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Cardiac function and phosphorylation of contractile proteins

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The primary regulation of cardiac contractility is probably through changes in the level of cytoplasmic free Ca^{2+}. In the stimulation of contraction by catecholamines, secondary controls may be present at the level of the contractile proteins. Troponin-I, a subunit of the troponin complex of the thin filament, and C-protein, a thick filament component, are both phosphorylated in perfused hearts in response to catecholamines over time courses similar to that for the increase in contraction. Both proteins are also phosphorylated rapidly *in vitro* by cyclic-AMP-dependent protein kinase. Phosphorylation of troponin-I causes a decrease in the sensitivity of both cardiac myofibrillar ATPase and tension development of skinned fibre preparations to Ca^{2+}, and also an increase in the rate of dissociation of Ca^{2+} from isolated troponin. These results support the hypothesis that the role of phosphorylation of cardiac troponin-I is to contribute to the increased rate of relaxation of the heart that is observed with catecholamines. C-protein is phosphorylated to a maximum of 4–5 mol phosphate per mole protein both *in vivo* and *in vitro*. At present, however, the functions of both C-protein itself and its phosphorylation are unknown.

Dephosphorylation of these contractile proteins after catecholamine stimulation is slow in perfused heart, although the rate can be increased by cholinergic agents. Phosphorylase, in contrast, is rapidly dephosphorylated under these circumstances. Phosphoprotein phosphatases relatively specific for phosphorylase have been identified in rat heart, whereas troponin-I appears to be dephosphorylated by general phosphatases. These observations account for the different rates of dephosphorylation of phosphorylase and the contractile proteins, but do not explain the slow dephosphorylation of the latter.

In control perfused hearts myosin P-light chain was 50 % phosphorylated, and this was not changed by perfusion with positive inotropic agents or by short-term ischaemia. It was also unchanged during long-term hormonal modifications. Perfusions with $^{32}P_i$ in rat heart give a half-time for the turnover of phosphate bound to the P-light chain of 2–4 min, showing that the myosin light chain kinase and phosphatase are active in the heart. It is hypothesized that under control conditions the kinase is already fully active, and that an increase in cytoplasmic Ca^{2+} cannot therefore cause further activation of the enzyme.

Introduction

Many hormones and pharmacological agents cause increases in the rate and extent of tension development in the heart. The primary regulation is most probably through changes in the concentration of cytoplasmic Ca^{2+}, controlled by the rates of transport of Ca^{2+} across the sarcolemmal and sarcoplasmic reticulum membranes (see Haiech & Demaille, this symposium). However, regulation may occur at the level of the contractile proteins through modification by phosphorylation. This is particularly so with those agents that elevate levels of adenosine $3':5'$-monophosphate (cyclic AMP). Catecholamines cause both an increase in maximum tension development and an increase in the rate of relaxation of the heart. Regulation of Ca^{2+} fluxes by membrane protein phosphorylation is clearly important in this effect (Haiech & Demaille, this symposium), but in addition there is phosphorylation of contractile proteins,

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which has a significant role in the response of the tissue to the hormone. Because catecholamines increase cytoplasmic concentrations of cyclic AMP and Ca^{2+}, there is the possibility of phosphorylation of proteins by both cyclic-AMP-dependent and Ca^{2+}-dependent protein kinases.

Contractile proteins that are phosphorylated may be divided into two categories. The first consists of those proteins that are rapidly phosphorylated and dephosphorylated *in vivo*, or whose phosphate groups are being turned over rapidly. Troponin-I, C-protein and myosin P-light chain are the three major contractile proteins in this group, and will be discussed below. The second category includes proteins in which the phosphate groups is metabolically inactive, at least over short time intervals. In many cases the phosphate group is probably incorporated either during or immediately after protein synthesis, and remains attached until the protein is degraded. Troponin-T is an example of this type of phosphorylated protein. Such phosphorylations obviously cannot participate in the acute regulation of contraction, and will not be discussed further in this paper.

PHOSPHORYLATION OF TROPONIN-I

Three subunits, troponin-C, troponin-I and troponin-T, make up the troponin complex, the function of which is to confer sensitivity to Ca^{2+} on striated muscle actomyosin (Ebashi 1966; Greaser & Gergely 1971). Cardiac troponin was shown to be a good substrate for cyclic-AMP-dependent protein kinase (cyclic AMP-PK) when present either as the isolated complex (Reddy *et al.* 1973; Cole & Perry 1975) or in intact myofibrils (Ray & England 1976). The phosphate was incorporated mainly into a specific serine residue (Ser-20) on troponin-I (Moir & Perry 1977), although phosphorylation of other sites on troponin-I was also observed. The K_m for troponin-I was 20 μM, and the V_{max} was 11 μmol phosphate transferred per minute per milligram of kinase (Blumenthal *et al.* 1978). These rates are comparable with those obtained with other physiological substrates.

Phosphorylation of troponin-I in perfused hearts in response to catecholamines has been shown in a number of species (England 1975, 1976, 1977; Solaro *et al.* 1976; Stull 1980). In control perfusions the level of troponin-I phosphorylation was 0.15–0.3 mol phosphate per mole troponin-I, but after maximal stimulation with catecholamine this increased to 1–1.5 mol per mole. The increase in phosphorylation was mainly into Ser-20 (Moir *et al.* 1980), and occurred over a similar time course to the increase in contractile force. If positive inotropic agents, which do not increase cyclic AMP levels, were used, there was no increase in troponin-I phosphorylation (Solaro *et al.* 1976; Ezrailson *et al.* 1977). Phosphorylation of troponin-I is therefore not obligatory for increased contraction, but is a specific response to increases in intracellular cyclic AMP.

The effect of phosphorylation of troponin-I is to decrease the affinity of the troponin complex for Ca^{2+}. With isolated cardiac myofibrils the effect of troponin-I phosphorylation was to double or treble the concentration of Ca^{2+} required for half-maximal activation of the actomyosin ATPase (Ray & England 1976; Reddy & Wyborny 1976; Bailin 1979; Holroyde *et al.* 1979*a*). A similar increase in the Ca^{2+} concentration required for development of half-maximal tension was also seen in skinned cardiac fibre preparations (McClellan & Winegrad 1980; Mope *et al.* 1980; Herzig *et al.* 1981). Using isolated troponin, Robertson *et al.* (1982) showed that phosphorylation of troponin-I decreased the affinity constant for Ca^{2+} binding, and that

this was a result of an increased rate of dissociation of Ca^{2+} from the complex. The half-time of dissociation was 50 ms when the troponin-I was dephosphorylated, and 30 ms when phosphorylated.

The importance of this change in the rate of dissociation is in its relation to the increased rate of relaxation of the heart in the presence of catecholamines. As shown in figure 1, the relaxation rate of the heart may be a function of both the rate of Ca^{2+} release from troponin
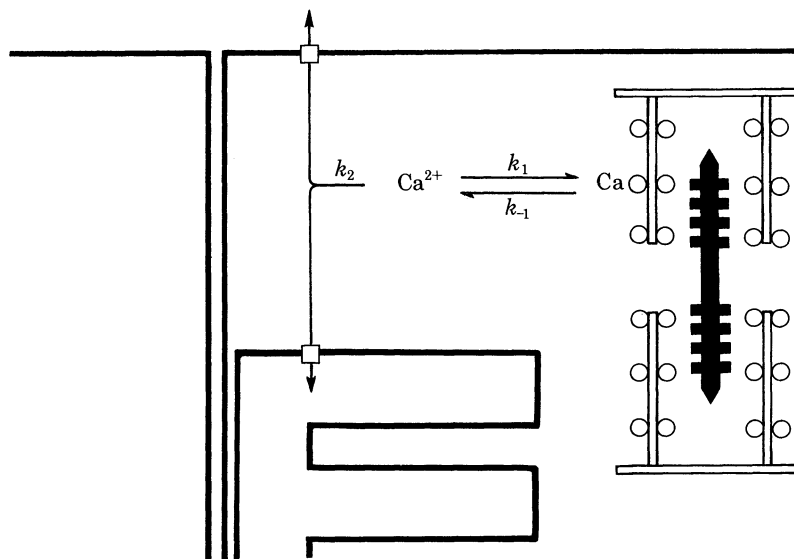

FIGURE 1. Schematic diagram showing the dissociation of Ca^{2+} from troponin, and its removal from the cytoplasm, during relaxation in the heart.

(k_{-1}) and the rate of removal of Ca^{2+} from the cytoplasm (k_2), depending on which is the slowest step in the sequence. After stimulation with catecholamines there is an increase in the concentration of cytoplasmic Ca^{2+} and in the amount of Ca^{2+} bound to troponin. As discussed by Haiech & Demaille (this symposium), mechanisms involving phosphorylation of membrane proteins are known that are thought to increase k_2. If k_2 is increased sufficiently, the possibility exists of k_{-1} becoming rate-limiting in the relaxation process. It is therefore advantageous for there to be an increase in k_{-1} in these circumstances, to allow for an increase in the overall rate of relaxation of the heart.

An unexplained observation in relation to troponin-I phosphorylation is that after stimulation of the heart with catecholamine there is a very slow dephosphorylation of troponin-I (figure 2) (England 1976; Stull *et al.* 1981). This is in contrast to the rapid dephosphorylation of phosphorylase under the same conditions. Dephosphorylation of troponin-I can, however, be stimulated by perfusion with cholinergic agents (England 1976). In rat heart we have identified five phosphoprotein phosphatase activities that show different degrees of specificity for protein substrates (D. Mills & P. J. England, unpublished observations). Figure 3 shows the separation obtained with DEAE-Sepharose. There are two peaks of activity that appear to be relatively specific for phosphorylase a, and two or three that have a much broader spectrum of substrates. All these phosphatases have molecular masses of 170–350 kDa. By including Mn^{2+} in all the buffers, disaggregation of all the phosphatase fractions to 60 kDa forms occurred. Interestingly, a very similar pattern of five separate phosphatases with different specificities was observed

after DEAE-Sepharose chromatography of the Mn^{2+}-treated enzyme. This suggests that the large molecular mass forms observed are separate enzymes, rather than merely different aggregation states.

The presence of different phosphatase activities towards phosphorylase and troponin-I in the heart explains the different rates of dephosphorylation of these proteins after stimulation by catecholamine. At present, however, we have no explanation of why the phosphatase activity towards troponin-I is generally low in these circumstances, nor a mechanism for its
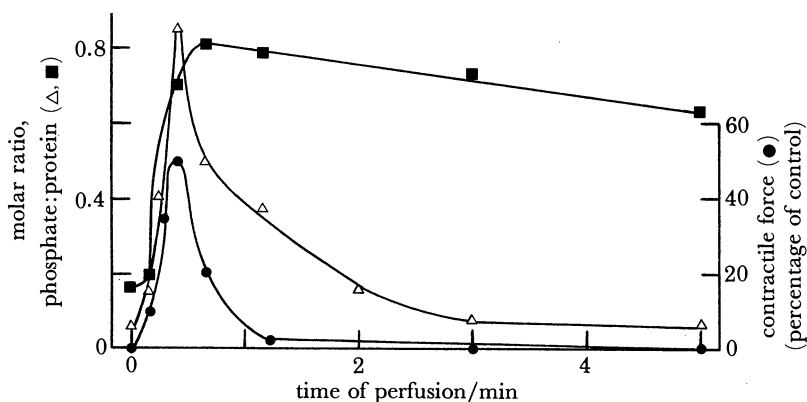

FIGURE 2. The slow dephosphorylation of troponin-I in perfused rat heart after a pulse of adrenalin. Rat hearts were pre-perfused with $^{32}P_i$ as described by England (1975), and given a 15 s pulse of 10^{-6} M adrenalin at time zero. Hearts were freeze-clamped at the times shown, and troponin-I phosphorylation measured either after purification of troponin-I (England 1975) or by radioautography after polyacrylamide gel electrophoresis of whole heart extracts (Jeacocke & England 1980a). The amount of phosphorylase in the a form was measured as described by England (1976). ■, Troponin-I phosphorylation; △, phosphorylase a; ●, contractile force.

stimulation after perfusion with cholinergic agents. There is some evidence that cyclic GMP may be involved in this latter response (England 1976; McClellan & Winegrad 1980; Hartzell & Titus 1982), although we have not found a direct effect of cyclic GMP on any of the phosphatase activities so far identified. It is unlikely that the phosphorylated phosphatase inhibitor-1 is implicated, as it does not appear to be present in rat heart (D. Mills & P. J. England, unpublished observations; J. G. Foulkes & P. Cohen, personal communication).

PHOSPHORYLATION OF C-PROTEIN

C-protein is a component of the thick filament that is located every 43 nm along the filament (cf. myosin spacing of 14.3 nm) (Craig & Offer 1976). In heart the protein has a molecular mass of 150 kDa, compared with 140 kDa in fast white skeletal muscle (Starr & Offer 1971; Offer et al. 1973; Yamomoto & Moos 1981). Its function is not known, although both structural and regulatory roles have been proposed (Offer et al. 1973; Moos et al. 1975, 1978; Starr & Offer 1978; Moos & Feng 1980; Miyahara & Noda 1980). Because of the 43 nm spacing of C-protein, however, not all the myosin molecules will interact with C-protein in an identical manner.

The first observation of phosphorylation of C-protein was made when isolated beef cardiac myofibrils were incubated with [γ-^{32}P]ATP and cyclic AMP-PK (Ray & England 1976), although C-protein was not identified as such at the time. ^{32}P was incorporated into two

proteins only, troponin-I and a protein of molecular mass 150 kDa. Prior treatment with phosphoprotein phosphatase increased ^{32}P incorporation into both proteins, indicating the presence of endogenous phosphate. Subsequently we have identified the 150 kDa protein as C-protein (Jeacocke & England 1980a), and have studied phosphorylation of the pure protein (H. T. Pask & P. J. England, unpublished observations). As isolated from beef heart, C-protein contains 2–3 mol phosphate per mole of C-protein. Incubation with cyclic AMP-PK and ATP increases the total phosphate content to 4–5 mol per mole. As shown in table 1, C-protein is an excellent substrate for cyclic AMP-PK. Because the C-protein used in these experiments was already partly phosphorylated, the use of dephosphorylated C-protein should give even more rapid rates of phosphorylation.
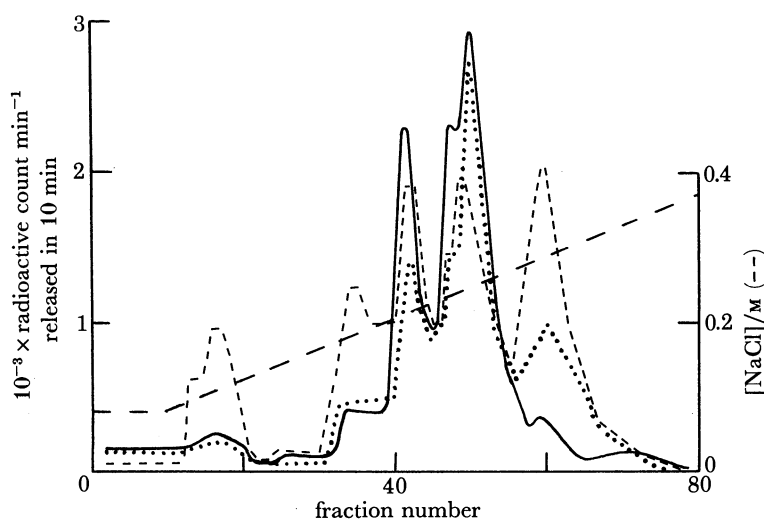

FIGURE 3. The separation of phosphoprotein phosphatases from rat heart on DEAE-Sepharose. A low-speed supernatant from a homogenate of rat heart was chromatographed on Sephacryl-200. The breakthrough peak, containing all the phosphoprotein phosphatase activity but no alkaline phosphatase, was chromatographed on DEAE-Sepharose in 25 mM Tris, pH 7.5, 15 mM 2-mercaptoethanol, 10 % (by volume) ethanediol, 80 mM NaCl. The column was developed with a linear gradient of NaCl from 0.08 to 0.4 M. Phosphoprotein phosphatase activity was assayed by ^{32}P$_i$ release from ^{32}P-labelled phosphorylase a (– – –), histone H$_1$ (——) and cardiac troponin ($\cdots$) (labelled on troponin-I). All substrates were initially phosphorylated with [γ-^{32}P]ATP of the same specific radioactivity, and used at approximately the same ^{32}P concentration.

C-protein has also been shown to be phosphorylated in perfused rat and frog hearts (Jeacocke & England 1980a; Hartzell & Titus 1982) and in isolated myocytes (Onorato & Rudolph 1981) in response to catecholamine. In control perfused rat hearts the level of C-protein phosphorylation was approximately 1 mol phosphate per mole of protein, which increased to approximately 5 mol per mole after 30 s of catecholamine (Jeacocke & England 1980a). The phosphorylation of C-protein and troponin-I followed almost identical time courses, suggesting a common phosphorylating enzyme, namely cyclic AMP-PK. C-protein was not phosphorylated in heart when contractility was increased by raising the extracellular Ca^{2+} concentration, nor was it phosphorylated in intact skeletal muscle in the presence of catecholamine (S. A. Jeacocke & P. J. England, unpublished observations). These last two observations indicate that, as with troponin-I, phosphorylation of C-protein is a specific response in heart to elevated cyclic AMP concentrations.

The function of C-protein phosphorylation is not known at present. The lack of any clear

indication as to the function of C-protein itself makes it difficult to suggest what properties may be altered by phosphorylation. Because there are thought to be three C-protein molecules at each 43 nm-spaced location, when the protein is fully phosphorylated *in vivo* there will be 12–15 phosphate groups in a highly localized region. At pH 7 this will mean an increase of *ca.* 20 negative charges after catecholamine stimulation. Although a direct charge effect on adjacent myosin molecules is unlikely, a profound conformational effect is to be expected. Preliminary experiments showed that C-protein phosphorylation did not affect the steady-state V_{max} rate of cardiac actomyosin ATPase, and experiments investigating more subtle parameters of actin–myosin interaction are obviously required.

TABLE 1. RATES OF PHOSPHORYLATION OF ISOLATED PROTEINS BY THE CATALYTIC SUBUNIT OF CYCLIC-AMP-DEPENDENT PROTEIN KINASE

substrate	concentration mg ml^{-1}	K_m mg ml^{-1}	rate of phosphorylation μmol min^{-1} mg^{-1} kinase
histone H$_1$	1.0	1.1	3.0
troponin-I†	4.0	3.6	11.0
C-protein	1.0	unknown	20.0

† As troponin complex.

MYOSIN P-LIGHT CHAIN PHOSPHORYLATION

As discussed elsewhere (Perry, this symposium), all muscle (and non-muscle) myosins contain a low molecular mass component, the P-light chain (P-LC), which can be phosphorylated by a specific, Ca^{2+}-dependent myosin light chain kinase (MLCK), and dephosphorylated by myosin light chain phosphatase. Recently a number of studies with skinned fibre preparations or cross-linked myofibrils have shown that in fast-twitch skeletal muscle, phosphorylation of the P-LC causes a decrease in the rate of ATPase at any given tension (Crow & Kushmerick 1981; Cooke *et al.* 1982). This is equivalent to a decrease in the rate of cross-bridge cycling, and makes the kinetics of the phosphorylated fast-twitch myosin similar to those of slow-twitch myosin. This effect of phosphorylation has also been observed in cardiac muscle (Ruegg *et al.* 1983), although dephosphorylated cardiac myosin already has a relatively slow rate of ATP hydrolysis.

Many studies have been made of the levels of P-LC phosphorylation in perfused heart. Most of these studies have found that in control perfusions there is 0.4–0.5 mol phosphate per mole of P-LC, and that this is unchanged during acute increases in contractility (Holroyde *et al.* 1979*b*; Perry *et al.* 1979; Jeacocke & England 1980*b*; High & Stull 1980). It is also unchanged in hearts from animals with altered cardiac myosin isoenzyme patterns (Litten *et al.* 1981; P. J. England, unpublished observations). The lack of increase in P-LC phosphorylation under conditions where an increased cytoplasmic Ca^{2+} concentrations would be expected to activate MLCK has been explained by Stull *et al.* (1981) in terms of the relative rates of activation and inactivation of MLCK. The rates of Ca^{2+} binding to calmodulin, and Ca$_4$-calmodulin binding to MLCK, are fast, whereas the dissociation of Ca$_4$-calmodulin from the complete active kinase complex is slow ($\tau_{\frac{1}{2}} = 1.3$ s). Therefore in hearts beating at frequencies of 2–5 Hz there would be insufficient time for the enzyme complex to dissociate during each diastole, and the enzyme would remain fully active even in control perfused hearts. Any increase in cytoplasmic Ca^{2+} would be unable to activate the kinase further.

Some evidence for this has come from calculations of the rates of MLCK and P-LC

phosphatase activities expressed in intact perfused heart. Perfusions of rat hearts with $^{32}P_i$ and measurement of rates of ^{32}P incorporation into P-LC (Jeacocke & England 1980b) permits calculation of phosphate turnover, and hence rates of MLCK activity. Values of 3–6 nmol min^{-1} g^{-1} heart were obtained, which should be compared with the total extracted MLCK activity of beef heart of approx. 5 nmol min^{-1} g^{-1} (Wolf & Hofmann 1980). Provided that the levels of MLCK are similar in beef and rat heart, these calculations support the hypothesis of Stull *et al.* (1981) that the MLCK is fully active in control hearts.

Some results have been published on the levels of P-LC phosphorylation in perfused heart that are not in agreement with those given above. Thus Kopp & Barany (1979) and Resink *et al.* (1981a, b) both found low levels of phosphorylation in control perfusions (0.07–0.1 mol phosphate per mole of P-LC), and increases of 1.5 to 3-fold after several minutes with catecholamine. The reasons for the discrepancies are not obvious. However, if phosphorylation of P-LC were occurring on stimulation with catecholamine, then according to the results of Ruegg *et al.* (1983), this would result in a decreased rate of cross-bridge cycling in the heart. However, this is not compatible with the increased rate of tension development observed in catecholamine-stimulated hearts. The studies from most laboratories indicate no change in the level of P-LC phosphorylation during acute changes in cardiac contraction induced by many different agents. It is therefore unlikely that phosphorylation of P-LC has a role in the acute regulation of cardiac contractility.

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