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The effect of adrenaline on the phosphorylation of the P light chain of myosin and troponin I in the perfused rabbit heart

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1. Two-dimensional electrophoresis has been used to study the extent of phosphorylation of the P light chain of myosin and troponin I in the rabbit beating heart. 2. A procedure has been developed that eliminates endogenous protein phosphatase activity during homogenization and sample preparation for electrophoresis. 3. Evidence has been obtained for two unphosphorylated forms of the P light chain in myosin from the ventricle of the rabbit, guinea pig and cow. 4. *In vivo* and in the rabbit perfused beating heart about 25% of the P light-chain fraction is in the phosphorylated form. 5. Intervention with adrenaline produced a slight increase in the extent of phosphorylation that reached a maximum after the peak in inotropic response. A similar increase was obtained with ischaemia in the absence of adrenaline. 6. The changes in phosphorylation of the major forms of troponin I identified by electrophoresis occurred after the peak of response to adrenaline and were compatible with previous results.

The role of phosphorylation of the P light chain of myosin in the regulation of contraction of striated muscle is far from clear. Actin-activation of the Mg^{2+}-stimulated ATPase of myosin from skeletal (Morgan *et al.*, 1976) and from cardiac muscle (Perry *et al.*, 1979) can occur when the P light chain is completely dephosphorylated. In this respect myosin from striated muscle differs from some preparations of myosin from smooth muscle. Some slight increase in the Mg^{2+}-stimulated ATPase of cardiac actomyosin was observed on phosphorylation of the myosin (Perry *et al.*, 1979), whereas Pemrick (1980) has reported much larger increases of ATPase activity in skeletal actomyosin after phosphorylation.

The results of studies on the role of phosphorylation of myosin in intact muscle are likewise not entirely consistent. In the original studies on the state of phosphorylation of the P light chain in the perfused rabbit heart we reported results that suggested that the P light chain was fully phosphorylated in the normal beating heart and that dephosphorylation accompanied intervention with adrenaline (Frearson *et al.*, 1976). The studies were carried out by total phosphate determination on an isolated light-chain fraction, but re-investigation of these findings by electrophoretic separation of the light chains suggested that in control hearts the P light chain was partially phosphorylated and that little change occurred on intervention with adrenaline (Perry *et al.*, 1979). These latter results were confirmed and extended by Holroyde *et al.* (1979) by study of the state of P light-chain phosphorylation in myofibrils isolated from the perfused rabbit heart and by Jeacocke & England (1980) with the perfused rat heart. On the other hand, Kopp & Barany (1979) report that changes in the phosphorylation of the P light chain accompany changes in the contractile state of the rat myocardium produced by adrenaline.

The various results reported by different workers reflect the difficulties in isolating the P light chain of cardiac myosin free from other proteins and accurately determining its covalently bound phosphate. One of the contaminating proteins of similar molecular weight to the P light chain is known to be phosphorylated (Stull *et al.*, 1980) and probably was present in the light-chain fraction isolated by Frearson *et al.* (1976). Thus phosphate analyses on this fraction gave erroneous values for the P light chain. Most studies so far reported have relied on electrophoresis in one dimension to separate the P light-chain fraction by molecular weight. The extent of phosphorylation has then usually been estimated either by direct analyses or by determination of ^{32}P content of the whole fraction rather than direct isolation of the phosphorylated P light chain.

In contrast with the situation with the P light

Abbreviation used: SDS, sodium dodecyl sulphate.

chain, the phosphorylation of troponin I in cardiac muscle in response to intervention with adrenaline is well-documented and most workers agree that this results in a decreased sensitivity of the actomyosin ATPase to Ca^{2+} (for review, see Perry *et al.*, 1979).

In the present investigation we have used two-dimensional electrophoresis of homogenates of perfused rabbit hearts to separate the phosphorylated forms of the P light chain and troponin I from the unphosphorylated forms and thus determine unambiguously the extent of phosphorylation of these proteins. The study provides evidence for the presence of two forms of the P light chain in rabbit ventricle. In contrast with troponin I, which is phosphorylated in response to adrenaline, only a slight increase in phosphorylation of the P light chain occurred simultaneously in the same muscle.

Materials and methods

Materials

Bovine cardiac cyclic AMP-dependent protein kinase (type 1), cyclic AMP, acrylamide, glycine, sodium dodecyl sulphate and Tris were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. [γ-^{32}P]ATP was purchased as the freeze-dried ammonium salt from The Radiochemical Centre, Amersham, Bucks., U.K. Serva Blau R was obtained from Serva, Heidelberg, Germany. Ampholines were purchased from LKB Products, Croydon, Surrey, U.K. All other reagents were purchased either from Fisons, Loughborough, Leics., U.K., or from BDH Chemicals, Poole, Dorset, U.K.

Preparation of myofibrils

Myofibrils were prepared from the rabbit heart as described by Solaro *et al.* (1971).

Preparation of myosin

Dephosphorylated myosin was prepared from rabbit white skeletal and cardiac ventricular muscles by extraction of the minced tissue with 2 vol. of 0.6 M-KCl/15 mM-2-mercaptoethanol, adjusted to pH 7.0 with 1 M-NaOH as described by Pires *et al.* (1974). After two precipitations the myosin was redissolved in 0.5 M-KCl and centrifuged at 55000 ***g*** for 45 min at 4°C. The clear myosin solution was then used in the phosphorylation experiments.

Preparation of myosin light chains

The whole light-chain fraction of myosin was prepared from rabbit cardiac muscle by the guanidine hydrochloride/ethanol fractionation procedure described by Perrie & Perry (1970).

Perfusion of rabbit hearts

Hearts were removed rapidly from rabbits killed by stunning and, after washing for 10–15 s in ice-cold 0.9% NaCl, were perfused as described by Moir *et al.* (1980). When required, adrenaline was added to the perfusion medium to a final concentration of 4 μM and perfusion was continued for various times after adrenaline administration. The force of contraction was maximal approx. 20–25 s after the addition of adrenaline.

Preparation of hearts for analysis

After perfusion. Hearts were cut down and the ventricular muscle (approx. 3 g) was homogenized within 10 s in a small Waring blender-type homogenizer with 25 ml of 15% (w/v) trichloroacetic acid at 0°C. The precipitated protein was centrifuged and the pellet was resuspended in 30 ml of 9 M-urea. Drops of 1 M-NaOH were then added until the pH of the resulting protein solution was approx. 7. Any insoluble protein was removed by filtration through glass wool. All samples were kept at 0°C until they were applied to the pH 4–6 isoelectric-focusing gels. Samples to be run in parallel on the pH 3.5–11 gradient were kept at −20°C and were applied to the isoelectric-focusing gels on the following day.

After anaesthesia. Each rabbit was anaesthetized with a gaseous mixture of fluothane and O_2. Anaesthesia usually took place within 10 min of administering the gaseous mixture. The thorax was opened, the heart was removed while still beating, rapidly homogenized in ice-cold 15% (w/v) trichloroacetic acid and prepared for isoelectric focusing as described for perfused rabbit hearts.

Beef. Hearts from freshly killed cattle were obtained from the slaughterhouse, kept on ice and processed the same day. A portion of the ventricles was homogenized in 8 vol. of 9 M-urea/15 mM-mercaptoethanol for two-dimensional gel electrophoresis.

Mouse, rat, guinea pig and neonatal rabbit. Hearts from animals killed by stunning were removed and washed in ice-cold 0.9% NaCl. The ventricles were then homogenized in 8 vol. of 9 M-urea/15 mM-mercaptoethanol before two-dimensional gel electrophoresis.

Myosin light-chain kinase assays

Myofibrils (3 mg/ml), myosin (1.5 mg/ml) and whole myosin light-chain fraction (1 mg/ml) from rabbit cardiac-muscle or skeletal-muscle myosin (5 mg/ml) were incubated with a crude preparation of skeletal-muscle myosin light-chain kinase (0.25 ml/1.5 ml final assay volume) under the conditions described by Pires & Perry (1977). The kinase preparation used was the eluate obtained from DEAE-cellulose, step 2 of the procedure of Pires & Perry (1977). The reactions were terminated by the addition of solid urea (approx. 50 mg/0.1 ml) if the sample was to be subjected to

isoelectric focusing or 2.5ml of 10% (w/v) trichloroacetic acid followed by the addition of 0.1ml of 1% bovine serum albumin to 0.1ml of the original sample if incorporation of ^{32}P radioactivity was to be measured.

Phosphatase activity in cardiac homogenates

Homogenates were prepared either in 9M-urea or in 15% trichloroacetic acid at 0°C as described above. The urea-treated homogenates were centrifuged at 1000*g* for 2min at 4°C to remove insoluble material and those precipitated by trichloroacetic acid were dissolved in 9M-urea as described for perfused hearts.

To 1ml of the homogenate was added 0.25ml of ^{32}P-labelled skeletal-muscle whole light-chain fraction in 8M-urea and the whole incubated for $4\frac{1}{2}$h at 0°C. After various intervals 0.1ml samples were removed and added to 3ml of ice-cold 15% trichloroacetic acid. The resulting suspension was centrifuged at 1000*g* for 5min at 4°C and ^{32}P was determined in the supernatant. P_i and peptide phosphate in the supernatant were fractionated by the procedure of Sugino & Miyoshi (1964) and determined by ^{32}P estimations.

Determination of ^{32}P

Protein precipitates and acrylamide gels were prepared for ^{32}P determination by the Čerenkov method as described by Perry & Cole (1973, 1974).

Estimation of P_i

Orthophosphate was precipitated in samples by the addition of triethylamine hydrochloride and ammonium molybdate after the procedure described by Sugino & Miyoshi (1964).

Protein determination

Protein was assayed by a micro method involving precipitation by tannin (Mejbaum-Katzenellenbogen & Dobryszycka, 1959) with bovine serum albumin as standard.

Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing and gradient SDS/polyacrylamide slab-gel electrophoresis of the proteins was performed essentially as described by Anderson & Anderson (1978*a*,*b*), except that SDS was not present in the solution used to dissolve the samples. It was found that if more than 30μl of 1% (w/v) SDS were present in the samples not all the SDS was stripped off the proteins during isoelectric focusing and an anomalous two-dimensional gel profile was obtained (S. A. Westwood, unpublished work). To avoid any possible interference of SDS in the interpretation of the protein profiles it was decided to use urea as the solubilizing agent in these studies. Either solid urea or a solution of freshly deionized 9M-urea was used to dissolve the proteins. Freshly dissolved samples were used whenever possible and were kept at 0°C until they were applied to the isoelectric-focusing gels to decrease the risk of carbamoylation of the proteins. Normally 10–30μl of urea solution containing protein representing 0.9–2.7mg wet wt. of muscle respectively was applied.

Isoelectric focusing was performed over the pH range 4–6 to separate the components of the myosin light-chain fraction, whereas a combination of Ampholines covering the pH range 3.5–11 was used to study the nature of troponin I in cardiac extracts. Owing to the very high pI of troponin I, non-equilibrium pH-gradient electrophoresis gave the best resolution of this protein.

The relative amounts of the P light chains and the different forms of troponin I were determined by scanning one- and two-dimensional gels with a Zeineh soft-laser densitometer (supplied by T. & J. Crump, Rayleigh, Essex, U.K.). The laser beam was set up to be slightly wider than the maximum width of the protein band or spot to be scanned. Areas under the peaks were determined by cutting out the traces and weighing. The area under any one peak was related to the total area under all peaks produced either by troponin I or the P light-chain fraction.

Results

Investigation of the dephosphorylation of the P light chain of myosin during sample preparation

When homogenates of rabbit hearts in 8M-urea were immediately chilled in ice and stored at 0°C, significant dephosphorylation of added ^{32}P-labelled whole light-chain fraction of rabbit skeletal-muscle myosin was observed. Experiments showed that dispersion of cardiac-muscle homogenates in 8M-urea/50mM-Tris adjusted to pH7.4 with 1M-HCl/10mM-magnesium acetate decreased the endogenous phosphatase activity on ^{32}P-labelled whole light-chain fractions of skeletal-muscle myosin by 97%. Nevertheless this residual activity produced significant dephosphorylation of the P light chain on storage even at 0°C, e.g. usually after 4.5h at 0°C about 50% of the ^{32}P was liberated as P_i. Dephosphorylation of the P light chain was confirmed by electrophoretic analysis of samples withdrawn during the course of the incubation. On addition of cardiac-muscle homogenates in 8M-urea to phosphorylated skeletal myosin a similar dephosphorylation was observed. This did not occur in control experiments carried out in the absence of added cardiac-muscle homogenates. It was therefore concluded that the dephosphorylation observed was due to phosphatase activity in the urea

homogenates of the cardiac muscle. These observations confirm the reports of Stull *et al.* (1980), who found that 8M-urea did not completely inactivate myosin light-chain phosphatase in the rabbit heart homogenate. If initial homogenization of the cardiac muscle was carried out in 15% (w/v) trichloroacetic acid and the protein subsequently dissolved in 8M-urea at pH7.0 (see the Materials and methods section), no detectable dephosphorylation of added phosphorylated whole light-chain fraction of rabbit skeletal-muscle myosin could be observed. This procedure was therefore routinely adopted for determining the state of P light-chain phosphorylation in perfused hearts. In studies of myofibrils and isolated myosin the protein was prepared for electrophoresis by dissolving in 8M-urea. No significant breakdown of P light chain was observed, indicating that the endogenous phosphatase activity of these preparations was negligible.

Phosphorylation of the P light chain of rabbit cardiac myosin

When rabbit cardiac myosin, isolated under conditions in which it would not normally be expected to be phosphorylated (see the Materials and methods section), was subjected to isoelectric focusing three major bands were observed in that region of the pH gradient normally occupied by the light chains (Fig. 1*b*). These bands were provisionally identified in order of increasing negative charge as the A1 light chain and two forms of the unphosphorylated P light chain, P1 and P2, by comparison with the electrophoretic behaviour of an isolated whole light-chain fraction of rabbit cardiac myosin. In addition a number of minor bands, which were presumed from their mobilities to correspond to contaminating myofibrillar proteins such as actin, tropomyosin etc., were present in amounts that varied from preparation to preparation. The myosin heavy chains did not migrate into the gel under these conditions. In addition to bands identical in mobilities with light-chains A1, P1 and P2, other bands increased in amounts on storage and were presumed to represent modified forms of the light chains, the so-called 'satellite' bands that were observed on isoelectric focusing of isolated whole light-chain fractions (see Perrie & Perry, 1970; Frearson & Perry, 1975).

When rabbit cardiac myosin was incubated before electrophoresis with a preparation of skeletal-muscle myosin light-chain kinase the modification of the band pattern observed was restricted to band P1 and band P2. As the incubation proceeded, two new bands of greater negative charge appeared in increasing amounts. These were designated band P3 and band P4 (see Fig. 1). Densitometric measurements of the band patterns indicated that the

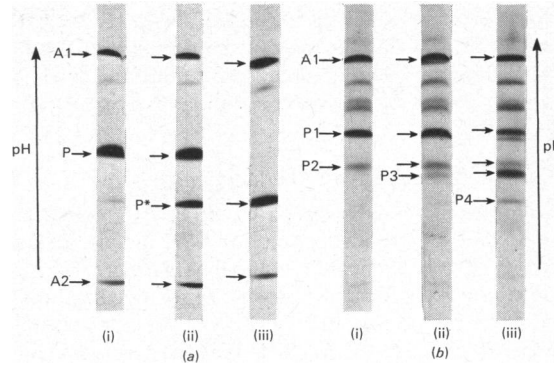

Fig. 1. *Effect of phosphorylation on the isoelectric-focusing pattern of the light chains of rabbit skeletal-muscle and cardiac myosins*

Dephosphorylated myosins incubated for the times indicated with partially purified rabbit skeletal-muscle myosin light-chain kinase as described in the Materials and methods section. The isoelectric-focusing gradient was in the pH range 4–6. A1 and A2 indicate alkali light chains. (*a*) 250μg of rabbit skeletal-muscle myosin: (i) zero time; (ii) 2min; (iii) 30min. P*, phosphorylated form of P, the P light chain. (*b*) 150μg of rabbit cardiac myosin: (i) zero time; (ii) 5min; (iii) 30min. P1, P2, P3 and P4 indicate the P light chains.

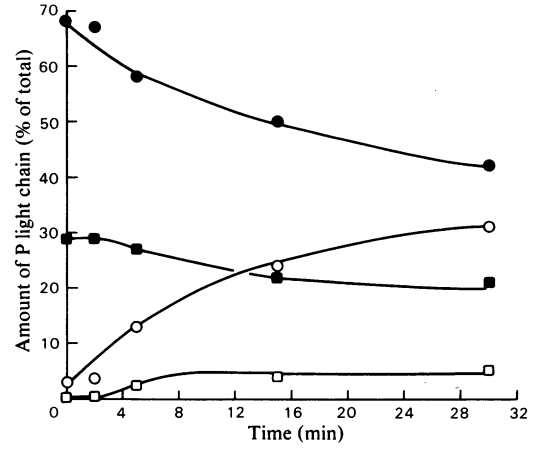

Fig. 2. *Changes in the amounts of the P light-chain components of rabbit cardiac myosin during incubation with myosin light-chain kinase*

Conditions of incubation and electrophoresis were as described in Fig. 1. Relative amounts of the P light chains were determined from densitometric tracings of the isoelectric focusing gels: ●, light-chain P1; ○, light-chain P3; ■, light-chain P2; □, light-chain P4.

Table 1. *Relative amounts of the P light-chain components in myosin and rabbit hearts perfused under various conditions*
Amounts of P light chains determined by densitometric scanning of isoelectric and two-dimensional gels. All hearts perfused for 20min before addition of 4 μM-adrenaline (see the Materials and methods section). Hearts homogenized in 15% trichloroacetic acid in series 1 and 2. Results are expressed as means ± S.E.M.

Condition	Number of experiments	Relative amounts of P light chains (% of total fraction) P1	P2	P3*	P4*	P1 + P3*	P3* + P4*
1. Anaesthetized rabbit hearts	4	59 ± 3	19 ± 2	18 ± 2	5 ± 1	77 ± 1	23 ± 3
2. Perfused hearts							
(*a*) Control	4	56 ± 2	19 ± 1	19 ± 2	6 ± 0.2	75 ± 1	25 ± 2
(*b*) + Adrenaline for 20–25 s: peak response	3	52 ± 3	20 ± 2	21 ± 2	7 ± 2	73 ± 3	29 ± 3
(*c*) + Adrenaline for 2 min	3	48 ± 2	22 ± 1	22 ± 2	8 ± 3	70 ± 4	30 ± 1
(*d*) + Adrenaline for 5 min	3	50 ± 2	17 ± 1	25 ± 1	8 ± 1	75 ± 2	33 ± 2
3. Hearts perfused ± adrenaline and homogenized in 8 M-urea	26	—	—	—	—	71 ± 1	—
4. Cardiac myosin at various extents of phosphorylation	10	—	—	—	—	72 ± 1	

* Phosphorylated forms.

amounts of band P1 and band P2 fell as the incubation progressed but the total amounts of band P1 and band P3 and the total amounts of band P2 and band P4 were constant (Table 1, Fig. 2). When incubations were carried out with [γ-^{32}P]ATP, estimations of radioactivity in the isolated bands cut out from the gels indicated that band P3 and band P4 contained ^{32}P. At early stages of incubation no radioactivity could be detected in band P1 or band P2. On prolonged incubation, slight radioactivity was detected in band P1, rising to about 15% of that present in band P3 after 30min [Fig. 1*b*(iii)]. Better resolution of bands P2 and P3 was obtained after isoelectric focusing than after two-dimensional electrophoresis. Apparently the electrophoresis in SDS caused some broadening of the sharp bands obtained on isoelectric focusing. Nevertheless, after two-dimensional electrophoresis the position of spot P3 was such to suggest that the protein had the same molecular weight as light-chain P1 and a slightly higher molecular weight than light-chain P2, although the spots were not completely resolved. It was concluded that components P1 and P2 were forms of the P light chain that were converted by myosin light-chain kinase into the phosphorylated forms P3 and P4 respectively.

When urea solutions of rabbit cardiac myofibrils were examined by isoelectric focusing, only band P1 and band P2 were observed. After the myofibrils were phosphorylated with myosin light-chain kinase, new bands corresponding to band P3 and band P4 were observed on electrophoresis as was the case with cardiac myosin.

Bands and spots corresponding to light-chain A1, P1, P2, P3 and P4 could also be observed on electrophoresis of homogenates of rabbit ventricular muscle. The identities of the components were confirmed by their co-migration with known samples of the light-chain fraction from partially phosphorylated cardiac myosin that were added to the system.

Relation of light-chain P1 to P2

In all cardiac P light-chain fractions studied the ratio of the amount of light-chain P1 + P3 to the amount of light chain P2 + P4 was remarkably constant (Table 1), i.e. light-chain P2 + P4 represented 28.6 ± 0.7% (mean ± S.E.M., $n = 48$) of the total myosin P light-chain fraction. This value was calculated from the collected results obtained with rabbit hearts treated in various ways, e.g. during anaesthesia, during perfusion in the presence or absence of adrenaline, in cardiac myofibrils and in cardiac myosin before and after phosphorylation. The proportion of the light-chain fraction represented by light-chain P2 + P4 was the same whether hearts were homogenized in urea or trichloroacetic acid.

The possibility that light-chain P2 was formed from light-chain P1 during the experimental procedures, either by charge modification or by partial proteolysis, was considered. Although the latter explanation would account for the slightly lower apparent molecular weight of light-chain P2, the constant ratio of the amounts of the two components in preparations obtained in a wide range of conditions suggested, however, that this was probably not the explanation. It is unlikely that the procedures involved in the electrophoresis of the P light chain caused such an interconversion. Further, such an interconversion did not occur in freshly prepared myosin from rabbit fast skeletal muscle, for

these preparations exhibited a single band of P light chain that was converted into a single, more negatively charged, phosphorylated form after incubation with myosin light-chain kinase (Fig. 1*a*). No detectable peptide phosphate was liberated when skeletal myosin light chains labelled with ^{32}P were incubated with either urea-treated or trichloroacetic acid-treated cardiac homogenates. This suggests that no significant proteolytic activity survives in homogenates of rabbit hearts in 8 M-urea or 15% trichloroacetic acid.

In studies with hearts from neonatal rabbits and other animals, differences in the relative amounts of light-chains P1 ± P3 to light-chains P2 + P4 were seen. In homogenates of ventricles from 3-day-old rabbits the amount of light-chains P2 + P4 (if present) was less than 10% (mean for four determinations) of the total P light-chain fraction. Light-chains P2 and P4 were absent in adult rat or mouse ventricles, but were present in adult guinea-pig and beef ventricles. The P light-chain pattern of the rabbit ventricle changed to the adult form 3–4 weeks after birth. In the studies on neonatal hearts homogenization was carried out in 8 M-urea. Although under these conditions some dephosphorylation of light-chains P3 and P4 may have occurred, it would not affect the relative amounts of light-chains P1 + P3 to light-chains P2 + P4.

State of phosphorylation of the myosin P light chain in the intact beating heart

Examination of homogenates of the ventricles after perfusing the rabbit heart for 15–20 min with normal Krebs–Henseleit solution gave reproducible two-dimensional patterns either with a pH gradient of 4–6 or with a pH gradient of 3.5–11 (Fig. 3).

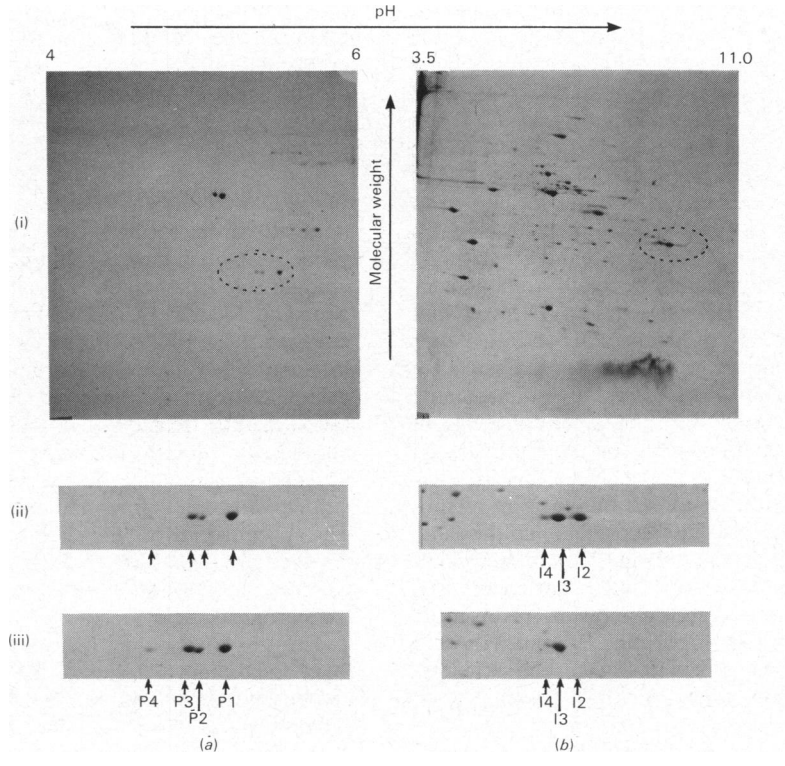

Fig. 3. *Two-dimensional gel electrophoresis of homogenates of rabbit hearts*
(*a*) P light chains; isoelectric focusing was carried out over the pH range 4–6. P light chains are indicated by P1, P2, P3 and P4. (i) Whole two-dimensional gel of heart perfused for 20 min in the absence of adrenaline. The area surrounded by the broken line enclosing the region occupied by the spots of the P light chains is illustrated in (ii) and (iii). (ii) Peak of inotropic response 23 s after addition of adrenaline; (iii) 5 min with adrenaline. (*b*) Troponin I; isoelectric focusing was carried out over the pH range 3.5–11. Troponin I is indicated by I2, I3 and I4; I1 was not apparent in this experiment. (i) Whole two-dimensional gel of heart perfused for 20 min in the absence of adrenaline. The area surrounded by the broken line enclosing the region occupied by spots of troponin I is illustrated in (ii) and (iii). (ii) Peak of inotropic response 23 s after addition of adrenaline; (iii) 2 min with adrenaline.

Similar results were obtained with hearts perfused for up to 60 min. In all cases four spots occupied the position of the P light-chain fraction. These were identified as light-chains P1, P2, P3 and P4 by comparison with the two-dimensional gel of rabbit cardiac myosin before and after phosphorylation with myosin light-chain kinase. From Table 1 it can be seen that in the normal beating perfused heart, approx. 25% of the total P light-chain fraction was in the phosphorylated forms P3 and P4. This would represent a value of 0.5 mol of phosphate/mol of cardiac myosin.

Examination of the P light-chain pattern of the heart after administration of 4 μM-adrenaline (Table 1) revealed that a greater proportion of light-chains P3 + P4 was present in hearts cut down and homogenized after the peak in the force developed than either before or at the peak of response (Fig. 4). If the heart was perfused with adrenaline for 5 min, light-chains P3 + P4 represented 33.0 ± 2% (mean ± S.E.M.; $n = 3$) of the total P light-chain fraction. A similar value was obtained in the absence of adrenaline when the heart was made ischaemic by clamping off the supply of oxygenated perfusate. In these circumstances, contrary to the situation with adrenaline, the force developed by the heart fell. In both control and adrenaline-treated hearts, the ratio of the amount of light-chain P1 + P3 to the amount of light-chain P2 + P4 was constant (Table 1). From these results it appeared that administration of adrenaline caused a relatively small increase in the extent of phosphorylation of the P light-chain fraction, but it cannot be concluded that the effect is unique to adrenaline as it was also obtained after ischaemia.

In hearts from anaesthetized rabbits the extent of phosphorylation of the P light chain was similar to control perfused hearts; light-chains P3 + P4 represented 23 ± 3% (mean ± S.E.M.; $n = 4$) of the total P light-chain fraction.

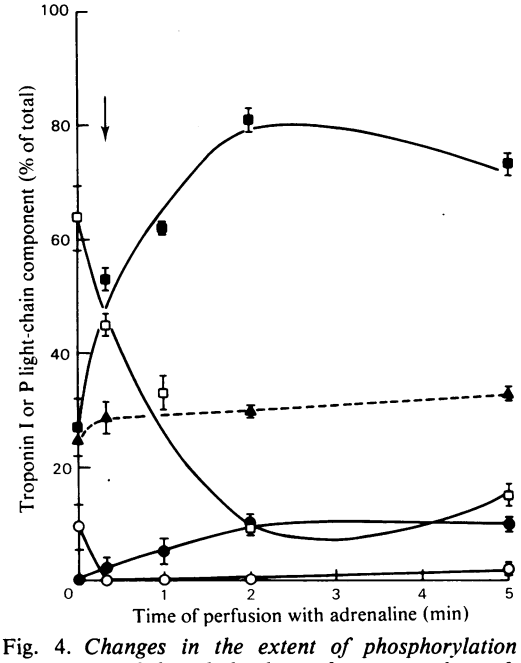

Fig. 4. *Changes in the extent of phosphorylation of troponin I and the P light chain of myosin in the perfused rabbit heart*

Hearts were perfused as described in the legend to Fig. 3 in the presence of 4 μM-adrenaline. Relative amounts of components were determined by densitometric tracing. The arrow indicates peak of inotropic response. Force increase at peak was 40% ± 3% ($n = 9$). ○, Troponin I1; □, troponin I2; ■, troponin I3; ●, troponin I4; ▲, myosin light-chains P3 + P4.

State of phosphorylation of troponin I in perfused hearts

The position of migration of troponin I on two-dimensional electrophoresis was established by comparison and co-electrophoresis with a sample of cardiac troponin I isolated by affinity chromatography (Syska *et al.*, 1976). In homogenates of perfused rabbit hearts four spots designated I1, I2, I3 and I4 in order of increasing negative charge were seen. The two main spots were I2 and I3, which always represented more than 90% of the total of I1, I2, I3 and I4 (Fig. 3). When cardiac myofibrils were incubated with cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP spot I2 decreased in size, whereas spot I3 increased and became radioactive. The nature of spot I1 and spot I4 is unclear as these protein spots were not apparent in all samples analysed. Spot I1 was sometimes seen as a minor spot in control perfused hearts and spot I4 was a relatively faint spot seen on some occasions after treating the heart with adrenaline.

When the relative proportions of the different forms of the troponin I were determined by densitometric tracing of the gels it was apparent that the amount of the phosphorylated form, troponin I3, reached a maximum 2–5 min after addition of adrenaline and not at the peak of the inotropic response. After 5 min perfusion in the presence of the drug the amount of troponin I3 had begun to decrease (Fig. 4).

Discussion

The two forms of the unphosphorylated P light chain seen on two-dimensional electrophoresis of homogenates of ventricular muscle from the rabbit, guinea pig and cow may be different gene products or the results of modification of a single gene

product, the extent of which varies during early neonatal development in the rabbit. Two forms of the unphosphorylated P light chain were also observed in previous studies on the adult rabbit heart, the so-called satellite forms (Frearson & Perry, 1975), but were then presumed to be modifications of the P light chain produced during isolation and purification. Cummins *et al.* (1980) have recently reported the presence of several P light-chain components in myosin isolated from the ventricle of man. They conclude that these components correspond to the two forms of the P light chain and their phosphorylated derivatives, which were described by Frearson & Perry (1975). These are identical with the P1, P2, P3 and P4 forms of the P light chain of the rabbit ventricle myosin described here. The explanation originally suggested for the origin of the P2 light chain (Frearson & Perry, 1975) does not appear to be valid in the current study in which electrophoretic analysis of the myosin light-chain fraction was made on freshly prepared homogenates of rabbit ventricular muscle under conditions where modification due to experimental manipulation would seem unlikely. Preliminary investigations indicate that the P1 light chain and the P2 light chain are present in the same relative proportions in different regions of the ventricle (S. A. Westwood & S. V. Perry, unpublished work). If the two forms of P light chain are different proteins, as the evidence suggests, myosin in each ventricular myocardial cell could exist in two forms differing only in the P light chains. Other workers have produced evidence for differences in the heavy chains of cardiac myosin (Hoh *et al.*, 1979), but to our knowledge so far there have been no reports of light-chain polymorphism in ventricular myosin from the adult animal.

The evidence indicates that components P3 and P4 represent the phosphorylated forms of the P1 and P2 light chains respectively. *In vivo* and in control perfused rabbit hearts the extent of phosphorylation of the P light chain was about 25%, a value that is similar to that reported by Holroyde *et al.* (1979) from studies on isolated rabbit cardiac myofibrils. Similar changes to those found after treatment with adrenaline were observed in ischaemic hearts, suggesting that the slight increase in phosphorylation that occurred after the peak of inotropic response to the drug may be a consequence of the increased metabolic demands on the tissue.

Two-dimensional electrophoretic studies also indicate that the composition of the troponin I in the rabbit heart may be more complex than other studies would suggest (Moir *et al.*, 1980). Nevertheless the two main forms of this protein identified by electrophoresis, and which together account for about 90% of the total, change in relative amounts in a manner that is completely compatible with one, troponin I3, being a more highly phosphorylated form of the other, troponin I2. The amounts of these two forms of troponin I determined by densitometric scanning of gels, agree well with the phosphate contents of rabbit cardiac troponin I before and after perfusion with adrenaline (Moir *et al.*, 1980). The increase in amount of the phosphorylated form occurred after the peak in inotropic response to adrenaline in the rabbit, in contrast with the situation in the rat where the increase in force and the increase in phosphorylation of troponin I are reported to occur simultaneously (England, 1975). The nature of the spots designated I1 and I4, which are also considered to be forms of troponin I, is not clear. It is possible that they represent forms of cardiac troponin I in which phosphorylation may or may not have occurred at sites other than serine-20. These sites, which have been described previously (Moir *et al.*, 1980; Moir & Perry, 1980), appear to be only slowly phosphorylated by cyclic AMP-dependent and other protein kinases.

Whereas phosphorylation of troponin I is a direct consequence of the action of adrenaline on cardiac muscle, the evidence would suggest that the small increase in phosphorylation of the P light chain is probably not a specific result of intervention with this drug. Phosphorylation of the P light chain occurs in control beating hearts to the extent that if both heads of each myosin molecule are identical, only one in four myosin molecules is phosphorylated in the ventricle of the normal rabbit beating heart. Thus the results obtained *in vivo* and with perfused hearts confirm the findings *in vitro* (Perry *et al.*, 1979) that phosphorylation of the P light chain is not a prerequisite for actin activation of the myosin ATPase.

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References

Anderson, N. G. & Anderson, N. L. (1978*a*) *Anal. Biochem.* **85**, 331–340

Anderson, N. L. & Anderson, N. G. (1978*b*) *Anal. Biochem.* **85**, 341–354

Cummins, P., Price, K. M. & Littler, W. A. (1980) *J. Muscle Res. Motility* **1**, 357–366

England, P. J. (1975) *FEBS Lett.* **50**, 57–60

Frearson, N. & Perry, S. V. (1975) *Biochem. J.* **151**, 99–107

Frearson, N., Solaro, R. J. & Perry, S. V. (1976) *Nature (London)* **264**, 801–802

Hoh, J. F. Y., Yeoh, G. P. S., Thomas, M. A. W. & Higginbottom, L. (1979) *FEBS Lett.* **97**, 330–334

Holroyde, M. J., Small, D. A. P., Howe, E. & Solaro, R. J. (1979) *Biochim. Biophys. Acta* **587**, 628–637

Jeacocke, S. A. & England, P. J. (1980) *Biochem. J.* **188**, 763–768

Kopp, S. J. & Barany, M. (1979) *J. Biol. Chem.* **254**, 12007–12012

Mejbaum-Katzenellenbogen, W. & Dobryszycka, W. M. (1959) *Clin. Chim. Acta* **4**, 515–522

Moir, A. J. G. & Perry, S. V. (1980) *Biochem. J.* **191**, 547–554

Moir, A. J. G., Solaro, R. J. & Perry, S. V. (1980) *Biochem. J.* **185**, 505–513

Morgan, M., Perry, S. V. & Ottaway, J. (1976) *Biochem. J.* **157**, 687–697

Pemrick, S. (1980) *J. Biol. Chem.* **255**, 8836–8841

Perrie, W. T. & Perry, S. V. (1970) *Biochem. J.* **119**, 31–38

Perry, S. V. & Cole, H. A. (1973) *Biochem. J.* **131**, 425–428

Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743

Perry, S. V., Cole, H. A., Frearson, N., Moir, A. J. G., Nairn, A. C. & Solaro, R. J. (1979) *Proc. FEBS Meet. 12th* **54**, 147–159

Pires, E. M. V. & Perry, S. V. (1977) *Biochem. J.* **167**, 137–146

Pires, E. M. V., Perry, S. V. & Thomas, M. A. W. (1974) *FEBS Lett.* **41**, 292–296

Solaro, R. J., Pang, D. C. & Briggs, F. N. (1971) *Biochim. Biophys. Acta* **245**, 259–262

Stull, J. T., Manning, D. R., High, C. W. & Blumenthal, D. K. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1552–1557

Sugino, Y. & Miyoshi, Y. (1964) *J. Biol. Chem.* **239**, 2360–2364

Syska, H., Wilkinson, J. M., Grand, R. J. A. & Perry, S. V. (1976) *Biochem. J.* **153**, 375–387