

Rapid Papers

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Phosphorylation of the Inhibitory Subunit of Troponin in Perfused Hearts of Mice Deficient in Phosphorylase Kinase

EVIDENCE FOR THE PHOSPHORYLATION OF TROPONIN BY ADENOSINE 3':5'-PHOSPHATE-DEPENDENT PROTEIN KINASE *IN VIVO*

By PAUL J. ENGLAND

Department of Biochemistry, University of Bristol Medical School,
University Walk, Bristol BS8 1TD, U.K.

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When hearts from control and phosphorylase kinase-deficient (I strain) mice were perfused with $0.1 \mu\text{M}$ -DL-isoprenaline, there was a parallel increase in contraction, cyclic AMP concentration and troponin I phosphorylation. However, there was no increase in phosphorylase *a* in the I-strain hearts, whereas the control hearts showed a large increase. Assays of I-strain heart extracts showed a normal cyclic AMP-dependent protein kinase activity but no phosphorylase kinase activity. It is concluded that troponin I is phosphorylated in intact hearts by protein kinase and not phosphorylase kinase.

Phosphorylation of the inhibitory subunit of troponin (troponin I) was first observed in a complex of troponin I and the tropomyosin-binding subunit (troponin T) from skeletal muscle when incubated with cyclic AMP-dependent protein kinase (Bailey & Villar-Palasi, 1971). Subsequent work (Stull *et al.*, 1972; England *et al.*, 1973) showed a rapid phosphorylation of troponin I by phosphorylase kinase. However, when the whole troponin complex (troponin I, troponin T and the Ca^{2+} -binding subunit, troponin C) from skeletal muscle was used, the rate of phosphorylation by both phosphorylase kinase and protein kinase was slow (Perry & Cole, 1973, 1974; England *et al.*, 1973). In contrast, whole troponin from cardiac muscle was rapidly phosphorylated in the presence of protein kinase (Reddy *et al.*, 1973; Cole & Perry, 1975; Ray & England, 1976), but only slowly phosphorylated by phosphorylase kinase (Cole & Perry, 1975). The phosphate was incorporated only into troponin I. Studies with perfused rat heart (England, 1975, 1976) showed that phosphorylation of troponin I occurred in response to stimulation by adrenaline and other β -adrenergic-receptor agonists. This phosphorylation occurred within 20s, and paralleled the increase in contraction induced by the catecholamines. In view of the results with isolated troponin *in vitro*, it is probably that the phosphorylation of troponin I *in vivo* is catalysed by protein kinase in response to the hormonally induced increase in cyclic AMP (England, 1976) and that in cardiac muscle there is no phosphorylation of troponin by phosphorylase kinase. The present results are an attempt to directly prove this point.

The strain of mice ICR/IAn (I strain) totally lacks phosphorylase kinase activity in skeletal muscle

(Lyon & Porter, 1963; Cohen & Cohen, 1973). If these mice are lacking phosphorylase kinase in cardiac muscle also, then phosphorylation of troponin I in perfused hearts from I-strain mice should be unaffected if the phosphorylation is catalysed by cyclic AMP-dependent protein kinase, but decreased or absent if the phosphorylation is catalysed by phosphorylase kinase. It was originally reported (Lyon & Porter, 1963) that cardiac muscle from I-strain mice contained phosphorylase kinase, on the basis of measured phosphorylase *a/b* ratios in hearts removed at various times after death. However, this method is very susceptible to overestimation of the amount of phosphorylase *a*, owing to activation by endogenous AMP in the assay (Cohen & Cohen, 1973). In the present paper, it is shown both *in vivo* and *in vitro* that cardiac muscle from I-strain mice does not catalyse the interconversion of phosphorylase *b* and *a*, indicating an absence of phosphorylase kinase. However, the phosphorylation of troponin I in perfused mouse hearts, when stimulated by isoprenaline, was the same in both control and I-strain mice, indicating that phosphorylation of troponin I *in vivo* is catalysed by cyclic AMP-dependent protein kinase.

Materials and Methods

A colony of ICR/IAn mice, carrying the X-linked gene for phosphorylase kinase deficiency, was established from three breeding-pairs kindly provided by Dr. Philip Cohen, University of Dundee, U.K. (Cohen & Cohen, 1973). A colony of C3H mice was used as a source of control mice.

Mouse hearts were perfused by the Langendorff technique as described previously for rat hearts (England, 1975, 1976). The hearts were perfused

for 20 min with $^{32}\text{P}_i$ -containing medium (Krebs & Henssleit, 1932), and then stimulated for 30 s with a continuous perfusion of $0.1\ \mu\text{M}$ -DL-isoprenaline, or given a control perfusion without the drug. Contractility was measured with a force-displacement transducer, and the hearts were rapidly freeze-clamped at the end of the perfusion (Wollenberger *et al.*, 1960). Owing to the small amount of tissue in each heart, for measurement of phosphorylase activity and cyclic AMP concentration hearts were perfused under identical conditions without $^{32}\text{P}_i$ in the medium. Methods for assay of phosphorylase activity and cyclic AMP concentration are described by England (1976). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ specific radioactivity was assayed by the method of England & Walsh (1976); troponin I was isolated by affinity chromatography (Syska *et al.*, 1974) and ^{32}P content determined as previously described (England, 1975, 1976). A molecular weight of 28000 was used for troponin I, as determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

Phosphorylase kinase activity was assayed in mouse hearts as follows. Groups of three fresh mouse hearts (approx. 300 mg wet wt. of tissue) were homogenized in 2 ml of 10 mM-Tris/HCl containing 2 mM-EDTA, pH 8.0, and centrifuged for 10 min at 3000g. Phosphorylase kinase was assayed by incubation of $20\ \mu\text{l}$ of supernatant in $250\ \mu\text{l}$ of 100 mM-Tris/HCl containing 10 mM-MgCl₂, 15 mM-2-mercaptoethanol, 0.1 mM-CaCl₂ and 1 mg of phosphorylase *b*/ml, 1.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 35 Ci/mol), pH 8.2, at 30°C. Portions ($50\ \mu\text{l}$) were taken at 4 min intervals, spotted on to 1 cm squares of filter paper, and ^{32}P incorporation into phosphorylase was assayed as described by Reimann *et al.* (1971). The assay was linear for at least 8 min, and was proportional to supernatant concentration. Addition of 1 mM-EGTA completely inhibited phosphorylation of phosphorylase *b*. For the assay of phosphorylase kinase activity towards troponin, the above conditions were used except that 1 mg/ml of bovine heart

native tropomyosin (troponin plus tropomyosin) was substituted for phosphorylase *b*.

Cyclic AMP activity towards lysine-rich histone (De Nooij & Westenbrink, 1962) or cardiac native tropomyosin was assayed in supernatants of mouse hearts made by homogenizing hearts singly in 1.5 ml of 10 mM-potassium phosphate containing 2 mM-EDTA, pH 6.8, and centrifuging at 3000g for 10 min. The assay medium was $250\ \mu\text{l}$ of 20 mM-potassium phosphate, pH 6.8, containing 20 mM-NaF, 2 mM-theophylline, 6 mM-magnesium acetate, 0.1 mM-cyclic AMP, 1.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 1 mg of protein substrate/ml and $20\ \mu\text{l}$ of heart supernatant. Protein-bound ^{32}P was assayed in portions ($50\ \mu\text{l}$) as described above. The assay was linear for at least 10 min and was proportional to supernatant concentration. However, there was a noticeable inhibition (approx. 50%) of exogenously added bovine heart protein kinase in assays of supernatants from both C3H- and I-strain mice, and the values were lower than those quoted for rat heart (Keely *et al.*, 1975). This is presumably owing to inhibitory factors (Ashby & Walsh, 1972) or adenosine triphosphatase activity (Corbin & Reimann, 1974) in the extracts. The values quoted in the Results and Discussion section should therefore only be used for comparison between the two strains, and not taken as a true determination of the total protein kinase activity in mice hearts.

Proteins and substrates were prepared by the following methods: phosphorylase *b*, Fischer & Krebs (1958); phosphorylase kinase, Cohen (1973); bovine heart protein kinase, Brostrom *et al.* (1970); bovine heart native tropomyosin, modified from Hartshorne & Mueller (1968) as described by Ray & England (1976); $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, Glynn & Chappell (1964).

Results and Discussion

Initial experiments were performed to show that conversion of phosphorylase *b* into *a* does not occur in I-strain mice. Table 1 shows that perfusion of

Table 1. Changes in contractility, cyclic AMP concentration, phosphorylase *a* activity and troponin I phosphorylation in mouse hearts perfused with $0.1\ \mu\text{M}$ -DL-isoprenaline

Mouse hearts were perfused for 20 min by the Langendorff technique, and then freeze-clamped (control perfusions) or perfused with $0.1\ \mu\text{M}$ -DL-isoprenaline for 30 s before freeze-clamping. Details of the subsequent analyses are given in the Materials and Methods section. The results are given as the means \pm S.E.M. with a minimum of four hearts in each group. ** $P < 0.01$; *** $P < 0.001$ [tested against values for control perfusions (Student's *t* test)].

	C3H-strain mice		I-strain mice	
	Control perfusion	Isoprenaline perfusion	Control perfusion	Isoprenaline perfusion
Contraction (% increase over control)	100	$171 \pm 7^{***}$	100	$168 \pm 6^{***}$
Cyclic AMP (nmol/g dry wt.)	1.61 ± 0.09	$3.40 \pm 0.31^{**}$	1.58 ± 0.18	$4.81 \pm 0.61^{**}$
Phosphorylase <i>a</i> (% of total phosphorylase)	16 ± 4	$70 \pm 7^{**}$	6 ± 2	10 ± 4
Troponin I phosphorylation (mol of ^{32}P /mol of troponin I)	0.34 ± 0.02	$1.17 \pm 0.12^{**}$	0.36 ± 0.07	$1.19 \pm 0.06^{***}$

Table 2. *Activities of phosphorylase kinase and cyclic AMP-dependent protein kinase in mouse heart extracts*
 Mouse hearts were homogenized in buffered medium, and supernatants from centrifugation at 3000g for 10 min were prepared. Enzyme activities were assayed in the supernatants by measuring the incorporation of ^{32}P into protein from [γ - ^{32}P]ATP. Phosphorylase kinase was assayed at pH 8.2 in the presence of 0.1 mM- Ca^{2+} . Protein kinase was assayed at pH 6.8 in the presence of 0.1 mM-cyclic AMP. In all assays the protein substrate concentration was 1 mg/ml. Other details of the assays are given in the Materials and Methods section. Results are given as the means \pm S.E.M., with a minimum of three determinations in each group.

Substrate	Phosphorylase kinase activity (nmol/min per g wet wt.)		Protein kinase activity (nmol/min per g wet wt.)	
	C3H strain	I strain	C3H strain	I strain
Phosphorylase <i>b</i>	1.53 \pm 0.18	0.045 \pm 0.035	—	—
Native tropomyosin	Not detectable	Not detectable	17.6 \pm 2.5	18.0 \pm 3.6
Histone (lysine-rich fraction)	—	—	34.1 \pm 4.5	42.3 \pm 7.4

hearts from C3H-strain mice with isoprenaline caused increases in contraction, cyclic AMP concentration and phosphorylase *a* activity that were very similar to those in rat hearts (England, 1976). With hearts from the I-strain mice, the changes in contraction and cyclic AMP concentration were the same as the control group, but there was no interconversion of phosphorylase *b* and *a*. The small amount of phosphorylase *a* measured is caused by activation of phosphorylase *b* by AMP carried over in the tissue extract. When phosphorylase kinase activity was measured in extracts of mouse hearts (Table 2), there was considerable activity in the C3H-strain mice, but no significant activity in the I-strain mice. This lack of activity was not caused by the presence of phosphorylase kinase inhibitors, as addition of purified rabbit skeletal-muscle phosphorylase kinase resulted in a rapid phosphorylation of phosphorylase in both C3H- and I-strain heart extracts. It therefore appears that in I-strain mice there is a deficiency of phosphorylase kinase in heart as well as skeletal muscle. As mentioned above, this is in contrast with the findings of Lyon & Porter (1963), but the more direct assays used in this study are probably less subject to errors in interpretation.

Table 1 shows that perfusion with isoprenaline caused an identical increase in troponin I phosphorylation in C3H- and I-strain mice. The amounts of phosphorylation in the unstimulated hearts and those perfused with isoprenaline are very similar to those found in rat heart (England, 1976). Owing to the small amount of troponin I isolated from a mouse heart (30 μg) it was not possible to assay for total phosphate in the protein, and so the values in Table 1 refer to the amount of ^{32}P per mol of troponin I. These results show that, in spite of the lack of phosphorylase kinase activity in the I-strain hearts, troponin I phosphorylation occurred normally in response to isoprenaline.

Further evidence for phosphorylation of troponin I by protein kinase is shown in Table 2. It can be seen that, whereas troponin I (as native tropomyosin)

is not a substrate for phosphorylase kinase, it is a good substrate for protein kinase, as previously reported (Cole & Perry, 1975). The activity of protein kinase towards troponin I was the same in both C3H- and I-strain mice, as would be expected from the phosphorylation seen *in vivo*. Gross & Mayer (1973) reported that skeletal muscle of I-strain mice, although lacking phosphorylase kinase activity, did possess a troponin I-phosphorylating activity which was one-half of that found in normal mice. This was interpreted as showing that phosphorylase kinase in I-strain mice retained activity towards troponin I. If the same situation prevailed in hearts from I-strain mice, the interpretation of the results above could be erroneous. However, cardiac troponin is a very poor substrate for phosphorylase kinase (Cole & Perry, 1975), and, as shown in Table 2, no phosphorylation of whole cardiac troponin was demonstrated under conditions that gave rapid phosphorylation of phosphorylase *b*. Also, the rate of troponin I phosphorylation found by Gross & Mayer (1973) in normal mice was considerably lower than that expected from the known activities of purified skeletal-muscle phosphorylase kinase (Stull *et al.*, 1972), suggesting that an enzyme other than phosphorylase kinase could have been phosphorylating troponin I (see also Moir *et al.*, 1977). It is therefore unlikely that, in the present study, the phosphorylation of troponin I in the hearts from I-strain mice could be attributed to a troponin-specific phosphorylase kinase activity.

The data in the present paper support the evidence of studies *in vitro* (Cole & Perry, 1975) that troponin I is phosphorylated by cyclic AMP-dependent protein kinase *in vivo*, in response to agents that increase cyclic AMP concentrations. Phosphorylation of troponin I has been identified in heart muscle in response to β -adrenergic stimulation in four different species (England, 1975, 1976; Solaro *et al.*, 1976; Ezrailson *et al.*, 1977; the present paper) and would appear to be a general phenomenon. Its exact role is unclear (Ray & England, 1976; England, 1976)

but it is very probable that it plays an important part in the modulation of cardiac contractility by catecholamines.

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