Myosin light chain phosphorylation during contraction of turtle heart

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The phosphorylation of myosin P-light chain was determined during the contraction cycle of turtle heart beating 5–8 times/min at 5°C. The hearts were freeze-clamped either in systole or diastole, then homogenized and washed in strong acids in order to completely inhibit myosin light chain kinase and phosphatase and isolate the total P-light chain of the heart. The phospho and dephospho forms of P-light chain were separated by two-dimensional gel electrophoresis and were quantitated by densitometry. Alternatively, the hearts were perfused with ^{32}P and the incorporation of [^{32}P]phosphate into the P-light chain was determined. Both methods demonstrated that in hearts frozen in systole more P-light chain was phosphorylated than in hearts frozen in diastole.

Myosin light chain *Light chain phosphorylation* *Cardiac muscle* *Contractility*

1. INTRODUCTION

Phosphorylation of the myosin P-light chain [1] occurs during electrical stimulation of skeletal muscle from frog [2], rabbit [3], mouse [4], and rat [5]. Similarly, phosphorylation of the P-light chain was shown in drug-or KCl-induced contractions of smooth muscle from porcine carotid arteries [6,7], rat uterus [8] and bovine trachea [9]. In contrast, attempts to demonstrate light chain phosphorylation correlated with contractility of mammalian heart gave variable results. Three different laboratories showed that 10–40 s exposure of rabbit or rat hearts to epinephrine or isoproterenol did not increase the phosphate content of the P-light chain under conditions when positive inotropy was observed [10–12], although in one case a 30 s isoproterenol treatment produced a significant increase in light chain phosphorylation [13]. Furthermore, when the hearts were perfused with isoproterenol or adrenaline for several minutes light chain phosphorylation was demonstrated [14,15].

It is difficult to measure light chain phosphorylation by manual freezing of mammalian hearts [10–12] because the hearts beat so quickly that freezing the hearts in a well-defined contractile state, systole or diastole, is almost impossible. We chose turtle hearts, because at cold room temperature (5°C) the turtle hearts beat only 5–8 times/min and can be manually freeze-clamped either in systole or diastole. We separated the phosphorylated and unphosphorylated forms of the P-light chain of turtle heart by two-dimensional gel electrophoresis [16] and found more phosphorylated light chain in heart frozen in systole than in diastole. Similar results were obtained when the hearts were perfused with ^{32}P and the incorporation of [^{32}P]phosphate into the P-light chain was calculated. Some of our findings have been presented [17].

2. METHODS

2.1. *Turtle heart preparation and perfusion*

Turtles *(Pseudemys scriptaelegans)* of 4–5 in. were used. The turtle was pithed and the bottom shell was removed. The innominate aorta was can-

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nulated, all the other vessels were cut, then the heart was removed from the turtle and placed in oxygenated Ringer's solution (145 mM NaCl, 5.6 mM KCl, 2.25 mM $CaCl_2$, 5.6 mM glucose and 2.4 mM Tris–HCl (pH 7.2) at 25°C). The blood was flushed out from the heart. The weight of the heart was under 0.5 g. The cannulated heart was attached to a column, mounted on a stand. The heart rate was 40–50 beats/min at room temperature. The stand with the cannulated heart was brought to a coldroom of 5°C. At 5°C the heart rate decreased to 5–8 beats/min and the heart was manually freeze-clamped with liquid nitrogen-cooled clamps either in the systolic or diastolic state. The freeze-clamped heart was then pulverized in a stainless steel mortar on the surface of frozen perchloric acid under liquid nitrogen [2] and the frozen powder was homogenized in an ice-jacketed Waring blender containing 3% perchloric acid. The residue was isolated by centrifugation at 40 000 × *g*, washed with a solution containing 2% trichloroacetic acid and 5 mM KH_2PO_4 several times and dissolved with the aid of a Brinkman Polytron in 0.2 M Na_2HPO_4 and 6% sodium dodecyl sulfate (SDS). The dissolved proteins were dialyzed against 1000 vol. 0.1% SDS overnight.

For radioactive experiments, a method of retrograde perfusion of turtle hearts was developed using a modified Langendorff perfusion system [18]. The hearts were first perfused with Ringer's solution containing 1–2 mCi carrier-free $^{32}P_i$ for 30 min and subsequently with non-radioactive Ringer's solution for an additional 30 min. The radioactivity of the fluid leaving the heart was monitored to assure that all the $^{32}P_i$ was removed from the extracellular space. Subsequently, the heart with the cannula was attached to the column and transferred to the coldroom of 5°C. The radioactive heart was freeze-clamped in systole or diastole as described before for the non-radioactive heart.

2.2. *Two-dimensional gel electrophoresis*

After the 0.1% SDS dialysis the heart preparations were clarified at 80 000 × *g* for 30 min and the supernatant protein was determined [19]. Aliquots of the supernatants were freeze-dried. The freeze-dried samples were dissolved in a solution containing 9.5 M urea, 2% Nonidet-P40, 0.2 M dithiothreitol, 2% Bio-Lyte, pH 4–6 or pH 3–10. Aliquots of 200 and 250 μg protein were isoelectrofocused in the first dimension and electrophoresed on 15% polyacrylamide slab gels in 0.1% SDS and 0.37 M Tris–HCl (pH 8.8) in the second dimension. Running buffer for the slab gels contained 0.1% SDS, 0.025 M Tris–HCl and 0.19 M glycine (pH 8.45). The gels were stained in 0.2% Coomassie blue, 50% methanol and 5% acetic acid and destained in 40% methanol and 5% acetic acid. The intensity of staining of the phosphorylated and unphosphorylated light chains was measured with a laser scanning densitometer equipped with an automatic integrator. In each light chain spot, the percentage of total intensity was determined from the number of integration units under the appropriate peak. It was assumed that the staining intensities are proportional to the percentage amount of P-light chain in the two different forms.

3. RESULTS AND DISCUSSION

Two-dimensional gel electrophoretic analysis of various hearts showed a large variation in the distribution of phosphorylated and unphosphorylated P-light chain [20]. Only 10–25% phosphorylated light chains was found in dog, cat, and chicken hearts, whereas 70% of the total P-light chain was phosphorylated in turtle heart. The extent of light chain phosphorylation in heart was correlated with the ratio of light chain kinase to phosphatase activity [20].

Fig. 1 compares the two-dimensional gel electrophoretic pictures of turtle hearts frozen in systole and in diastole. The staining patterns (upper panel) illustrate that the P-light chain, at the M_r 19 000 level, is well separated from the other muscle proteins in a trichloroacetic acid-insoluble residue preparation. The P-light chain consists of two forms, phosphorylated (more acidic) and unphosphorylated (more alkaline); they are well separated from each other. The phosphorylated light chain was previously identified by autoradiography [17]. It may be seen that in the heart frozen in systole (left) less light chain remains in the unphosphorylated form than in the heart frozen in diastole (right). The lower panel shows the scans of the P-light chain. From the integration of the areas of the P-light chain spots, 80% of the total P-light chain was found to be phosphorylated

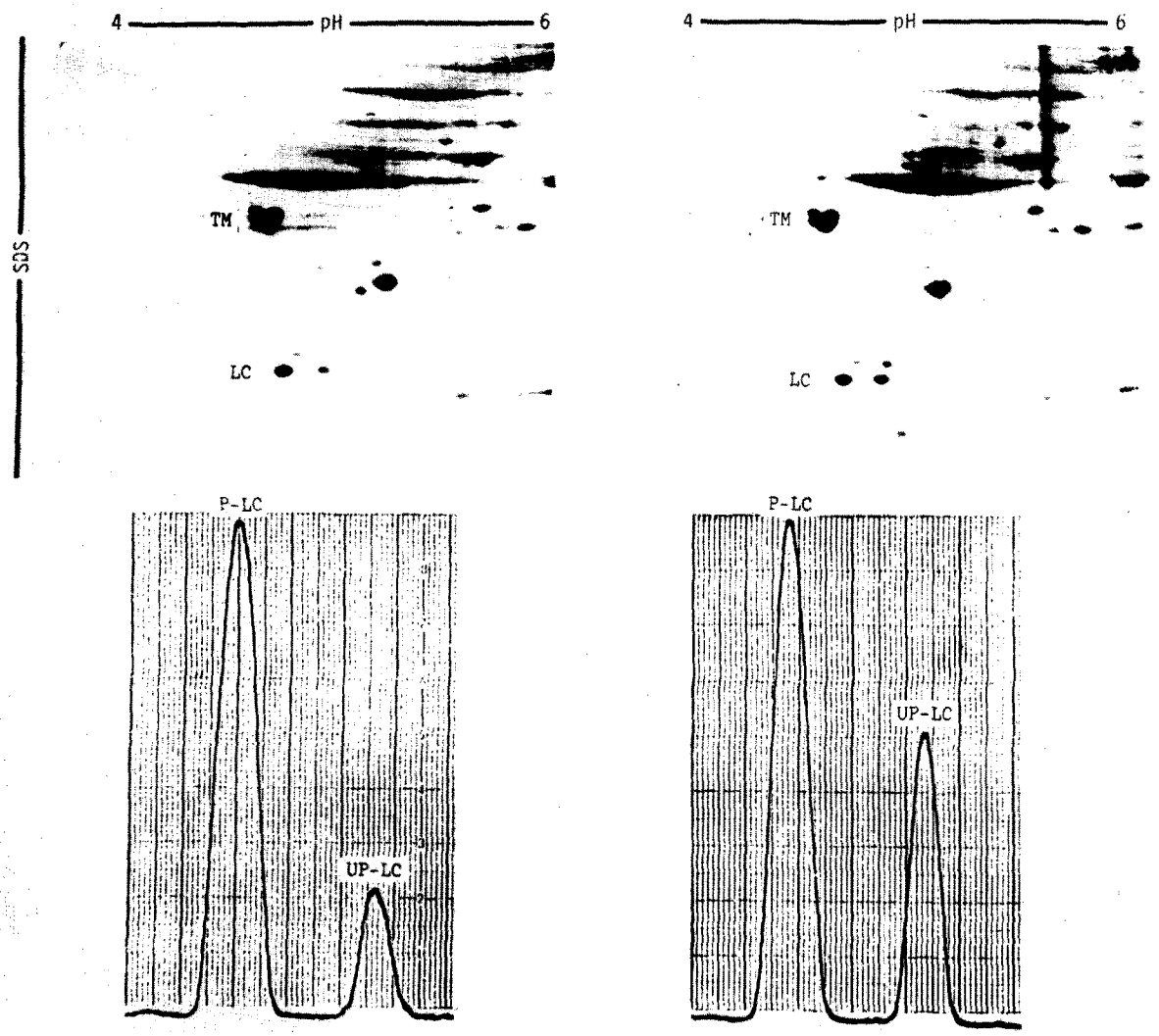

Fig. 1. Two-dimensional gel electrophoretic comparison of the distribution of phosphorylated (P-LC) and unphosphorylated (UP-LC) myosin light chains in trichloroacetic acid-insoluble residues of turtle hearts frozen in systole (left) and in diastole (right). Upper panels show the gel staining profiles and lower panels show the densitometric tracings of the P-light chains (LC). TM refers to tropomyosin.

in the heart frozen in systole (left) and 67% of the P-light chain was phosphorylated in the heart frozen in diastole (right).

Homogenization of frozen hearts in perchloric acid and washing the insoluble residues in trichloroacetic acid completely inhibit myosin light chain kinase and phosphoprotein phosphatase activities. Furthermore, homogenization and washing in these acids precipitate all muscle proteins, leading to isolation of the total P-light chain of the heart.

Table 1 summarizes the results of several experiments in which the percentage phosphorylated light chain of the total P-light chain is compared from hearts frozen in systole and in diastole as determined by densitometry. Each sample was analysed on two-dimensional gels in duplicate and the average values were taken. The data of table 1 show an 11% increase in phosphorylation of the P-light chain from hearts frozen in systole as compared to hearts frozen in diastole. Statistical analysis, by Student's *t*-test of the means revealed

Table 1

Phosphorylated myosin light chain content of turtle hearts frozen in systole and in diastole

Frozen in	Phosphorylated light chain of the total P-ligth chain[a]	(*n*)	mol [^{32}P]phosphate/mol P-light chain	(*n*)
Systole	75.1 ± 7.6%	28	0.75–0.85	3
Diastole	67.4 ± 7.3%	23	0.60–0.70	3

[a]Percentage phosphorylation is given as mean ± standard deviation

this difference was significant to $P<0.001$.

In fig. 2, we compare staining patterns and autoradiograms of total proteins, separated by two-dimensional gel electrophoresis over pH 3–10, from hearts frozen in systole (left) and diastole (right). A large number of protein spots may be discerned in the upper panel, as compared with the very few radioactive spots in the lower panel. (The broad zones in the autoradiograms at the acidic end of the gels are due to labeling of the phospholipids.) The highest amount of radioactivity is confined to the light chain, other radioactive spots are from phosphorylated membrane pro-

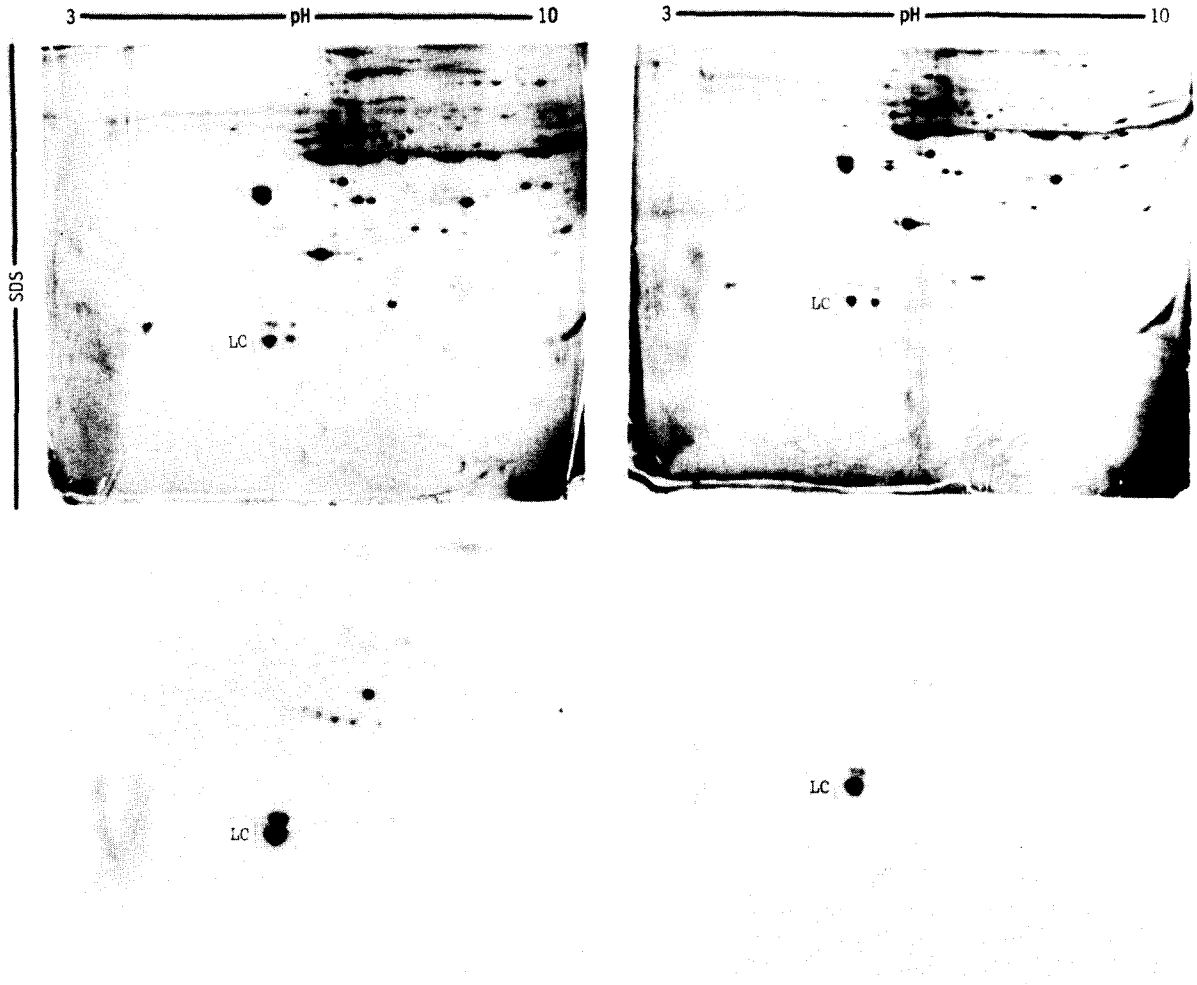

Fig. 2. Two-dimensional gel analysis of trichloroacetic acid-insoluble turtle heart proteins prepared from two comparable hearts, perfused with the same ^{32}P-containing solution. One heart was freeze-clamped in systole (left) and the other in diastole (right). Upper panels show gel staining profiles and lower panels show autoradiographic profiles.

teins. Above the M_r 19 000 light chain a radioactive satellite doublet appears. Its app. M_r 20 000 suggested that it may correspond to phosphorylated smooth muscle myosin light chain; i.e., turtle heart may be composed of both striated and smooth muscle fibers. However, electron microscopy showed no smooth muscle to be present in turtle heart. Visual inspection of the autoradiograms shows more radioactivity in the light chain and satellite spot from heart frozen in systole that that frozen in diastole.

In 3 radioactive experiments, the P-light chain spots were dissected from the two-dimensional gels, dissolved by digestion in 30% H_2O_2 at 110°C, and the radioactivity was determined by liquid scintillation counting. The values were normalized using the specific activity of ^{32}P-labeled phosphocreatine isolated from the same heart [14]. In these experiments, the incorporation of $^{32}P_i$ into P-light chain was 0.75–0.85 mol [^{32}P]phosphate/mol P-light chain in the hearts frozen in systole and 0.60–0.70 mol [^{32}P]phosphate/mol P-light chain in the hearts frozen in diastole (table 1). The percentage phosphorylated light chain of the total P-light chain and the mol [^{32}P]phosphate/mol P-light chain values of table 1 corroborate each other.

These results suggest a role for myosin light chain phosphorylation in turtle heart. The unusually high level of phosphorylation in diastole, that we call the basal level of light chain phosphorylation, may be responsible for the slow contractility of turtle heart. In [3], light chain phosphorylation was shown to reduce the actomyosin ATPase activity in vivo; heart muscles which contract slowly possess a low myosin ATPase [21,22]. The increases in light chain phosphorylation during the systolic state of turtle heart may parallel the increases in phosphorylation found during the contraction of skeletal and smooth muscles. Although the increase in systolic phosphorylation amounts to only 11% relative to that in diastole (table 1), X-ray diffraction studies of beating canine heart show that only 18–19% of the myosin heads are transferred from the vicinity of the thick filaments to that of the thin filaments when the heart enters systole from diastole [23]. Thus, the observed small difference in light chain phosphorylation is compatible with the idea that phosphorylation is involved in the contractile activity of turtle heart.

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