Phosphorylation of Myosin Light Chains in Perfused Rat Heart

EFFECT OF ADRENALINE AND INCREASED CYTOPLASMIC CALCIUM IONS

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1. A method was developed for the isolation of essentially pure myosin light chains from perfused rat heart. The phosphorylation of the P-light chains was estimated by hydrolysis and measurement of phosphate released, by electrophoresis in 8M-urea and by ^{32}P incorporation in perfusion with $[\text{}^{32}\text{P}]\text{P}_i$. 2. In control perfusions there was 0.5–0.6 mol of phosphate/mol of P-light chain. This was not changed by perfusion with 5 μM -adrenaline for 10–40 s. Perfusion for 1 min with medium containing 7.5 mM- CaCl_2 , or for 30 s with medium containing 118 mM-KCl, also did not change the phosphorylation of P-light chains. 3. It is concluded that phosphorylation of P-light chains is not important in mediating the action of inotropic agents in the heart.

Mammalian myosin contains two classes of low-molecular-weight proteins, the light chains, in addition to the high-molecular-weight component. One class of these light chains is easily dissociated from the myosin molecule, and was previously known as DTNB-light chain or LC_2 (Weeds & Frank, 1973). However, this light chain has been shown to be phosphorylated by a specific light-chain kinase in all mammalian muscle types, and has been named the P-light chain (Perrie *et al.*, 1973; Pires *et al.*, 1974; Frearson & Perry, 1975; Frearson *et al.*, 1976a). The light-chain kinase is activated by Ca^{2+} (Pires *et al.*, 1974), and has been shown to be composed of a catalytic subunit and a regulatory subunit that is identical with the calcium-binding protein calmodulin (Dabrowska *et al.*, 1977; Yazawa & Yagi, 1977; Yazawa *et al.*, 1978). In addition all muscles contain a specific light-chain phosphatase (Morgan *et al.*, 1976) that removes phosphate from the P-light chain. At present, however, the significance of phosphorylation of the P-light chain in mammalian striated muscle is unclear. It was originally reported that phosphorylation had no effect on ATPase activity of skeletal-muscle myosin alone, or in the presence of actin (Morgan *et al.*, 1976). More recent reports have indicated increases in actin-activated myosin ATPase of 20–60% on phosphorylation of P-light chains (Pemrick, 1976; Perry *et al.*, 1979).

Phosphorylation of P-light chain has been shown to occur *in vivo* in both skeletal and cardiac muscle. In unstimulated skeletal muscle of frog and rabbit there was approx. 0.5 mol of phosphate/mol of P-light chain, which increased to 0.9 mol/mol after 30 s of tetanus (Bárány & Bárány, 1977; Stull &

High, 1977). In rat skeletal muscle there was an increase in the phosphate content of P-light chain from 0.1 mol/mol to 0.7 mol/mol after a 1 s tetanus (Manning & Stull, 1979). However, this increase occurred subsequent to the period of tetanus, and suggested that phosphorylation of the P-light chain was not an essential part of the contractile process.

In perfused rabbit heart it was reported that in control perfusions the P-light chain was fully phosphorylated (1 mol of phosphate/mol of P-light chain) and that adrenaline caused a 50% decrease in the phosphate content (Frearson *et al.*, 1976b). As adrenaline causes an increase in cardiac intracellular Ca^{2+} (Van Winkle & Schwartz, 1976), which would be expected to increase the activity of the light-chain kinase, this result was rather surprising. We have therefore re-investigated the phosphorylation of P-light chain in perfused heart under a number of conditions that increase contractility. A preliminary report of some of these findings has been presented previously (Jeacocke & England, 1980).

Methods and Materials

Heart perfusions

Hearts from Wistar rats (250–300 g) were perfused by the Langendorff technique with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 11 mM-glucose, gassed with O_2/CO_2 (19:1), at a pressure of 7 kPa. After preperfusion for 10 min the hearts were perfused for various times up to 1 min with either control medium, or medium modified as described in the Results section before being freeze-clamped (Wollenberger *et al.*, 1960). Perfusions with $[\text{}^{32}\text{P}]\text{P}_i$ were performed as described

by England (1975). Changes in contractility were measured by a type 4-422 pressure transducer (Bell and Howell, Basingstoke, Hants., U.K.) connected to the perfusion cannula and an MX-2 recorder (Devices Instruments, Welwyn Garden City, Herts., U.K.).

Frozen hearts were powdered under liquid N₂ in a mortar and pestle and stored at -40°C until extraction.

Purification of P-light chain from perfused hearts

This was modified from the method described by Frearson *et al.* (1976b). All procedures were carried out at 0–4°C. Each heart was homogenized in 10 ml of 5 M-guanidinium hydrochloride, 100 mM-glycine, 5 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride, 15 mM-2-mercaptoethanol, pH 1.5, centrifuged at 10000g for 20 min and the supernatant was dialysed against the same buffer for 3 h. The dialysed extract was neutralized with KOH and diluted with an equal volume of water and 4 vol. of ethanol. The precipitated protein was removed by centrifugation and the supernatant was dialysed overnight against 1 M-trichloroacetic acid. The precipitate was collected by centrifugation, redissolved in 2 ml of 0.1 M-NaOH and dialysed against 4 M-urea/10 mM-4-morpholinopropanesulphonate (MoPS)/60 mM-KCl/15 mM-2-mercaptoethanol (pH 7.2) (buffer A). The dialysed extract was fractionated by chromatography on a column (5 cm × 1 cm) of DEAE-cellulose equilibrated with buffer A. The column was washed with 30 ml of buffer A, and a crude light-chain fraction was eluted with 25 ml of buffer A containing an additional 50 mM-KCl. The protein was precipitated with 1 M-trichloroacetic acid, redissolved in 2 ml of 0.1 M-NaOH and dialysed against 4 M-urea/20 mM-acetate/20 mM-KCl/15 mM-2-mercaptoethanol (pH 5.4). Further purification was obtained by chromatography on a column (2.5 cm × 1 cm) of CM-cellulose equilibrated with the same buffer. The unbound fraction contained P-light chain accompanied by a certain proportion of the alkali or A-light chain (Weeds & Frank, 1973). The only other contaminating protein was a protein of apparent mol.wt. 17000 (see the Results and Discussion section).

Preparation of proteins

Myosin was prepared from rat skeletal muscle by the method of Uchida *et al.* (1977). A light-chain fraction (containing A- and P-light chains) was prepared from myosin by the method of Perrie & Perry (1970). Myosin light-chain kinase was prepared as described by Pires & Perry (1977).

Phosphorylation of the P-light chain was carried out in the presence of [γ -³²P]ATP as described by Pires & Perry (1977). Approx. 0.8 mol of ³²P was incorporated/mol of P-light chain.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the method of Laemmli (1970). Protein was stained with Coomassie Brilliant Blue, and the relative amounts of protein were estimated by densitometric scanning. The P- and A-light chains were assumed to be equally stained by Coomassie Blue (Fenner *et al.*, 1975).

Polyacrylamide-gel electrophoresis in the presence of 8 M-urea was performed as described by Perrie & Perry (1970). The relative proportions of proteins in each band were estimated as described above.

Analytical methods

[γ -³²P]ATP specific radioactivity in perfused hearts was estimated as described in England & Walsh (1976). Total protein-bound phosphate was estimated in purified light-chain fractions by hydrolysis in 2.5 M-NaOH (Meisler & Langan, 1969), followed by measurement of released phosphate by the method of Itaya & Ui (1966). Protein was measured by the method of Lowry *et al.* (1951).

Materials

Urea (as 8 M solution) was deionized before use by treatment with Amberlite monobed resin MB-3 (BDH Chemicals, Poole, Dorset, U.K.) and stored at 4°C. [γ -³²P]ATP was prepared as described by England (1979). [³²P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Biochemicals were obtained from Boehringer Corporation, London, U.K. DEAE- and CM-cellulose were from Whatman, Maidstone, Kent, U.K. All other chemicals were obtained either from BDH Chemicals, or Sigma (London) Chemical Co., Poole, Dorset, U.K.

Results and Discussion

Purification of light-chain fraction

Initial attempts to prepare a pure light-chain fraction were made by using the method of Frearson *et al.* (1976b), in which hearts were homogenized in 8 M-urea buffered at pH 8.0, unwanted protein was removed by precipitation with ethanol and the light chains were obtained by chromatography on DEAE-cellulose. However, this method proved to be unsuitable for use with rat heart. During the initial homogenization and dialysis in 8 M-urea there was extensive proteolysis of the P-light chain. This was investigated by adding ³²P-labelled P-light chains to non-radioactive hearts during the initial homogenization. Proteolysis was followed by loss of ³²P from the material precipitated with 1 M-trichloroacetic acid. It was found that more than 90% of the ³²P would not precipitate after 4 h of dialysis at 4°C. This was probably not due to removal of ³²P as P_i by

a phosphatase during dialysis. It was shown that the ^{32}P not precipitated by trichloroacetic acid was not present as P_i , as after treatment of the supernatant with triethylamine hydrochloride/ammonium molybdate (Sugino & Miyoshi, 1964), which selectively precipitates P_i , all the ^{32}P remained in solution.

A number of modifications were made to the homogenization buffer to inhibit the proteolytic activity. Addition of 0.1 mM-phenylmethanesulphonyl fluoride resulted in 40% of the ^{32}P in the P-light chain being recovered in the trichloroacetic acid-precipitated material. Reduction of pH to 1.5 (+0.1 mM-phenylmethanesulphonyl fluoride), however, gave 100% recovery of ^{32}P in the trichloroacetic acid-precipitated material. Homogenization and subsequent dialysis were therefore carried out at pH 1.5.

The presence of a proteinase that retained a relatively high activity in 8 M-urea was surprising. Apart from general proteinases, such as cathepsins, the presence of a proteinase that appears to be relatively specific for the P-light chain has been reported in heart muscle (Uchida *et al.*, 1977; Murakami & Uchida, 1978). In addition, rat heart is especially rich in proteinases that are active towards muscle protein at pH 8–9 (Drabikowski *et al.*, 1977). The stability of any of these proteinases in 8 M-urea is not known. However, it is apparent that at least one of them retains sufficient activity to hydrolyse most of the P-light chain in a few hours, even at 4°C. The

P-light chain will also be in a denatured state, which may also make it more susceptible to proteolysis (Hilz & Fanick, 1978). This proteolytic activity does not act equally on all proteins, however. There was a selective loss of P-light chain relative to A-light chain during dialysis. In addition, troponin-I was not hydrolysed during dialysis for 15 h under the same conditions (England, 1976). This proteolysis may be particularly marked in rat heart (Drabikowski *et al.*, 1977), and was not reported in rabbit heart (Frearson *et al.*, 1976b).

The original homogenization medium of Frearson *et al.* (1976b) was further modified by the substitution of 5 M-guanidinium HCl for 8 M-urea, as this was found to give a better recovery during the subsequent ethanol precipitation (Holt & Lowey, 1975). The homogenization medium finally used is described in the Methods and Materials section. After dialysis, unwanted protein was removed by precipitation with ethanol, and the light chains were precipitated with trichloroacetic acid. Recovery of P-light chain at this stage was 80%, as measured by the addition of ^{32}P -labelled P-light chain.

Chromatography on DEAE-cellulose gave a light-chain fraction that was still significantly contaminated with other proteins (Fig. 1b). Approx. 30% of the A-light chain was eluted in the initial wash (Fig. 1a), whereas all of the P-light chain was recovered in the elution with 110 mM-KCl (Fig. 1b). In the study by Frearson *et al.* (1976b) a fraction

Fig. 1. Polyacrylamide-gel electrophoresis and radioautography of light-chain fractions

Light-chain fractions were subjected to electrophoresis on 12.5% (w/v) polyacrylamide in the presence of sodium dodecyl sulphate (a–c) or on 10% (w/v) polyacrylamide in the presence of 8 M-urea (e). The proteins were detected by staining with Coomassie Brilliant Blue. Other details are given in the text. (a) Fraction eluted from DEAE-cellulose by 60 mM-KCl; (b) fraction eluted from DEAE-cellulose by 110 mM-KCl; (c) fraction obtained from CM-cellulose; (d) radioautograph of gel c; (e) fraction obtained from CM-cellulose; (f) radioautography of gel e. The positions of the P- and A-light chains are shown by P and A respectively. P1 indicates the position of the non-phosphorylated P-light chains, and P2 the phosphorylated form. C indicates the position of the 17000-mol.wt. contaminant.

that was eluted from DEAE-cellulose at a similar ionic strength was used to estimate the phosphate content of the P-light chain. However, the fraction obtained in the present study was considered too impure for total phosphate analysis. In particular, traces of troponin-I and other phosphorylated proteins were observed when this fraction was prepared from hearts perfused with [^{32}P]P_i and analysed by electrophoresis and radioautography.

Further chromatography on CM-cellulose gave a fraction that contained three proteins only (Fig. 1c). The P-light chain comprised $55 \pm 4\%$ (mean $\pm$ S.D.; $n = 20$) of the protein and the A-chain $41 \pm 5\%$. The only contaminant was an acidic protein of apparent mol.wt. 17000, which was present at approx. 5% of the amount of light chains. This fraction was used for the estimation of total phosphate in P-light chain. It was assumed that only the P-light chain contained protein-bound phosphate. This was tested by preparation of light chains from hearts perfused with [^{32}P]P_i. Fig. 1(d) shows that ^{32}P was present only in the P-light chain. If either of the other proteins contained phosphate that was not labelled with ^{32}P then the total phosphate present in the P-light chain would be overestimated. However, the A-light chain is not phosphorylated (Perrie *et al.*, 1973) and the 17000-mol.wt. contaminant would only affect the result significantly if it were phosphorylated at several sites.

Phosphorylation of P-light chain in perfused hearts

In hearts perfused with control medium there was 0.66 mol of phosphate/mol of light chain measured by hydrolysis of covalent bound phosphate (Table 1, column A). The amount of P-light chain in the phosphorylated form can be determined independently by polyacrylamide-gel electrophoresis in 8M-urea, which separates the phosphorylated and non-phosphorylated forms (Perrie *et al.*, 1973). A typical separation is shown in Fig. 1(e). The pattern is complicated by the presence of 'satellite' bands (Perrie *et al.*, 1973), which appear to be formed by

reaction of the light chains with contaminants in the urea solution used in their purification. The proportion of P-light chain in the phosphorylated form was found from the relative intensities of staining of the protein bands (including 'satellite' bands). Fig. 1(f) shows the incorporation of ^{32}P into the two bands of the phosphorylated form of the P-light chain isolated from hearts perfused with [^{32}P]P_i. Column B of Table 1 shows that in control perfusions the proportion of the P-light chain in the phosphorylated form was 0.47. This value is somewhat lower than that measured on the basis of total phosphate content (Table 1, column A). In general, Table 1 shows that measurement of total phosphate gave a slightly higher estimate of the phosphorylation of the P-light chain than that obtained from electrophoresis. However, this was probably due to problems of trace contamination of glassware, chemicals and protein samples with P_i, rather than to contamination of the purified light chains with another phosphorylated protein (see above). When hearts were perfused with [^{32}P]P_i for 15 min, the light chain was found to contain between 0.3 and 0.5 mol of ^{32}P /mol of P-light chain, a value approaching that obtained by electrophoresis.

When hearts were perfused with 5 μM -adrenaline for 30s there was an increase in contraction of 192%. However, the phosphorylation of the P-light chain was unchanged by the hormone (Table 1). The same result was obtained with hearts that had been perfused with 5 μM -adrenaline for 10–40s. Perfusion with [^{32}P]P_i for 15 min before addition of adrenaline for 30s gave a ^{32}P incorporation into the light chain that was very similar to that obtained in control perfusions. However, there was an increase in the ^{32}P content of troponin I (results not shown), indicating that the adrenaline was effective at an intracellular level (England, 1976).

Contraction was also increased by raising the extracellular Ca^{2+} to 7.5 mM (Table 1), but this caused no change in the phosphorylation of the

Table 1. Degree of phosphorylation of P-light chain in perfused rat heart

Light chains from hearts perfused under the conditions shown were purified as described in the Methods and Materials section. Column A: total phosphate was measured after alkaline hydrolysis of the light-chain fraction (Meisler & Langan, 1969) by the method of Itaya & Ui (1966). Column B: the proportion of P-light chain in the phosphorylated form was measured after polyacrylamide-gel electrophoresis in 8M-urea (Perrie & Perry, 1970) by densitometric scanning. All results are quoted as means $\pm$ S.D.

Perfusion condition	Change in contraction (%)	Total phosphate (mol/mol of P-light chain) (A)	Proportion of P-light chain in phosphorylated form (B)
Control	0	0.66 ± 0.14 (13)	0.47 ± 0.06 (27)
30s, 5 μM -adrenaline	192 ± 86 (14)	0.60 ± 0.11 (14)	0.51 ± 0.02 (11)
60s, 7.5 mM-CaCl ₂	61 ± 20 (9)	0.53 ± 0.14 (9)	0.46 ± 0.04 (6)
30s, 118 mM-KCl	(Cardiac arrest)	0.60 ± 0.18 (12)	0.50 ± 0.05 (5)

P-light chain. There was also no change in phosphorylation when the hearts were perfused with medium containing 118 mM-KCl/29 mM-NaCl (Table 1). A high-KCl medium causes depolarization of the cell membrane and is thought to increase intracellular Ca^{2+} (Bianchi & Shanes, 1959).

General discussion

These results show that in rat heart approx. 50% of the P-light chain is in the phosphorylated form, and that a number of interventions that increase intracellular Ca^{2+} do not change this value. This is in contrast with the results of Frearson *et al.* (1976b), who reported that in rabbit hearts perfused with control medium the P-light chain was fully phosphorylated, and that adrenaline caused a decrease in phosphorylation. However, a recent report from this group (Perry *et al.*, 1979) has cast doubt on the original observations of Frearson *et al.* (1976b), and the higher phosphate content reported in this study may have been due to contamination of the light-chain fraction with other phosphorylated proteins. A recent brief report (Meshkov *et al.*, 1979) also suggested that phosphorylation of the P-light chain could be changed in heart during alterations in contraction. However, the phosphate content reportedly approached 2 mol of phosphate/mol of P-light chain, whereas no other reports have found more than 1 mol of phosphate incorporated either *in vivo* or *in vitro*. This suggests that the light-chain fraction used by Meshkov *et al.* (1979) could also have been contaminated with a phosphorylated protein.

The results presented in this paper are in contrast with those reported for skeletal muscle, where increases in phosphorylation of P-light chain were associated with increases in intracellular Ca^{2+} (Bárány & Bárány, 1977; Stull & High, 1977; Bárány *et al.*, 1979; Manning & Stull, 1979). When skeletal muscle was placed in tetanus, there was an increase in the phosphate content of P-light chain. However, phosphorylation appeared to be much slower than the development of tension (Manning & Stull, 1979), suggesting that phosphorylation is not an obligatory step in the interaction of myosin with actin. The differences between heart and skeletal muscle may be related to the different concentrations of light-chain kinase and phosphatase in these muscles (Frearson *et al.*, 1976a). In heart the activity of the kinase is approx. one-twentieth of that found in skeletal muscle. The activity of the phosphatase is very similar in the two muscles, however, and in the heart there is more phosphatase activity than kinase. It may be that in control hearts there is sufficient intracellular Ca^{2+} to totally activate the kinase during each systole, and that the ratio of activities of the kinase and phosphatase *in*

vivo results in the P-light chain being 50% phosphorylated. Any increase in intracellular Ca^{2+} would then be unable to activate the kinase further, and as the phosphatase does not appear to be inhibited by Ca^{2+} (Morgan *et al.*, 1976), no change in phosphorylation would occur. During a 15 min perfusion with [^{32}P]P_i the amount of ^{32}P incorporated into the P-light chain was similar to the total phosphate incorporated. This indicates that the phosphate in the light chain is turning over at a reasonable rate during control perfusions, and that the kinase and phosphatase are both active. However, in view of the results of the present study, it is unlikely that phosphorylation of P-light chains is important in mediating the inotropic response of heart to adrenaline and other agents that increase intracellular Ca^{2+} .

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