Ala-Tyr shown to be present in the N-terminal region of rabbit cardiac-muscle troponin I (Grand *et al.*, 1976). In view of the isolation of ^{32}P -labelled phosphoserine after hydrolysis of ^{32}P -labelled troponin I in 5.7 M-HCl (see above) the serine residue in peptides 2C and 3C, which corresponds to that at position-20 in the intact molecule, is presumed to be the residue phosphorylated. It was therefore concluded that the phosphate group on serine-20 was exchanging, presumably by enzymic reactions, with the phosphate pool of the control perfused heart.

Two other experiments were performed to locate the sites of phosphorylation of troponin I in the hearts not treated with adrenaline. In one case the ^{32}P -labelled troponin I isolated by affinity chromatography was digested directly with trypsin, whereas in the other, the protein was digested with CNBr in 0.1 M-HCl and the peptide containing residues 1-48 subsequently digested with trypsin. In both experiments the only ^{32}P -labelled peptides that could be identified contained serine-20.

Identification of residues that are phosphorylated in the heart perfused in the presence of adrenaline

^{32}P -labelled troponin I isolated from rabbit hearts perfused in the presence of both ^{32}P -labelled orthophosphate and adrenaline was digested with trypsin and the ^{32}P -containing peptides were purified by gel filtration on a column (2.5 cm $\times$ 110 cm) of Sephadex

Fig. 3. Distribution of covalently-bound phosphate in the CNBr peptides of troponin I prepared from the perfused rabbit heart

Samples of troponin I prepared from perfused rabbit hearts were digested with CNBr and carboxymethylated with iodoacetic acid (Moir *et al.*, 1974). The digests were applied to a column (2.2 cm $\times$ 150 cm) of Sephadex G-75 equilibrated and eluted with 0.01 M-HCl. Consecutive fractions were pooled as indicated by the histograms, dried *in vacuo* and the total phosphate content of each was determined. Histograms represent the total phosphate content (nmol) of each pooled fraction. —, A_{215} . (a) Troponin I prepared from control hearts. (b) Troponin I prepared from hearts perfused in the presence of adrenaline (4 μ M).

G-25 equilibrated and eluted with 50 mM- NH_4HCO_3 (Fig. 5) followed by paper electrophoresis at pH 6.5 (Fig. 6b), pH 2.0 and 3.5. The total recovery of radioactivity was 56%, of which 61% was associated with peptide 2A and 22% with peptide 3A (Table 2). These peptides were identical with the radioactive peptides 2C and 3C isolated from the control perfused heart. Several other ^{32}P -containing bands were located, but could not be characterized because of the very small amounts obtained. None

Fig. 4. Gel filtration of CNBr digests of ^{32}P -containing troponin I prepared from the perfused rabbit heart. CNBr digests of cardiac-muscle troponin I prepared from rabbit hearts perfused with Krebs-Henseleit buffer containing ^{32}P (approx. 10 $\mu\text{Ci/ml}$) were carboxymethylated (Moir *et al.*, 1974) and applied to a column (2.5 cm $\times$ 110 cm) of Sephadex G-75 equilibrated and eluted with 0.01 M-HCl. Fractions (5 ml) were collected and the A_{215} was measured and radioactivity determined in alternate fractions by the Cerenkov procedure (Gould *et al.*, 1972). —, A_{215} ; ---, radioactivity. (a) Troponin I prepared from control hearts; (b) troponin I prepared from hearts perfused in the presence of adrenaline (4 μ M).

of these minor bands contained more than 5% of the recovered radioactivity. It was concluded that in the adrenaline-stimulated heart, as with the control per-

Fig. 5. Gel filtration of enzymic digest of ³²P-containing cardiac-muscle troponin I prepared from the perfused rabbit heart

Samples of troponin I prepared from adrenaline-stimulated rabbit hearts perfused with Krebs-Henseleit buffer containing ³²P were digested with trypsin and applied to a column (2.5 cm × 110 cm) of Sephadex G-25 equilibrated and eluted with 50 mM-NH₄HCO₃. Fractions (5 ml) were collected and the A₂₁₅ was measured and radioactivity in alternate fractions determined by the Cerenkov procedure. —, A₂₁₅; ----, ³²P radioactivity.

Fig. 6. Radioautography of peptides of ³²P-labelled troponin I from perfused rabbit hearts

Pooled fractions of ³²P-containing peptides obtained by gel filtration (Fig. 5) were further fractionated by paper electrophoresis at pH 6.5. Radioautograms were developed after exposure for approx. 40–60 h. (a), Peptides from control heart; (b), peptides from heart treated with adrenaline (4 μM). Abbreviation: O, origin.

Table 2. Amino acid composition of ³²P-containing peptides isolated from troponin I prepared from perfused rabbit hearts
Control and adrenaline-treated hearts were perfused with ³²P-labelled orthophosphate, homogenized in urea buffer and troponin I isolated by affinity chromatography and subsequently digested with enzymes as indicated in the text. Amino acid compositions are expressed as mol/mol of peptide. Values in parentheses are the nearest whole numbers of residues. For significance of peptide nomenclature see the text and Fig. 6. Mobility at pH 6.5 is expressed relative to Asp = -1 (Offord, 1966).

		Amino acid content			
		Control digest		Adrenaline-treated digest	
Peptides ...		2C	3C	2A	3A
Asp		0.9 (1)	0.7 (1)	0.9 (1)	1.0 (1)
Ser		1.3 (1)	1.3 (1)	1.2 (1)	1.2 (1)
Ala		1.0 (1)	0.9 (1)	0.9 (1)	0.8 (1)
Tyr		0.6 (1)	0.6 (1)	1.0 (1)	0.6 (1)
Arg		2.0 (2)	1.1 (1)	2.0 (2)	1.2 (1)
Mobility		+0.09	0	+0.11	0

fused hearts, virtually all of the ³²P incorporated into the troponin I was located at serine-20.

Four other experiments were performed to study the incorporation of ³²P into the adrenaline-treated perfused heart. In two experiments the ³²P-containing troponin I was digested with trypsin, in another with trypsin and pepsin and in the fourth by CNBr in 0.1 M-HCl, followed by tryptic digestion of fragments containing residues 1–150 and 1–48 of the molecule. In all cases the only ³²P-containing peptides that could be identified contained serine-20.

Estimation of the extent of phosphorylation of serine-20 in the perfused heart

The experiments described above indicated that the amount of radioactivity at serine-20 increased

when the heart perfused with ³²P-labelled phosphate was treated with adrenaline. Despite the fact that the total covalently bound phosphate of troponin I clearly increases on adrenaline treatment, from the evidence provided so far it cannot be unequivocally concluded that this is due to an increase in phosphorylation at serine-20. The results from the studies with the CNBr peptides could be explained on the basis of increased exchange of phosphate with the intracellular pool. This might apply particularly if the increase in covalently bound phosphate of the intact troponin I due to adrenaline action took place on some other site that was relatively labile and lost during digestion with CNBr or enzymes.

Direct determination of the phosphate content of the N-terminal CNBr peptide comprising residues

Table 3. *Phosphate contents of N-terminal CNBr peptides from troponin I of perfused rabbit heart*
Troponin I was isolated as indicated in Table 2 and CNBr peptides were isolated as indicated in the text. Values are means $\pm$ S.E.M. Abbreviation: N.D., value not determined. Values in parentheses indicate the numbers of experiments performed.

	Treatment ...	Phosphate content (mol/mol)	
		Control	Adrenaline-stimulated
Peptide CCN3 (residues 1–48)		0.47, 0.49 (2)	1.01 $\pm$ 0.15 (3)
Peptide CCN1 (residues 1–150)		N.D.	1.08 $\pm$ 0.18 (3)

Table 4. *Specific radioactivity of peptides isolated from hearts perfused in the presence of ^{32}P -labelled orthophosphate*
Peptides were isolated as described for Table 2.

	Treatment ...	$10^{-5} \times$ Specificity radioactivity (d.p.m./ μmol)	
		Control	Adrenaline-stimulated
Troponin I		0.35	2.4
Tryptic peptide 2		0.88	2.8
Tryptic peptide 3		0.97	1.9
Extent of phosphorylation of serine-20 (mol of phosphate/mol)		0.38	1

* Average value.

1–48 confirmed that a real increase in phosphate content had occurred (Table 3).

Further evidence that a real increase in phosphorylation of serine-20 had occurred was obtained by analysis of the radiochemical data. Since the incorporation of ^{32}P into troponin I in the perfused heart appears to be confined almost exclusively to one residue, it is possible to obtain an approximate value for the extent of phosphorylation of this residue in the control and adrenaline-treated hearts by comparing the specific radioactivity of the isolated protein to that of the small ^{32}P -containing fragments isolated by paper electrophoresis. If the residue is completely phosphorylated, the specific radioactivity of the whole protein and of the fragment will be similar; if phosphorylation is partial, the specific radioactivity of the small peptide will be higher than that of the whole protein (see Moir & Perry, 1977).

It can be seen from Table 4 that in the control heart, the specific radioactivity of the isolated peptides was 3-fold higher than the activity of the intact protein, indicating incomplete phosphorylation of serine-20. In hearts treated with adrenaline, the specific radioactivities of the tryptic peptides were similar to that of the troponin I, demonstrating that the extent of phosphorylation of serine-20 approaches completion.

Conclusions

The results indicate that in the normal perfused rabbit heart serine-20 of troponin I is about 30–40% phosphorylated and that phosphate on this site is exchanging with the phosphate pool, presumably in the form of ATP, of the myocardium. This phosphorylation site appears to be the only one that is in equilibration during the perfusion experiments with the intracellular phosphate pool. Phosphate at serine-20 appears to account for the total phosphate content of the *N*-terminal peptide consisting of residues 1–48 that is isolated after CNBr digestion and would in the normal perfused heart contribute 0.3–0.4 mol of phosphate/mol of troponin I. The fact that intact troponin I isolated from control perfused hearts contains a total of 1.15 mol of phosphate/mol suggests that other phosphorylation sites exist on the molecule. Nevertheless no significant amounts of phosphate could be detected in the CNBr peptides other than those containing residues 1–48. It seems likely that this discrepancy in phosphate content is due to the presence of a particularly labile site that is lost during the CNBr-digestion procedure. We have been unable so far to locate this site. As very little ^{32}P was lost from labelled troponin I during CNBr digestion we conclude that the phosphate at serine-20 was stable to those conditions and furthermore

that the labile site is not in dynamic equilibrium with the phosphate pool.

The increase in covalently bound phosphate of troponin I that occurs on perfusion with adrenaline is completely accounted for by the increase in phosphorylation at serine-20, the phosphate content of which changes from 0.3–0.4 to about 1.0 mol of phosphate/mol when the rabbit heart is exposed to 4 μ M-adrenaline in the perfusate. The increase in incorporation of 32 P is due to the increased amount of phosphorylation at this site and as in the case of the control perfused heart is restricted to serine-20.

The studies in the perfused heart confirm the studies *in vitro* that showed serine-20 as the major site of phosphorylation when rabbit cardiac-muscle troponin I is incubated *in vitro* with cyclic AMP-dependent protein kinase (Moir & Perry, 1977). Thus it would appear that this increase in the extent of phosphorylation is due to the increased activity of 3':5'-cyclic AMP-dependent protein kinase in the perfused rabbit heart as a result of the administration of adrenaline.

Rabbit cardiac-muscle troponin I is also a substrate *in vitro* for phosphorylase *b* kinase (Cole & Perry, 1975). Since the extent of phosphorylation of the phosphorylase kinase-specific sites of troponin I does not change in the perfused heart in response to adrenaline, it seems unlikely that the activity of this enzyme towards cardiac-muscle troponin I alters during the inotropic response. This supports the observation of England (1977) that phosphorylation of troponin I occurs in the perfused heart of phosphorylase kinase-deficient mice. The work of England (1976) with the rat and of this laboratory with the rabbit (C. Jones, R. J. Solaro & S. V. Perry, unpublished work) has demonstrated that the peak of the force increase in the perfused heart is reached before the phosphorylation of troponin I is maximal. Furthermore, phosphorylation of troponin I in cardiac-muscle natural actomyosin leads to a decrease in the sensitivity of the ATPase activity towards Ca^{2+} (Solaro *et al.*, 1976; Ray & England, 1976; Reddy & Wyborny, 1976; Perry *et al.*, 1978, 1979; Bailin, 1979). Thus the consequence of phosphorylation of cardiac-muscle troponin I at position-20 is not to increase directly the ATPase activity of the heart. Instead, the role of phosphorylation of troponin I is probably to smooth out the contractile response of the heart produced by the transient increase in Ca^{2+} concentrations that result from the administration of adrenaline or other β -adrenergic agents (Perry, 1979). Thus phosphorylation of cardiac-muscle troponin I represents a negative-feedback control mechanism on the ATPase activity of the heart.

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