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The turnover of phosphate bound to myosin light chain-2 in perfused rat heart

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The rate of exchange of phosphate bound to ventricular myosin light chain-2 (LC_2-*P*) was measured in rat hearts perfused with [^{32}P]P_i at various levels of perfusate Ca^{2+}. Computer simulations of the light-chain labelling suggested the presence of two isotopically distinct pools of LC_2-*P*, one large pool comprising 90% of the total and a small pool consisting of the remaining 10%. At control levels of perfusate Ca^{2+} the phosphate of the large pool turned over very slowly ($t_{\frac{1}{2}} \simeq 250$ min), whereas that of the small pool turned over much more rapidly ($t_{\frac{1}{2}} \simeq 1$ min). At high levels of perfusate free Ca^{2+} (5 mM) the turnover of the phosphate of the small pool decreased markedly, whereas that of the large pool remained little changed. Conversely, at low perfusate free Ca^{2+} (0.2 mM), the turnover of the large pool decreased, whereas that of the small pool remained unchanged. The possible identity of these two pools is discussed. The total myosin-light-chain kinase activity of rat ventricle was found to be only 2–3-fold higher than the kinase activity expressed in the heart under control conditions. This, coupled with the very low turnover of most of the LC_2-bound phosphate, implies that, in heart, there is insufficient myosin-light-chain kinase activity to cause a rapid rise in the overall level of light-chain phosphorylation, even under conditions of increased cytoplasmic Ca^{2+}.

INTRODUCTION

The 19 kDa light-chain component of myosin (LC_2) was first shown to be phosphorylated *in vitro* by a myosin-light-chain kinase (MLCK) isolated from white skeletal muscle (Pires *et al.*, 1974). Subsequently the presence of MLCK in cardiac muscle was also demonstrated (Frearson & Perry, 1975). The activation of MLCK was shown to occur by reversible binding of Ca^{2+}–calmodulin at Ca^{2+} concentrations similar to those required for activation of myofibrillar ATPase (Walsh *et al.*, 1979, 1980).

Myosin-light-chain phosphorylation has been shown to play a key role in the regulation of the actomyosin interaction in smooth muscle (see Kamm & Stull, 1985). Its role in striated muscle, in which troponin mediates the Ca^{2+}-sensitivity of the myofibrils, is, in contrast, poorly understood. Myosin phosphorylation is not an obligatory step in the production of force in striated muscle (Manning & Stull, 1979); however, it has been suggested to play a modulatory role. Crow & Kushmerick (1982) found that increased myosin-light-chain phosphorylation was associated with a decrease in the actomyosin ATPase rate in fast-twitch muscle. Similarly Cooke *et al.* (1982) found that thiophosphorylation of LC_2 in glycerine-permeabilized fibres caused a decreased actomyosin ATPase rate. However, a recent report by Sweeney & Kushmerick (1985) suggested that the decreased actomyosin ATPase rate previously reported may not have been caused by LC_2 phosphorylation but by some other factor. In support of this, Barsotti & Butler (1984) and Butler *et al.* (1983) have found no effect of myosin-light-chain phosphorylation on the actomyosin ATPase rate in fast-twitch muscle.

In cardiac myofibrils lightly cross-linked with glutaraldehyde, increased myosin-light-chain phosphorylation has been shown to cause a decrease in actomyosin ATPase rate (Franks *et al.*, 1984). It has also been recently reported that increased LC_2 phosphorylation caused a 50% increase in isometric tension at submaximally activating levels of Ca^{2+} in skinned heart fibres (Morano *et al.*, 1985). However, no effect was observed at saturating Ca^{2+}. This would suggest that phosphorylation of LC_2 increases the sensitivity of cardiac myofibrils to Ca^{2+}.

Much controversy exists over the factors that regulate the levels of myosin-light-chain phosphorylation in the heart. As MLCK is activated by Ca^{2+} one might expect its activity to be increased by elevated levels of cytoplasmic calcium, e.g. during stimulation by catecholamines. However, perfusion of rabbit or rat hearts with high Ca^{2+} or positive inotropic agents for 30 s was shown to cause no change in the levels of light-chain phosphorylation (Holroyde *et al.*, 1979; High & Stull, 1980; Jeacocke & England, 1980).

Although no net change in the level of light-chain phosphorylation occurred in rat hearts perfused with inotropic agents, Jeacocke & England (1980) observed a significant incorporation of [^{32}P]P_i from the perfusate medium into myosin light chains. This would indicate that the phosphate bound to LC_2 was turning over at a significant rate. We have therefore investigated the turnover of the LC_2-bound phosphate at various levels of perfusate Ca^{2+} and correlated this with the total

Abbreviations used: LC_1, myosin light chain 1; LC_2, myosin light chain 2; LC_2-*P*, the phosphorylated form of myosin light chain 2; MLCK, myosin-light-chain kinase; DABTH-, 4-*NN*-dimethylaminoazobenzene-4′-thiohydantoin-.

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available MLCK activity of rat ventricle, in order to elucidate the importance of myosin-light-chain phosphorylation in cardiac muscle.

METHODS

Heart perfusions

Hearts from Wistar rats (250–300 g) were perfused by the Langendorff technique, at a pressure of 6 kPa, with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 11 mM-glucose and 0.234 mM-P_i, gassed with O_2/CO_2 (19:1). Hearts were pre-perfused by drip-through for 5–10 min with control medium, and then perfused by recycling for periods between 10 and 60 min with medium containing 20 μCi of $^{32}P_i$/ml and various Ca^{2+} concentrations as described in the Results and Discussion section. At the end of the recycling period the hearts were briefly perfused (30 s) with non-radioactive medium and freeze-clamped at −196 °C (Wollenberger *et al.*, 1960). Unless otherwise indicated, the atria and large vasculature were removed from the top of each heart and pooled for each time point. The ventricles from each heart or pooled atria were powdered at −196 °C and stored at −75 °C.

Measurement of ATP specific radioactivity

The ATP specific radioactivity was determined in each heart by the method of England & Walsh (1976). The exact $[^{32}P]P_i$ content of the medium for each perfusion was measured, and the ATP specific radioactivity normalized to an average medium specific radioactivity.

Measurement of the LC_2-$[^{32}P]P$ specific radioactivity

Crude myosin was extracted from the frozen heart powders using a modification of the method of Perrie *et al.* (1973). Approx. 100 mg of powder was homogenized (Polytron, PT 10 probe; 15 s at setting 5) in 1 ml of Guba–Straub buffer (0.25 M-KCl/0.1 KH_2PO_4/0.05 M-K_2HPO_4/1 mM-EDTA/50 mM-NaF, pH 6.5) and left at 0 °C for 15 min. The homogenate was centrifuged for 5 min at 2000 *g* and the supernatant was diluted with 10 ml of water. The crude myosin precipitate was collected by centrifugation and dissolved in 1 ml of electrophoresis buffer (8 M-urea/2% Ampholines (LKB Instruments Ltd.)/15 mM-2-mercaptoethanol).

The phosphorylated and dephosphorylated forms of LC_2 were separated by isoelectric focusing in polyacrylamide tube gels over the pH range 4–6 as described by Silver & Stull (1982). The myosin from each heart was run on three separate isoelectric-focusing gels. Each group of 20 gels (5 mm × 150 mm) were run at 500 V for 16 h by using a Pharmacia GE2/4 polyacrylamide-gel-electrophoresis apparatus. Gels were stained with 0.2% Fast Green in acetic acid/methanol/water (1:5:4, by vol.) for approx. 6 h, and subsequently destained in acetic acid/methanol/water (1:4:5, by vol.) The ratio of phosphorylated to dephosphorylated LC_2 in each gel was determined by densitometric scanning of the gel, using a Joyce–Loebl Chromoscan-3 coupled to a Hewlett–Packard 9845 desk-top computer. The positions of the bands were determined by comparison with pure LC_2 standard.

The amount of protein in the phosphorylated LC_2 (LC_2-*P*) band of each gel was determined by densitometric scanning and compared with the absorbance of a known amount of pure LC_2 standard run in parallel. The LC_2-*P* band was then carefully excised and digested in 1 ml of H_2O_2 (100 vol.) at 120 °C for 2 h. The dried residue was redissolved in 10 ml of water and the ^{32}P measured by Čerenkov radiation. For calculation of specific radioactivities the M_r of the LC_2 was taken as 19000 (Frearson & Perry, 1975). The LC_2-*P* specific radioactivities were normalized to an average perfusion-medium $[^{32}P]P_i$ specific radioactivity.

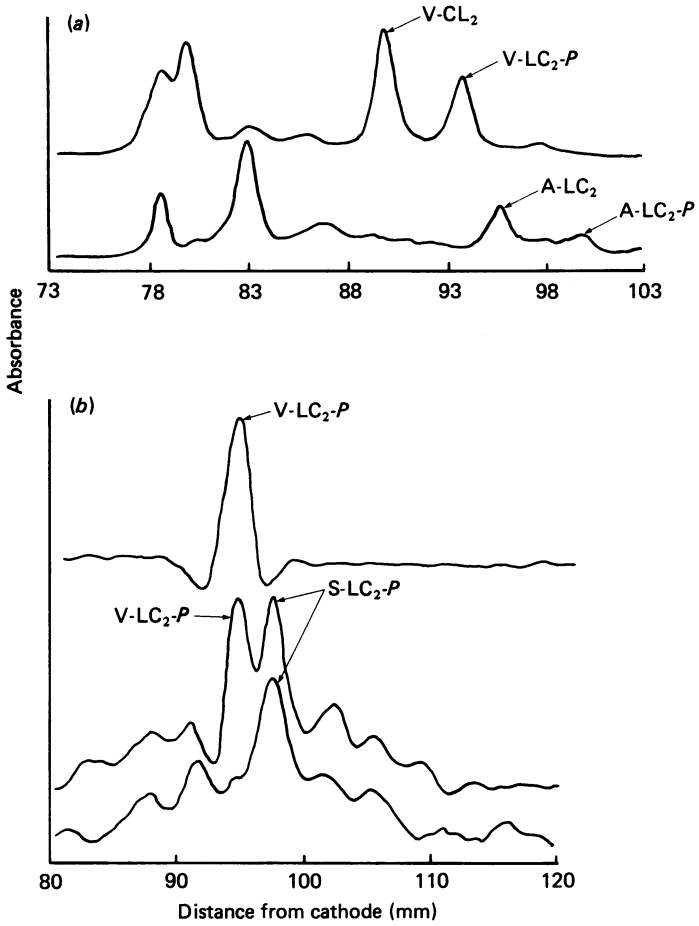


Fig. 1. Separation of ventricular, atrial and smooth-muscle LC_2 by isoelectric focusing

(*a*) Densitometric scans of isoelectric-focusing gels of rat cardiac ventricular (upper line) and atrial (lower line) myosin, stained for protein with Fast Green. (*b*) Densitometric scans of autoradiographs from isoelectric-focusing gels of rat ventricular (upper line), aortic + ventricular (middle line) and aortic (lower line) myosin from tissues incubated with $[^{32}P]P_i$. Abbreviations used: V-LC_2, ventricular LC_2; V-LC_2-*P*, ventricular LC_2-*P*; A-LC_2, atrial LC_2; A-LC_2-*P*, atrial LC_2-*P*; S-LC_2-*P*, aortic LC_2-*P*.

Preparation of proteins

Ventricular LC_2 was prepared from ox heart by using a method adapted from Perrie & Perry (1970) and Jeacocke & England (1980). Crude ox ventricular myosin was prepared as described above, extracted in 5 M-guanidinium chloride, the heavy chain precipitated by ethanol and the light chains purified by chromatography on DEAE-cellulose as described by Jeacocke & England (1980), except that EDTA was omitted from the

extraction buffer. The pooled light-chain fraction was extensively dialysed against water followed by dialysis against 20 mM-BisTris/HCl/4 M-urea/50 mM-KCl/1 mM $MgCl_2$/0.1 mM-EGTA/1 mM-dithiothreitol, pH 6.0. Further purification was carried out by chromatography on an 8 ml Mono Q ion-exchange column (equilibrated with the same buffer) in conjunction with a Pharmacia Fast Protein Liquid Chromatography system. Before application to the column the sample was centrifuged at 20000 *g* for 20 min, and filtered through a 0.22 μm-pore-size filter (Millipore GSWP). Proteins were eluted with a 50–250 mM-KCl gradient in the column buffer. The gradient was controlled manually in such a way that each time an increase in the A_{280} of the eluate was detected, the concentration of KCl was held constant until the absorbance had returned to baseline. A total gradient of about 400 ml was required for each run. The column fractions were analysed by SDS/polyacrylamide-gel electrophoresis, and those containing LC_2 (eluted at approx. 80 mM-KCl) pooled, dialysed against water and stored at −20 °C. As assessed by polyacrylamide-gel electrophoresis the sample contained > 95% LC_2, with the remainder being LC_1.

Phosphorylase *b* and phosphorylase kinase, prepared from rabbit skeletal muscle by the methods of Fischer & Krebs (1958) and Cohen (1973) respectively, were generously donated by Mr. David Mills, Department of Biochemistry, University of Bristol.

Polyacrylamide-gel electrophoresis

Isoelectric focusing in the presence of urea was carried out as described by Silver & Stull (1982). Polyacrylamide-gel electrophoresis in the presence of SDS was performed by the method of Laemmli (1970) and the proteins stained with Coomassie Blue R 250. Two-dimensional electrophoresis was performed by isoelectric focusing in the first dimension as described above, and SDS/polyacrylamide-gel electrophoresis in the second dimension as described by Laemmli (1970).

Phosphoamino acid analysis

Phosphoamino acids were separated by h.p.l.c. on a C_{18} reverse-phase column following derivatization with 4-dimethylaminoazobenzene-4′-sulphonyl chloride (Chang *et al.*, 1983; Chang, 1984). The column was initially equilibrated with 10 mM-potassium phosphate, pH 6.1, 20% (v/v) acetonitrile in water, and was then developed with an increasing gradient of acetonitrile, the phosphoamino acid derivatives being eluted between 25 and 31% acetonitrile.

Hydrolysates of LC_2-*P* for determination of phosphoamino acid composition were obtained as follows. The LC_2-*P* bands were excised from six or seven isoelectric-focusing tube gels and washed for 1 h in a large volume of water. The protein was electro-eluted from the gel in 100 mM-NH_4HCO_3 into a dialysis bag. This eluted protein was dialysed against water overnight and dried by rotary evaporation. The protein residue was hydrolysed in 200 μl of 6 M-HCl at 110 °C under N_2 in sealed glass tubes for 2 h. After rotary evaporation the residue was redissolved in 100 μl of 100 mM-NH_4HCO_3, pH 9, and derivatized as previously described (Chang *et al.*, 1983; Chang, 1984). A typical separation of the phosphoamino acids is shown in Fig. 1.

MLCK

Immediately after decapitation of a 250–300 g Wistar rat the heart was removed and the ventricle excised, weighed and then homogenized (Polytron PT 10; 15 s at setting 4, 5 s at setting 5) in 2 ml of the following buffer: 50 mM-sodium phosphate/10 mM-$MgCl_2$/50 mM-NaCl/0.5 mM-EGTA/10 mM-NaF/1 mM-dithiothreitol/

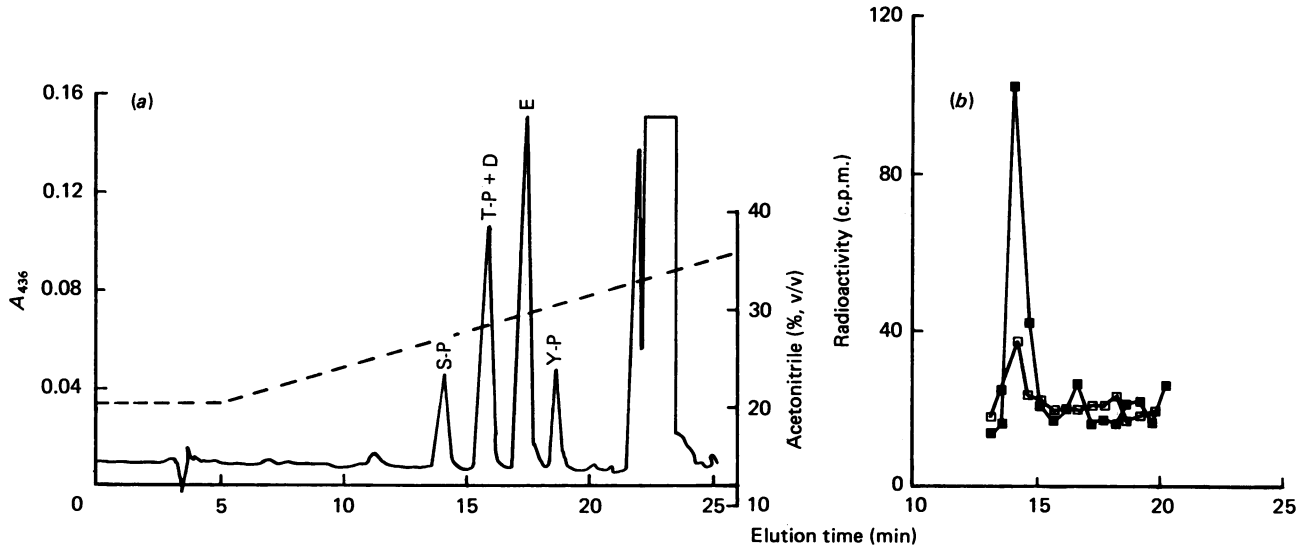


Fig. 2. H.p.l.c. separation of phosphoamino acid standards and hydrolysates of ^{32}P-labelled LC_2 from rat hearts

Phosphoamino acids or LC_2 hydrolysates were derivatized with 4-dimethylaminoazobenzene-4′-sulphonyl chloride (to give the DABTH derivatives) as described in the Methods section. After derivatization a mixture of the hydrolysate plus DABTH-phosphoserine (S-P), DABTH-phosphothreonine (T-P) and DABTH-phosphotyrosine (Y-P) was applied to a reverse-phase C_{18} column. The column was developed with a gradient of acetonitrile/10 mM-potassium phosphate, pH 6.1. (*a*) A_{436}; further abbreviations: D, DABTH-aspartate; E, DABTH-glutamate. (*b*) ^{32}P in fractions collected over 30 s intervals, LC_2-*P* was isolated from hearts perfused for 10 min (□) or 60 min (■) with [^{32}P]P_i.

Table 1. Size and turnover rates of pools LC_2-*P*

The values of the proportion of LC_2 (mean ± S.D., $n > 20$) in the phosphorylated state were measured as described in the Methods section. The values for pool sizes and rates of turnover were computed from the two-pool model (Scheme 2) as described in the Results and Discussion section. Each row is the result of a completely separate experiment. The value of total LC_2 in rat heart was taken as 127.8 nmol·g wet wt.$^{-1}$ (Everett *et al.*, 1983).

Perfusate free [Ca^{2+}] (mM)	LC_2 in phosphorylated form (±S.D.)(%)	LC_2-*P* pool size (nmol·g wet wt.$^{-1}$)			Rates of flux of ^{32}P(nmol·min^{-1}·g wet wt.$^{-1}$)	
		Total	P_a	P_b	R_a	R_b
5.00	34±4	44.73	40.26	4.47	0.10	0
4.00	32±4	50.49	45.44	5.05	0.10	1.0
0.75	37±4	47.30	42.57	4.73	0.10	2.5
0.75	44±4	58.80	52.92	5.88	0.10	2.5
0.20	45±4	57.51	51.76	5.75	0.05	2.5
0.20	45±2	56.23	50.61	5.62	0	2.5

0.3 mM-digitoxigenin/1 mM-ouabain/oligomycin (1 μg/ml) 1 mM-Na_3VO_4/1 mM-phenylmethanesulphonyl fluoride/soya-bean trypsin inhibitor (10 μg/ml)/leupeptin (1 μg/ml)/pepstatin (1 μg/ml)/antipain (1 μg/ml), pH 7.5. The resulting homogenate was diluted 10-fold in this buffer in the final assay mixture.

Each assay was performed (at 30 °C) in quadruplicate in the presence of 0.1 mg of calmodulin/ml, approx. 0.5 mM-free Ca^{2+}, with or without added LC_2 (final concn. 2 mg/ml). The reaction was started by addition of [γ-^{32}P]ATP (2 mM final concn. and approx. 1 Bq/pmol). Aliquots (50 μl) of the assay mix were spotted on to filter paper (Whatman 3MM) at 0.25, 0.5 and 1.0 min, and placed in 10% (w/v) trichloroacetic acid. After extensive washing the papers were dried, placed in 10 ml of water and the ^{32}P radioactivity counted by Čerenkov radiation. Results were corrected for non-specific binding of [γ-^{32}P]ATP and [^{32}P]P_i to the papers. The MLCK activity was determined from the difference in ^{32}P incorporation in assays performed with or without added LC_2. The time course was linear over the first 30 s of the incubation, and the rates were calculated from this period. The ^{32}P incorporation into endogenous LC_2 in the tissue extract was examined by SDS/polyacrylamide-gel electrophoresis followed by autoradiography of samples from an incubation carried out in the absence of added LC_2. This was found to be negligible and was therefore ignored in the calculations.

RESULTS AND DISCUSSION

Purity and analysis of phosphorylated light chain

Possible contamination of the ventricular LC_2-*P* band obtained by isoelectric focusing by other phosphoproteins, particularly atrial and smooth-muscle light chains, was assessed as follows. For atrial LC_2 contamination, samples of non-radioactive atrial and ventricular myosin were run together on the same isoelectric-focusing gel, and also separated on parallel gels. For smooth-muscle myosin the LC_2 was labelled with ^{32}P by incubating rat aortic strips with [^{32}P]P_i as described by Murray & England (1980), and crude myosin similarly subjected to isoelectric focusing followed by autoradiography. Considerable separation of the ventricular, atrial and smooth-muscle light chains was observed, with a minimum distance between the ventricular LC_2-*P* and any other LC_2 of 4 mm (Fig. 1). Sections of ventricular LC_2-*P* carefully cut from the gels were therefore most unlikely to be contaminated with other light chains.

In addition, two-dimensional electrophoresis (isoelectric focusing in first dimension, SDS/polyacrylamide-gel electrophoresis in the second) showed no other ^{32}P-containing protein migrating with the same isoelectric point as the ventricular LC_2*P* (results not shown).

It has been reported (Cole *et al.*, 1985; Ikebe & Hartshorne, 1985) that threonine residues in LC_2 can be phosphorylated in smooth muscle by MLCK. In view of this, and the results of the computer modelling discussed below, the possible presence of phosphorylated threonine residues in ventricular LC_2-*P* was investigated. Fig. 2 shows the h.p.l.c. separation of a hydrolysate of ventricular LC_2-*P* prepared from hearts perfused with [^{32}P]P_i for 10 min. It can be seen that all of the ^{32}P was associated with phosphoserine, no other phosphoamino acids being detected. Identical results were obtained with hearts perfused for 60 min with [^{32}P]P_i.

There was no statistically significant change in the proportion of LC_2 in the phosphorylated form at various perfusate free [Ca^{2+}] (Table 1). In addition, at any [Ca^{2+}] this proportion remained constant throughout the perfusion period. The lack of change is consistent with previously published studies on the lack of effect of a range of agents on the phosphorylation state of cardiac LC_2 (Holroyde *et al.*, 1979; High & Stull, 1980; Jeacocke & England, 1980; Westwood & Perry, 1981).

Phosphate incorporation in [^{32}P]P_i-perfused hearts

Fig. 3 shows the rate of incorporation of [^{32}P]P_i into the γ-phosphate of ATP in perfused hearts. As previously described (England & Walsh, 1976) there was a slow incorporation of label, which as been attributed to a slow exchange of phosphate across the cell membrane. For the purposes of subsequent analysis by computer simulation, the [γ-^{32}P]ATP specific radioactivity was fitted by least-squares regression analysis to single exponentials, as shown in Fig. 3. Table 2 gives the parameter values for the fitted lines at each of the perfusate Ca^{2+} concentrations used. It can be seen that there is no obvious effect of [Ca^{2+}] on the rate of labelling of [γ-^{32}P]ATP.

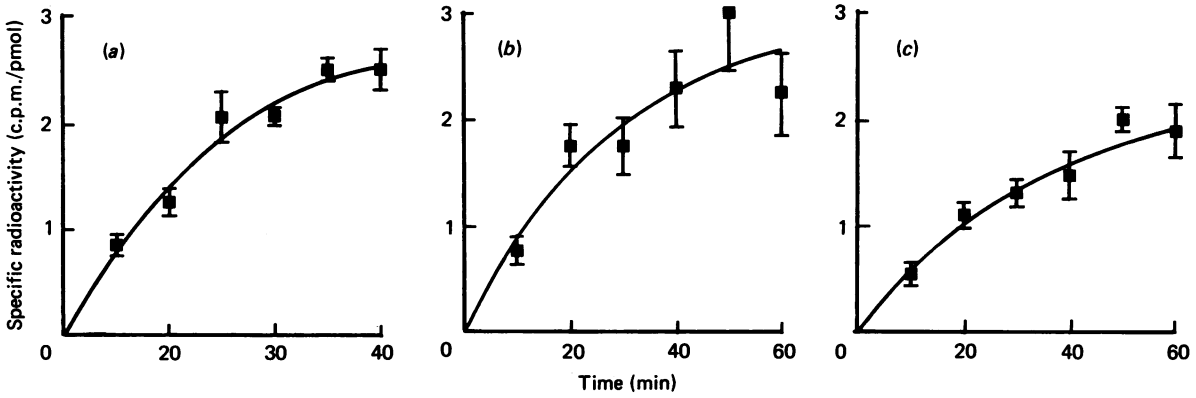


Fig. 3. Labelling of [γ-^{32}P]ATP in hearts perfused with [^{32}P]P_i

Hearts were perfused for the times shown with [^{32}P]P_i, and [γ-^{32}P]ATP specific radioactivity was assayed as described in the Methods section. The concentration of free Ca^{2+} in the perfusate was: (*a*) 0.7 mM; (*b*) 4.0 mM; (*c*) 0.2 mM. Each point is the mean for three or four hearts. Error bars are ±S.D. The lines drawn are those fitted by non-linear exponential regression (see Table 2).

Table 2. Parameter values for the regression of [γ-^{32}P]ATP specific radioactivity as a function of time in perfused hearts

The data of [γ-^{32}P]ATP specific radioactivity measured in hearts after perfusion with [^{32}P]P_i at different times (see Fig. 3) were fitted to single exponentials of the form:

Sp. radioactivity of [γ-^{32}P]ATP $= a\,(1-e^{-b\cdot time})$

Perfusate free $[Ca^{2+}]$ (mM)	a (c.p.m.·pmol^{-1})	$10^2 \times b$ (min^{-1})
5.0	9.82	3.53
4.0	3.02	3.60
0.75	2.97	3.40
0.75	3.01	2.95
0.2	2.37	2.85
0.2	8.55	1.67

Examples of the ^{32}P incorporation into ventricular LC_2 at various perfusate $[Ca^{2+}]$ are shown in Fig. 4. Note that the lines drawn are those derived from the computer simulations described below and are not 'best' lines fitted by eye. There were significant differences in the rates in incorporation of ^{32}P into LC_2-*P* at different $[Ca^{2+}]$, and also in the shape of the time courses of the LC_2-*P* specific radioactivity.

Several qualitative conclusions can be drawn from the data shown in Figs. 3 and 4. The most significant feature is that the specific radioactivity of the LC_2-*P* at all perfusate $[Ca^{2+}]$ was $< 20\%$ of the [γ-^{32}P]ATP. This implies a slow turnover of the phosphate bound to the LC_2. In view of this, and the relatively slow labelling of [γ-^{32}P]ATP, the shape of the time course of LC_2P specific radioactivity would be expected to be concave (i.e. showing a lag phase), assuming a simple model of LC_2-*P* labelling (Scheme 1). However, in perfusions at control levels of free $[Ca^{2+}]$ an unexpectedly rapid initial rate of labelling of LC_2-*P* was observed (Fig. 4*a*). This result cannot be explained by the simple model, and further analysis was carried out by computer simulation of a more complicated model (see below).

At a perfusate free $[Ca^{2+}]$ of 5 mM (Fig. 4*b*), the labelling of the phosphate in LC_2-*P* showed a time course which was compatible with the simple model in Scheme 1. A very slow rate of turnover of the phosphate was suggested from the long lag period of labelling when compared with the rate of [γ-^{32}P]ATP labelling.

At low concentrations of perfusate free $[Ca^{2+}]$ (0.2 mM; Fig. 4*c*), the time course of labelling was similar to that at control $[Ca^{2+}]$, and would not fit the simple model of Scheme 1. The shape of the curve resembled that of the ATP labelling, except that the specific radioactivity of the LC_2-*P* was approx. 20% of the ATP specific radioactivity. This result could be explained if only a small proportion of the phosphate on LC_2-*P* was turning over rapidly, with the remainder having either slow or no phosphate turnover.

Computer modelling of light-chain labelling

Because the labelling of the phosphate in LC_2-*P* did not generally appear to fit the simple model of Scheme 1, the incorporation of ^{32}P into ventricular LC_2 was analysed by computer simulation (described in more detail below). Fig. 5 shows the simulations obtained by using the single-pool model of Scheme 1 with the data obtained with 0.7 mM free Ca^{2+}. As expected from the qualitative analysis, a poor fit was obtained when all of the LC_2-*P* phosphate was assumed to be turning over (continuous lines), whatever the rate of turnover. Although a better fit could be obtained when only a fraction of the LC_2-*P* was assumed to be turning over (broken line), a satisfactory fit over the whole time course could not be obtained.

A model containing two pools of LC_2-*P* (Scheme 2) was chosen as the simplest alternative to the one-pool model. The modelling was carried out on a Hewlett–Packard 9845 desk-top computer using a BASIC version of the program described by England (1970). The differential equations for the models were written in explicit form into the program and solved by a fifth-order Runge–Kutta method. The results of the simulations were displayed graphically and visually compared with the perfusion data. The variable parameters (R_a, R_b, P_a and P_b) in the equations were altered manually to obtain the best fit. R_a and R_b are the turnover rates of the LC_2-*P*

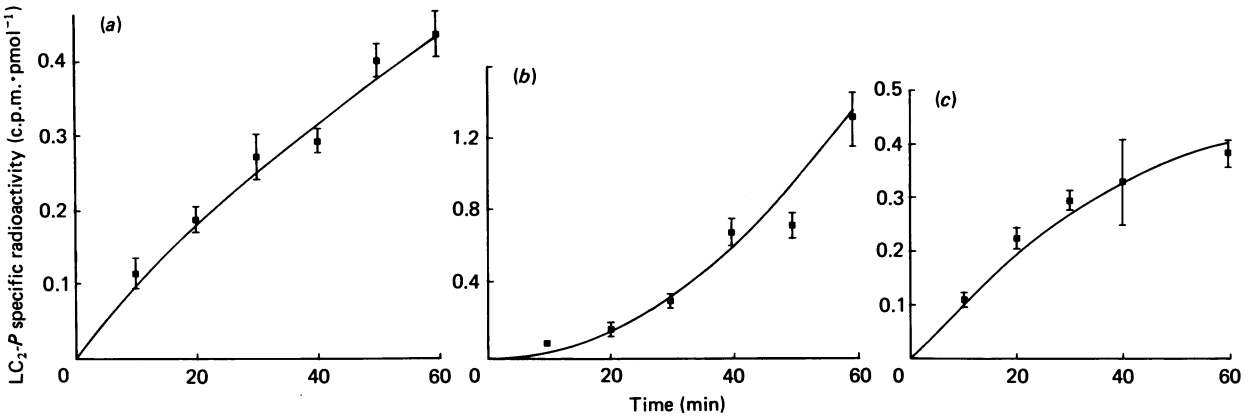
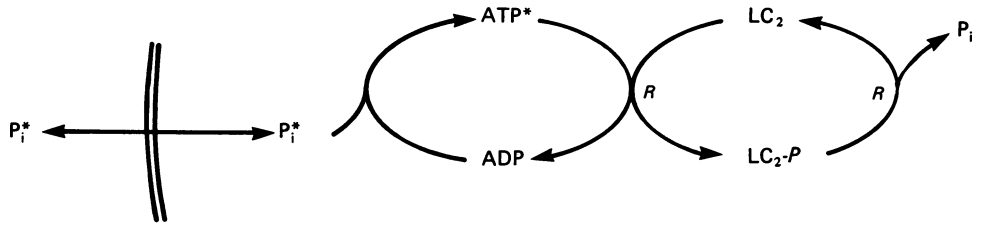


Fig. 4. Specific radioactivity of myosin LC_2 in hearts perfused with [^{32}P]P_i

The specific radioactivity of LC_2 was determined in hearts perfused with [^{32}P]P_i as described in the Methods section. The concentration of Ca^{2+} (free) in the perfusate was: (*a*) 0.7 mM; (*b*) 5.0 mM; (*c*) 0.2 mM. Each point is the mean for three or four hearts. Error bars are ±S.D. The lines drawn are those obtained by computer simulation using the model shown in Scheme 2 and the values of Table 1.



Scheme 1. One-pool model of LC_2-*P* labelling

The scheme shows the exchange of [^{32}P]P_i (P_i*) across the cell membrane (slow) and its incorporation into the γ-phosphate at ATP (rapid). *R* is the rate of kinase and phosphatase expressed in the tissue (nmol·min^{-1}·g wet wt.$^{-1}$).

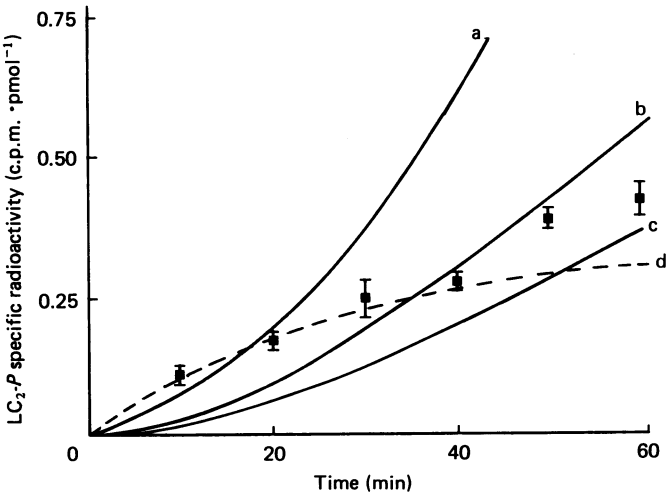


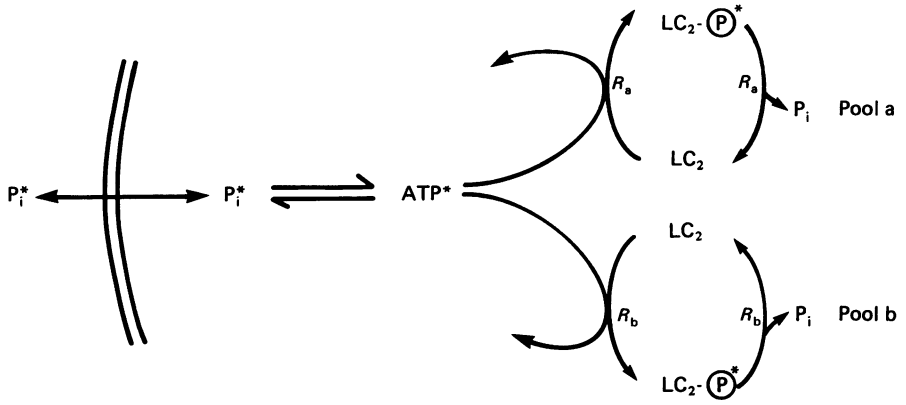
Fig. 5. Examples of simulations using a one-pool model

Simulations were performed using the model shown in Scheme 1. a: $r = 0.7$ nmol·min^{-1}·g wet wt.$^{-1}$, P (pool size) = 55 nmol·g wet wt.$^{-1}$; b: $R = 0.35$, $P = 55$; c: $R = 0.24$, $P = 55$; d: $R = 5.0$, $P = 8$. The data points are those of Fig. 4(*a*).

phosphate (expressed as nmol·min^{-1}·g wet wt.$^{-1}$), and P_a and P_b are the concentrations (expressed as nmol·g wet wt.$^{-1}$) of LC_2-*P* associated with Pool a and Pool b respectively in Scheme 2. The best fit could be approximately checked by calculating the sum of squares of the differences between the fitted line and the means of the experimental points. Rather than attempting to simulate the labelling of the γ-phosphate of ATP, the regression lines (Fig. 3 and Table 2) were used as direct inputs to the models. The simulations were subsequently used to give estimates of the expressed activities of MLCK and phosphatase in intact heart.

An additional value required by the model is the concentration of LC_2-*P* in heart muscle. The concentration of LC_2 was estimated by Everett *et al.* (1983) as 0.127 μmol·g wet wt.$^{-1}$. The actual [LC_2-*P*] in the hearts was calculated by multiplying this total [LC_2] by the mean proportion of LC_2 in the phosphorylated state in each set of perfusions (see Table 1).

The initial qualitative analysis above suggested that most of the LC_2-*P* phosphate was turning over slowly, but there was an indication from the simulation in Fig. 5 (broken line) that a small component could be turning over much faster. The starting values for the two pool



Scheme 2. Two-pool model of LC_2-*P* labelling

The metabolites in this scheme are the same as those in Scheme 1. R_a and R_b are the rates of kinase and phosphatase activities associated with Pool *a* and Pool *b* respectively. The differential equations used to stimulate this model (England, 1970) are:

$$\frac{d(s.a._{LC_2\text{-}P(a)})}{dt} = \frac{R_a(s.a._{ATP} - s.a._{LC_2\text{-}P(a)})}{[P_a]}$$

$$\frac{d(s.a._{LC_2\text{-}P(b)})}{dt} = \frac{R_b(s.a._{ATP} - s.a._{LC_2\text{-}P(b)})}{[P_b]}$$

where $s.a._{LC_2\text{-}P()}$ etc. refer to the specific radioactivities of the various components, and P_a and P_b are the concentrations of LC_2-*P* in pools a and b respectively.

simulations were therefore chosen such that the small pool was approx. 10% of the total LC_2-*P*, with a turnover rate approx. 10 times faster than the large pool. The best fit to the experimental data at a perfusate free $[Ca^{2+}]$ of 0.7 mM is shown in Fig. 4(*a*), with the parameter values used in the simulation given in Table 1. The model gave a ratio of the sizes of the large to small pools of 9:1. The rate of turnover of the phosphate in the large LC_2-*P* pool was very slow, with a $t_{\frac{1}{2}}$ calculated from the turnover rate of > 200 min. In contrast, the $t_{\frac{1}{2}}$ of the small pool was calculated to be approx. 1 min, although, as discussed below, this is a maximum estimate. This difference in half-times was not caused solely by the difference in pool sizes, but reflected a large difference in the rates of the kinase and phosphatase activities associated with the two pools. Two complete experiments were carried out at this $[Ca^{2+}]$, and the computer modelling gave identical rates of turnover and ratio of pool sizes in each experiment.

The simulation gives a minimum total expressed MLCK activity in perfused rat heart of 2.6 nmol·min^{-1}·g wet wt.$^{-1}$. This should be compared with the total kinase activity measured in homogenates of rat hearts as described in the Methods section of 5.7±2.0 (mean±S.D., $n = 5$) nmol·min^{-1}·g wet wt.$^{-1}$. It would therefore appear that, in these perfusions, the expressed MLCK activity is approx. 50% of the total activity present in the tissue. This high level of expressed activity of the kinase is consistent with the calculations of Stull *et al.* (1981), which showed that there is a rapid association (and hence activation) of Ca^{2+}–calmodulin with MLCK, but a slow dissociation and inactivation. Thus, under the rapid oscillations of Ca^{2+} occurring in a beating heart, the kinase would be expected to remain active throughout the cardiac cycle. These results explain why no short-term changes in LC_2 phosphorylation have been observed in perfused hearts during increases in cytoplasmic $[Ca^{2+}]$ (England, 1984).

The data at elevated perfusate Ca^{2+} were simulated by using the same two-pool model as that described above. The simulated best fit gave a significantly lower rate of turnover of the small LC_2-*P* pool, becoming zero with 5 mM free perfusate Ca^{2+} (Fig. 4*b* and Table 1). This latter result is equivalent to fitting the data to a single-pool model and could be interpreted as a dephosphorylation of the small pool rather than a decrease in its rate of turnover. It can be seen from Table 1 (column 2) that the measured level of LC_2 phosphorylation was possibly decreased by some 10–20% after perfusions with elevated free Ca^{2+}, in support of this latter interpretation. However, because these experiments were done over a period of 15 months, it is not possible to exclude variations between groups of animals causing these small changes in the levels of LC_2 phosphorylation. The turnover of the large pool did not appear to be significantly altered by the elevation in perfusate Ca^{2+}.

Two complete sets of perfusions were also carried out at 0.2 mM free Ca^{2+}, and one such set of experimental data are shown in Fig. 4(*c*). Simulations were again carried out with the same P_a/P_b ratio. Under these conditions the simulations indicated that the turnover of the small pool remained the same as with 0.7 mM free Ca^{2+} in the perfusate. However, there was a decrease of the turnover of the large pool, and in the case of one experiment this decreased to zero (Table 1).

The overall results of the simulation indicate that two isotopically distinct pools of LC_2-bound phosphate exist in perfused rat hearts. There is a large pool accounting for some 90% of the total LC_2-*P*, with a low turnover rate, and a smaller pool comprising the remaining 10% with a high turnover rate. At elevated levels of perfusate free Ca^{2+} the turnover of the phosphate of the small pool was either much decreased or became zero. In comparison, at low levels of Ca^{2+}, the turnover of the phosphate associated with the large pool of LC_2-*P* is much decreased, and that of the small pool remains unchanged.

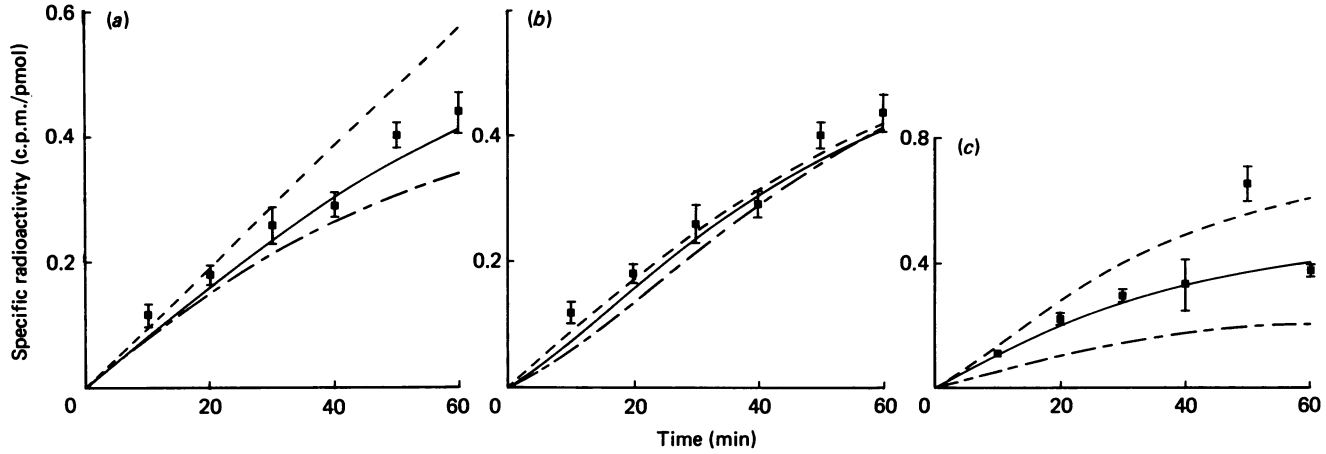


Fig. 6. Examples of varying the parameter values on the simulations using the two-pool model

(*a*) 0.7 mM-Free [Ca^{2+}]:——, the simulation obtained by using the values in Table 1; ----, the simulations by halving (lower line) or doubling (upper line) the value of R_a. (*b*) 0.7 mM-free [Ca^{2+}]:——, the simulation obtained using the values in Table 1; ----, the simulations obtained by halving (lower line) or doubling (upper line) the value of R_b. (*c*) 0.2 mM-free [Ca^{2+}]:——, the simulation obtained using the values in Table 1; ----, simulations obtained by varying the ratio of P_a/P_b from 17:3 (upper line) to 19:1 (lower line).

Reliability of stimulated parameter values

The parameter values shown in Table 1 are the values obtained from the simulated curves that best fitted the experimental points using the criteria discussed above. In order to obtain an assessment of the reliability of these values, for each simulation each variable parameter (pool size and rate) was varied in turn, with the other parameter values being held constant. The effect of this on the fit to the experimental data was then assessed.

Equivalent percentage changes in each of the parameters did not produce the same change in the simulated curve, and different parameters were more sensitive at the various perfusate free [Ca^{2+}]. Fig. 6(*a*) shows the effect of doubling or halving the value of R_a at 0.7 mM free Ca^{2+}. The simulated line was very sensitive to changes in this parameter, and the value calculated was therefore well defined. When R_b was varied to the same extent (Fig. 6*b*), there was much less change in the simulated line, particularly when the value was increased. This is because the turnover of the small pool is already rapid enough to ensure near-equilibration with [γ-^{32}P]ATP, and any increase in R_b can have little effect. Therefore the simulation can give only a minimum value for R_b. When the ratio P_a/P_b was altered from 9:1 to 19:1 or 17:3 the results (not shown) were very similar to those of Fig. 6(*a*). It was possible in this case to obtain a reasonable fit by varying R_a and R_b at the same time. However, the ratio of 9:1 for P_a:P_b was necessary to obtain a good fit to the data for perfusate free Ca^{2+} of 0.2 mM (Fig. 6*c*).

At the lower free medium [Ca^{2+}], because the value of R_a is low or zero, the shape of the simulated curve is very sensitive to small changes in its value. As with the model at a free Ca^{2+} of 0.7 mM, increases in R_b have little effect on the simulation, but a decrease of 50% in the value caused a change very similar to that shown in Fig. 6(*b*).

With the stimulation at a perfusate free Ca^{2+} of 5 mM, a good fit could only be obtained with $R_b = 0$. Any other value of R_b caused a marked deviation of the simulation from the experimental data (result not shown). Small (10%) changes in the size of the large pool (P_a) had little effect on the fit, and therefore the value obtained for the simulations at 0.7 mM free Ca^{2+} was used. The fit was, however, very sensitive to changes in R_a, since this was the major parameter determining the simulated curve. A 50% increase or decrease in the quoted value of R_a caused marked and obvious deviations from the experimental data.

General discussion

The results of the simulations have shown that, in rat hearts perfused with [^{32}P]P_i, two isotopically distinct pools of ventricular LC_2-bound phosphate could be distinguished: a large pool consisting of 90% of the total LC_2-*P*, in which the phosphate was turning over very slowly ($t_{\frac{1}{2}} > 200$ min), and a small pool comprising the remaining 10% with a much more rapid turnover. The $t_{\frac{1}{2}}$ for this latter pool was estimated to be approx. 1 min. However, this value cannot be accurately determined from the simulations, owing to the fast turnover coupled with a slow incorporation of label into [γ-^{32}P]ATP. The $t_{\frac{1}{2}}$ of 1 min is therefore a maximum value. The MLCK activity required to give the combined turnover rate of both pools accounts for approx. 50% of the total measurable MLCK activity in rat heart, and, given that this total activity is low, explains why rapid increases in phosphorylation of LC_2 are not seen during increased inotropy (England, 1984). Calculations show that even if there were full activation of the kinase, coupled with no change in the phosphatase rate, a 50% increase in the level of LC_2 phosphorylation would require at least 10 min.

The results obtained from perfusions at low [Ca^{2+}] indicate the presence of a Ca^{2+}-sensitive phosphatase acting on the ventricular LC_2-*P in vivo*. The decreased turnover rate of the large pool of LC_2-bound phosphate under these conditions must result from a decrease in activity of both the MLCK and a myosin LC_2 phosphatase, as no net change in the overall level of light-chain phosphorylation occurred. This supports the evidence obtained by Stewart *et al.* (1983), which

demonstrated that isolated myosin light chains are a good substrate of the Ca^{2+}/calmodulin-dependent protein phosphatase 2B *in vitro*. However, this does not exclude the presence of other phosphatases acting on LC_2-*P* which are not Ca^{2+}-regulated.

The physical identity of the two pools of LC_2-*P* is unclear at present. We have eliminated the possibility of contamination by atrial or vascular LC_2-*P*, or any other phosphoprotein with same isoelectric point but a different M_r. It is possible that the two pools represent two different phosphorylation sites on the light chains. Both phosphorylation sites must be on serine residues, however, as only phosphoserine was detected after acid hydrolysis. Also both sites must be mutually exclusive, as no evidence for the presence of diphosphorylated LC_2 was obtained from two-dimensional polyacrylamide-gel electrophoresis (results not shown). Since such constraints make this a very unlikely explanation for our results, other possibilities are discussed below.

Two pools of LC_2-*P* could be produced by two different cell types within the ventricle, each containing a distinct, but electrophoretically identical, LC_2. Alternatively, the two cell types could contain MLCK phosphatase systems of very different activity. It is also possible that the two LC_2 pools could be compartmented within the same cell. Although none of these can easily be discounted as an explanation, little evidence for them has been reported in the literature.

The two pools of LC_2-*P* may be a result purely of kinetic constraints. There is much argument as to the mechanism of phosphorylation of intact myosin, and the significance of co-operativity of phosphorylation between the two heads (e.g. Trybus & Lowey, 1985). However, it is difficult to see how one could generate two kinetic pools of LC_2-bound phosphate by evoking the co-operativity between the two heads unless they do not behave identically (e.g. Furukawa *et al.*, 1980).

Finally, the possibility exists that the different myosin heavy-chain isoenzymes present in the heart (D'Albis *et al.*, 1979) may influence the phosphorylation of the associated light chains. Lompre *et al.* (1981) have shown that, in rats of the same age as used in this study (3 months), the predominant ventricular myosin present is the high-ATPase-activity V_1 form (using the nomenclature of Hoh *et al.*, 1978). However, significant amounts of the low-ATPase (V_3) and hybrid V_2 forms are also present (approx. 10%). Thus if the LC_2 kinase and phosphatase have widely different activities towards the light chains associated with the different heavy chains, two pools of LC_2-bound phosphate would result. In such a model, the phosphate bound to the LC_2 of the V_3 myosin would be turning over much more rapidly than that associated with the V_1 form, and hence would form the small pool of LC_2-*P*. The values of Table 1 predict that, after perfusion of hearts for 10 min with [^{32}P]P_i, there would be approx. 5 times more ^{32}P associated with the V_3 isoenzyme than with the V_1 form. After 60 min of perfusion approximately equal amounts of ^{32}P would be associated with each isoenzyme form. We have tested this hypothesis by separating V_1 and V_3 myosin isoenzymes from [^{32}P]P_i perfused hearts by non-denaturing polyacrylamide-gel electrophoresis (Hoh *et al.*, 1978; D'Albis *et al.*, 1979), followed by autoradiography of the myosin bands (results not shown). After perfusion for both 10 and 60 min with [^{32}P]P_i the proportion of the LC_2-[^{32}P]*P* associated with the V_3 isoenzyme was 0.14 ± 0.04 ($n = 4$). This proportion is very close to that reported for the ratio of V_3 to V_1 isoenzymes (0.11; Lompre *et al.*, 1981), and indicates that the turnover of LC_2-bound phosphate is not influenced by the heavy-chain isoenzyme with which the LC_2 is associated.

Although we have been unable to establish the identity of the two pools of LC_2-associated phosphate, the results do show that there is insufficient MLCK activity in rat ventricle to produce a rapid rise in the level of LC_2 phosphorylation. It is therefore very unlikely that this phosphorylation has any role in the short-term regulation of contraction, although there may be a long-term regulatory function for LC_2 phosphorylation. The intriguing possibility remains, however, that there may be a significant regulatory role for the rapidly exchanging small LC_2-*P* pool, since changes in the level of phosphorylation of this pool could occur within a very short period.

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