A comparative study of myosin and its subunits in adult and neonatal-rat hearts and in rat heart cells from young and old cultures

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A possible explanation for the decrease in myosin Ca^{2+} -dependent ATPase activity as rat heart cells age in culture is presented. The subunit structure and enzyme kinetics of myosin from adult and neonatal rat hearts and from rat heart cells of young and old cultures are compared. These studies indicate that the loss in Ca-ATPase activity of myosin from older cultures was an intrinsic property of the myosin itself. Myofibrillar fractions from the indicated four sources showed no qualitative or quantitative differences in electrophoretic patterns. Myosin from older cultures was more sensitive to alkaline denaturation than was myosin from younger cultures, as indicated by its more accelerated loss of $\text{K}^+(\text{EDTA})$ -dependent ATPase activity after 10 min of incubation at pH 10. Furthermore, myosin from older cultures was more temperature-sensitive, as indicated by a more rapid loss of Ca-ATPase with decrease in assay temperature. It is suggested that there is either a change in conformation of myosin molecules at or near the active site of the enzyme or alternatively there is a change in light chain 1–light chain 2 and/or light-chain–heavy-chain interaction(s) in the myosin molecules under study.

Cardiac myosin and skeletal myosin have similar hexameric structures, but differ in other specific structural and functional features. Structurally, skeletal myosin contains three classes of light chains, whereas cardiac myosin has only two classes of light chains (Ellenbogen *et al.*, 1960; Perrie & Perry, 1970; Weeds & Frank, 1973). Under physiological conditions and in an intact muscle fibre, myosin is associated with other myofibrillar proteins. The major myofibrillar proteins are myosin, actin, tropomyosin and troponin. Actin binds to myosin to form actomyosin and activates myosin Mg-ATPase activity. Calcium control of ATPase is obtained by the presence of tropomyosin and troponin on the actin filament (Huxley, 1969). Troponin itself is composed of three subunits. These are: troponin T (mol.wt. 31 000), which interacts strongly with tropomyosin; troponin I (mol.wt.

21 000), which inhibits actomyosin ATPase in the absence of Ca^{2+} ; and troponin C (mol.wt. 18 000), which binds four Ca^{2+} ions/molecule *in vitro* (Greaser & Gergely, 1971, 1973). Cardiac myosin subunits are: heavy chain, mol.wt. 200 000; light chain 1, mol.wt. 27 000; and light chain 2, mol.wt. 19 000. The molar ratio heavy chain/light chain 1/light chain 2 in myosin is 2:2:2 (Weeds & Lowey, 1971).

Any changes in ATPase activity with age of enzyme source could be in part accounted for by possible changes in either myosin structure, molecular ratio of its subunits, conformation, and/or turnover of myofibrillar proteins and their subunits. Moreover, mechanical properties of muscles are related to biochemical activity of contractile within the muscle cells (Heller & Whitehorn, 1972). Bhan & Scheuer (1975) and Wilkerson & Evonuk (1971) have reported a 30% increase in Ca-ATPase activity of cardiac myosin from rats conditioned by physical training as compared with sedentary rats. Pressure-overload cardiac hypertrophy as related to dog cardiac myosin ATPase and subunit composition has been reported by Siemankowski & Dreizen (1978*a,b*). Age-dependent decreases in Ca-ATPase activity of rat cardiac myosin (up to 20%) have also been reported (Heller & Whitehorn, 1972). It

Abbreviations used: Ca-ATPase, Ca^{2+} -dependent ATPase; $\text{K}^+(\text{EDTA})$ -ATPase, $\text{K}^+(\text{EDTA})$ -dependent ATPase; Mg-ATPase, Mg^{2+} -dependent ATPase; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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should be noted that Ca-ATPase activity is generally used as a test of myosin integrity *in vitro*, although this activity has little physiological significance. In muscle, only actin-stimulated Mg-ATPase activity is important.

Cardiac myosin ATPase activity may be subject to hormonal control. An increase in cardiac myosin ATPase activity from guinea pigs and rabbits treated with thyroxine has been reported (Hjalmarsen *et al.*, 1970; Goodkin *et al.*, 1974). Hypophysectomized rats show a decrease in myosin ATPase activity that correlates with decrease in cardiac function; the decrease in myosin activity and decrease in cardiac performance have been restored to normal values by thyroxine treatment. Catecholamine and dibutyryl cyclic AMP have been noted to increase Ca-ATPase activity of myosin in cultured rat heart cells (Rovetto *et al.*, 1972; Yazaki & Raben, 1975).

McCarl *et al.* (1969) and Desmond & Harary (1972) have reported a decrease in Ca-ATPase activity per mg of protein of myosin from cultured rat heart cells as the cells age in culture. Cultured rat heart cells are in a non-physiological hormonal state and are exposed to an 'abnormal' work load. Moreover, as these cells get older, a decrease in the rate and strength of beating is observed. This study attempts to explain why myosin from older cultured heart cells has a lower Ca-ATPase activity.

Experimental

Materials

Dithiothreitol, ATP, Tris and Tes were obtained from Sigma. All materials for SDS/polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories. All other chemicals were reagent grade and were obtained from Fisher Scientific Co. Rats were supplied from the Centralized Biological Laboratory, Pennsylvania State University, University Park, PA 16802, U.S.A.

Method of tissue culture and culture characterization

Rat heart cells were cultured as described by Ghanbari & McCarl (1976). Cell growth can be determined by measuring protein content per dish versus time in culture. Cell number per dish and thymidine incorporation per dish can also be measured. During the first several days of culture there is some thymidine incorporation into cultures, but by day 7 these cultures are composed of post-mitotic cells. Protein content per plate also increases until day 7 or 8 and remains constant for the next 10–12 days. Beating rate and beating intensity are optimal by day 2 and remain so up to days 9–12. Beating in cells studied in the present work slowed after day 12 and eventually ceased. We refer to cells 7 and 14 days old as 'younger' and 'older' cells respectively.

Preparation of tissue homogenate

Hearts from 6–18-month-old rats (adult rats) and 3–5-day-old rats (neonatal) were washed in cold iso-osmotic saline (0.155 M-NaCl) solution. Connective tissue was removed and the washed hearts were then minced. Minced tissue was washed in cold iso-osmotic saline solution, after which excess solution was removed from the tissue by blotting. The tissue was weighed and added to 10 vol. of cold 'wash' solution. 'Wash' solution consisted of 80 mM-KCl, 1.0 mM-dithiothreitol and 5.0 mM-Tes buffer, pH 7.0. All steps were carried out at 0–4°C. The pH of Tes and Tris buffers was adjusted using HCl and KOH.

Cultured cells were scraped off the plates with cold saline solution after removing the medium overlaying the cells. The scraped cells were put in a centrifuge tube containing cold iso-osmotic saline solution and centrifuged at 3000g for 5 min. The packed cells were then weighed and resuspended in 10 vol. of wash solution. Minced tissue and cells in wash solution were then homogenized in a like manner by using a Polytron homogenizer at full speed for three bursts of 5 s each. Portions of the homogenates were mixed with equal volume of 0.92 M-KCl and 0.10 M-Tris buffer, pH 7.5, before use in the high-salt Ca-ATPase assay.

Preparation of myofibrillar fraction

Tissue homogenates (in wash solution) were filtered through two layers of cheesecloth into centrifuge tubes. Crude myofibrillar proteins were sedimented by centrifugation of the filtrate at 12000g for 10 min. The pellet was homogenized in 10 vol. of wash solution with a motor-driven Teflon/glass tissue homogenizer. The homogenate next was centrifuged at 12000g for 10 min. The homogenization step followed by centrifugation was repeated four times. The wash solution contained 0.2% Triton X-100 only in the first two homogenizations. Finally, the pellet was homogenized in 3 vol. of wash solution. A portion of this homogenate, the purified myofibrillar fraction, was centrifuged and the pellet was dissolved in 'sample buffer' for gel electrophoresis. Portions of the myofibrillar fraction in wash solution were adjusted also to the conditions of the Ca-ATPase and Mg-ATPase assays. All the above steps were performed at 0–4°C.

Purification of myosin

Portions of the purified myofibrillar fraction in wash solution were adjusted to 0.3 M-KCl (by using 2.0 M-KCl), 5.0 mM-sodium pyrophosphate (by using 0.1 M-sodium pyrophosphate, pH 7.0), and 5.0 mM-MgCl₂ (by using 1.0 M-MgCl₂). Myosin was extracted from the resulting solution by stirring for 15 min. Sodium pyrophosphate and MgCl₂ help

to dissociate myosin from actomyosin. The short extraction time and relatively-low-ionic-strength conditions prevent extensive extraction of contaminating actin (Weeds & Frank, 1973). After 15 min of stirring, the preparations were centrifuged at 12000 *g* for 10 min. The supernatant solution then was centrifuged at 100000 *g* for 2 h. Myosin in the supernatant solution was precipitated by slowly adding 10 vol. of 2.0 mM-EDTA, pH 7.0, to supernatant solutions and stirring for 10 min, and allowing the solutions to set overnight. Precipitated myosin was collected by centrifugation at 12000 *g* for 10 min and the myosin pellet was dissolved in 1.0 M-KCl/0.10 M-Tris buffer solution, pH 7.5. A Teflon/glass tissue homogenizer was used to effect solution. The solution was adjusted to 2.0 mg of protein/ml, 0.5 M-KCl, 0.05 M-Tris, 1.0 mM-dithiothreitol, 5.0 mM-ATP, 5.0 mM-MgCl₂, pH 7.5. Myosin solutions were stirred for 10 min and centrifuged at 70000 *g* for 1 h to remove any actomyosin still present.

Myosin was re-precipitated again by slowly adding 9 vol. of 2.0 mM-EDTA to the supernatant solutions with stirring for 10 min and then leaving the solutions to precipitate for 3 h. Re-precipitated myosin was collected by centrifugation at 12000 *g* for 10 min. The pellets were dissolved in 0.5 M-KCl/1.0 mM-dithiothreitol/1.0 mM-EDTA/5.0 mM-sodium pyrophosphate, pH 7.0, with the aid of a Teflon/glass homogenizer.

In order to remove any contaminating actin still remaining, the myosin solutions were brought to room temperature and adjusted to 0.3 M-KCl with 0.15 M-phosphate buffer, pH 6.5. After 20 min stirring at room temperature, the preparations were centrifuged at 70000 *g* for 1 h. The myosin in the supernatant solution was re-precipitated again and dissolved in 0.5 mM-KCl/1.0 mM-dithiothreitol/1.0 mM-EDTA/0.15 M-Tris buffer, pH 7.5. This myosin solution was centrifuged at high speed to precipitate any polymerized myosin; myosin was precipitated again, washed in properly diluted desired buffer (low ionic strength; 1.0 M), and finally dissolved in the desired buffer and used as a highly purified myosin solution. For (NH₄)₂SO₄ fractionation the desired buffer was 1.0 mM-dithiothreitol/1.0 mM-EDTA/0.05 M-sodium pyrophosphate, pH 7.5, and the final protein concentration was 2.0 mg/ml. The solution was made 2 mM-in ATP just before fractionation with (NH₄)₂SO₄. Myosin precipitated between 38 and 43% (NH₄)₂SO₄ saturation (Wikman-Coffelt *et al.*, 1973) and was then dissolved in 0.5 M-KCl/1.0 mM-dithiothreitol/1.0 mM-EDTA/0.05 M-Tris buffer, pH 7.5. It was then dialysed against the same buffer, and again centrifuged at high speed before it was called 'purified myosin'. This myosin solution was precipitated, washed in properly diluted desired buffer (1.0 M),

and finally dissolved in the desired buffer before use. Myosin solution when mixed with an equal volume of glycerol may be stored at -40°C.

All the above steps were carried out at 0-4°C, except where indicated otherwise.

ATPase assays

The ATPase activity of myosin was assayed by measuring the P_i released from ATP in the presence of enough myosin to liberate 30-150 nmol of P_i in 5 min. The reaction rate is linear with time during this initial period (Fenner *et al.*, 1973).

Ca-ATPase. The total final volume of reaction mixture was 1.0 ml and routinely contained 10 mM-CaCl₂, 0.1 M-KCl, 5 mM-ATP and 0.05 M-Tris buffer, pH 7.5. The reaction was initiated with addition of myosin and continued for 5 min at room temperature. The reaction was stopped by adding 0.5 ml of cold 20% (w/v) trichloroacetic acid solution and precipitated protein was removed by centrifugation. A 1 ml portion of the supernatant solution was used for P_i determination. Variations in pH and/or temperature are presented in the Results section. High-salt Ca-ATPase activity was determined in 0.5 M-KCl, but other conditions were unchanged.

Mg-ATPase. The total final volume of reaction mixture was 1.0 ml and contained 0.1 mM-CaCl₂, 0.1 M-KCl, 5 mM-MgCl₂, 5 mM-ATP and 0.05 M-Tris, pH 7.5. The reaction was initiated with addition of myosin and continued for 5 min at room temperature. The reaction was stopped by adding 0.5 ml of cold 20% trichloroacetic acid solution and the precipitated protein was removed by centrifugation. A 1.0 ml of portion of the supernatant solution was used for P_i determination.

K(EDTA)-ATPase. The total final volume of reaction mixture was 1.0 ml and routinely contained 1.0 mM-EDTA, 0.5 M-KCl, 5 mM-ATP and 0.05 M-Tris, pH 7.5. The reaction was initiated with addition of myosin and continued for 5 min at room temperature. The reaction was stopped by adding 0.5 ml of cold 20% trichloroacetic acid solution and precipitated protein was removed by centrifugation. A 1.0 ml portion of the supernatant solution was used for P_i determination. Variations in pH and/or temperature are explained in the Results section.

Phosphate determination

Inorganic phosphate was determined by the method of Marsh (1959) with minor modifications: (a) the assay was performed in 12.0 ml graduated glass-stoppered centrifuge tubes; (b) the total volume of reaction mixture was 10.0 ml; (c) phase separation was achieved by low-speed centrifugation.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Gel electrophoresis

SDS/polyacrylamide-gel disc electrophoresis of samples was performed by using a method described by Maizel (1971). Densitometry of the Coomassie Brilliant Blue R-250-stained gels was performed on a Gilford spectrophotometer (model 240) equipped with a linear-transport gel carrier.

Precipitin test

This test was performed by the procedure of Lowey & Steiner (1972).

Results

Yield of myosin

Some physical properties of myosin, such as viscosity, solubility in high- or low-ionic-strength buffers and degree of association with other myofibrillar proteins, partially determine the yield in any isolation procedure. Adult- and neonatal-rat heart tissues yielded 2–4 mg of myosin per g of wet tissue, whereas cultured rat heart cells yielded 1–2 mg of myosin per g of wet packed tissue (spun at 3000 g for 5 min). However, when the yields were expressed as myosin per unit of protein, all yields were in the range of 20–40 mg per g of cellular protein. This would indicate that the packed cultured cells have a higher water content than has the intact tissue.

ATPase activity of tissue homogenates

Myosin ATPase activities in tissue homogenates were determined by utilizing a high-salt (0.5 mM-KCl) and Ca^{2+} (10 mM)-containing assay mixture (see the Experimental section). High-salt conditions result in the dissociation of actin from the acto-myosin complex, so that actin activation of myosin Ca-ATPase activity is eliminated. The K-(EDTA)-ATPase activity may not be totally myosin-related, owing to the possible presence of other K^{+} -dependent ATPases. However, high-salt Ca-ATPase activity is myosin-specific. Care was taken to eliminate interfering effects of endogenous P_i in the

samples from different sources by running a control with each assay. In control experiments, myosin (the enzyme) was trichloroacetic acid-inactivated before the addition of ATP substrate, whereas in a normal assay, myosin was trichloroacetic acid-inactivated 5 min after the addition of ATP (see the Experimental section).

Three independent experiments were performed by using adult-rat hearts (from 6- to 18-month-old rats) and neonatal-rat hearts (from 3- to 6-day-old rats) and rat heart cells from young and older cultures (6 and 12 days old respectively). A minimum of six adult-rat hearts, 20 neonatal-rat hearts and 120 plates of cultured cells were used in each experiment. The results are shown in Table 1. The Ca-ATPase activity per mg of protein of rat heart cells from older cultures is significantly lower ($P < 0.05$) than those of adult- and neonatal-rat hearts as well as that of cultured rat heart cells from young cultures. These data verify previous work in this laboratory. However, this experiment alone does not indicate why and where this decrease in Ca-ATPase activity occurs. The following experiments were designed to examine possibilities for this decreased enzyme activity.

ATPase activity of myofibrillar proteins

The values of ATPase activity of myofibrillar fractions from adult- and neonatal-rat hearts and heart cells from young and old cultures are shown in Table 2. The Ca-ATPase activity per mg of myofibrillar protein of cultured rat heart cells from older cultures is again significantly lower ($P < 0.05$) than those of adult and neonatal rat hearts as well as that of cultured rat heart cells from young cultures. This indicates that, if any 'inhibitor' factor is present, it is in the myofibrillar fraction itself, since exhaustive washing should have eliminated any exogenous ATPase inhibitor. Moreover, when Mg-ATPase activity was determined in the myofibrillar fractions from the four sources, all had similar activities, as is shown in Table 2 ($0.20 \pm$

Table 1. Ca-ATPase activity of tissue homogenates from adult- and neonatal-rat hearts and heart cells from younger and older cultures

High-salt Ca-ATPase activity was determined in 1 ml of 0.05 M-Tris buffer, pH 7.5, containing 0.5 M-KCl, 10 mM- CaCl_2 , 5 mM-ATP, and enough myosin to liberate 30–150 mmol of P_i in 5 min at room temperature. The reaction was stopped by adding 0.5 ml of cold 20% trichloroacetic acid solution, and P_i was determined by using a method described by Marsh (1959) with minor modifications. Values are given as means $\pm$ S.D. ($n = 3$).

Source of tissue	Ca-ATPase activity (high-salt) (μmol of P_i /min per mg of total protein)
Adult-rat hearts	0.18 ± 0.024
Neonatal-rat hearts	0.20 ± 0.035
Heart cells from younger cultures	0.17 ± 0.031
Heart cells from older cultures	$0.10 \pm 0.020^*$

* Significantly different from other values ($P < 0.05$) as determined by Student's t test.

0.03 μmol of P_i /min per mg of protein). It follows that a change in Ca-ATPase activity in older cultures is due to a change within the myofibrillar fraction and possibly within the myosin molecule itself. The following series of experiments were designed to answer more specifically the question of how the inactivation occurs.

ATPase activity of purified myosin

Myosins from adult- and neonatal-rat hearts, and cultured rat heart cells from younger and older cultures, were purified according to the procedure described in the Experimental section. The myosin preparations were highly pure, as indicated by precipitin test, A_{280}/A_{260} ratio and SDS/polyacrylamide-disc-gel electrophoresis. The A_{280}/A_{260} ratio was 1.40 or higher. These highly purified myosins were compared kinetically by utilizing both K-(EDTA)- and Ca-ATPase activities.

K-(EDTA)-ATPase activity. Although the physiological importance of K-(EDTA)-ATPase is not clear, it is nevertheless one of the properties of the myosin molecule and should not be ignored when a comparative study is attempted. K-(EDTA)-ATPase activities of highly purified myosins from adult- and neonatal-rat hearts and rat heart cells from younger and older cultures were determined, and the results are shown in Table 3. There were no significant differences among the K-(EDTA)-ATPase activities of myosin from the four sources (0.55 μmol of P_i /min per mg of myosin).

When the temperature-dependence of K-(EDTA)-ATPase of the myosins was tested, myosins from all four sources followed the same curve (from 10 to 35°C) and showed no segregation (within experimental error) (results not shown). However, when the myosins were incubated in 0.1M-Tris/glycine buffer, pH9.0, the myosin from rat heart cells of older cultures lost more than 50% of its K-(EDTA)-ATPase activity after 10min incubation at room temperature, whereas myosins from rat heart cells

of young cultures as well as myosins from adult- and neonatal-rat hearts showed less than 10% loss of the same period of incubation. The effect of incubation at pH9.0 on K-(EDTA)-ATPase activity of myosins at room temperature from the four sources is shown in Fig. 1. This experiment

Fig. 1. Effect of incubation at pH9.0 on K-(EDTA)-ATPase activity of myosin from different sources K-(EDTA)-ATPase activity was determined as explained in Table 3. The time in the Figure refers to the duration of incubation of purified myosin in 0.1M-Tris/glycine buffer, pH9.0, at room temperature. Each point represents an arithmetic mean of values obtained in three determinations. ○, Myosin from neonatal-rat hearts; ●, myosin from adult-rat hearts; □, rat heart cell myosin from younger cultures; △, rat heart cell myosin from older cultures.

Table 2. ATPase activity of myofibrillar fractions from adult- and neonatal-rat hearts and heart cells from younger and older cultures

High-salt Ca-ATPase activity was determined as described in Table 1; Mg-ATPase activity was determined in the same way, except that the reaction buffer was 0.05M-Tris, pH7.5, containing 5mM-MgCl₂, 0.1mM-CaCl₂, 0.1M-KCl and 5mM-ATP. Values are given as means $\pm$ s.d. (n = 3).

Source of tissue	Activity (μmol of P_i /min per mg of myofibrillar protein)	
	Ca-ATPase (high-salt)	Mg-ATPase
Adult-rat hearts	0.28 $\pm$ 0.026	0.20 $\pm$ 0.036
Neonatal-rat hearts	0.30 $\pm$ 0.033	0.21 $\pm$ 0.039
Heart cells from younger cultures	0.26 $\pm$ 0.039	0.19 $\pm$ 0.038
Heart cells from older cultures	0.17 $\pm$ 0.021*	0.20 $\pm$ 0.033

* Significantly different from other values (P < 0.05) as determined by Student's t test.

Table 3. *ATPase activity of purified myosins from adult- and neonatal-rat hearts and heart cells from younger and older cultures*

K-(EDTA)-ATPase and Ca-ATPase activities were determined as explained in Table 1, except that the incubation mixture (0.05M-Tris, pH 7.5) contained 1.0mM-EDTA, 0.5M-KCl and 5mM-ATP for K-(EDTA)-ATPase and 10mM CaCl₂, 0.1 M-KCl and 5mM-ATP for Ca-ATPase. Values are given as means $\pm$ S.D. ($n = 3$).

Source of tissue	Activity ($\mu\text{mol of P}_i/\text{min per mg of myosin}$)	
	K (EDTA)-ATPase	Ca-ATPase
Adult-rat hearts	0.56 ± 0.065	0.90 ± 0.083
Neonatal-rat hearts	0.57 ± 0.063	1.15 ± 0.103
Heart cells from younger cultures	0.54 ± 0.075	0.80 ± 0.085
Heart cells from older cultures	0.53 ± 0.078	$0.55 \pm 0.075^*$
Neonatal-rat hearts and heart cells from older cultures mixed in ratio of 1 : 1	0.53 ± 0.091	0.88 ± 0.098

* Significantly different from other values ($P < 0.05$) as determined by Student's t test.

indicates that there is a change in the myosin molecule of rat heart cells from older cultures with respect to those from the other sources that renders it more sensitive to alkaline pH.

Ca-ATPase activity. Ca-ATPase activities of highly purified myosin from adult- and neonatal-rat hearts and rat heart cells from young and old cultures were determined and the results are shown also in Table 3. Myosin from adult heart and heart cells from younger cultures had similar Ca-ATPase activities, which were lower than Ca-ATPase activity of myosin from neonatal-rat hearts. The myosin from rat heart cells of older cultures had lower Ca-ATPase activity than myosin from any of the three other sources ($P < 0.05$). Moreover, when myosin from rat heart cells of older cultures was mixed (1:1) with myosin from neonatal-rat hearts, the resultant Ca-ATPase activity was additive and no synergistic effect was seen (Table 3). This eliminates the possibility of the involvement of any activator of Ca-ATPase activity.

When the temperature-dependence of Ca-ATPase of the myosins was examined, it was observed that myosin from rat heart cells of older cultures behaved differently than myosins from the other three sources. The apparent enthalpies of Ca-ATPase activation at 25°C were 24.3, 23.9, 26.0 and 29.3 kJ (5.8, 5.7, 6.2 and 7.0 kcal/mol) for myosin from adult- and neonatal-rat hearts and myosin from rat heart cells in younger and older cultures respectively. These values were calculated from the data in Fig. 2 by using the following formula (Yazaki & Raben, 1975):

$$\Delta H^* = R[d(\ln V)/d(T^{-1})] - RT$$

where ΔH^* is the apparent enthalpy of activation, R is the molar gas constant, V is the Ca-ATPase activity and T is the absolute temperature. These data indicate that the kinetically less active myosin

Fig. 2. *Temperature-dependence of Ca-ATPase activity of myosin from different sources*

Ca-ATPase activity was determined as explained in Table 3, except that the reaction mixture was equilibrated at the specified temperature before adding myosin and the temperature was held throughout the reaction. Each point represents an arithmetic mean of values obtained in three determinations. Each value varied from the mean by less than 10%. ○, Myosin from neonatal-rat hearts; ●, myosin from adult-rat hearts; □, rat heart cell myosin from younger cultures; △, rat heart cell myosin from older cultures.

from older cultured rat heart cells is more temperature-sensitive.

It has been observed that myosin lability at pH 9.0 is minimal in the presence of Ca²⁺ (Yazaki & Raben, 1975), and, furthermore, Ca²⁺ solubility in

the presence of phosphate decreases at high pH values. Hence it was not feasible to determine the differential inactivation at alkaline pH for Ca-ATPase activity of myosin from different sources.

Gel electrophoresis

Electrophoresis experiments were performed on the myofibrillar fraction and myosin from adult- and neonatal-rat hearts and from rat heart cells from younger and older cultures concomitant with kinetic studies.

The SDS/polyacrylamide-disc-gel-electrophoretic patterns of the myofibrillar fraction from adult and neonatal rat hearts and from rat heart cells from younger and older cultures were similar with respect to the bands corresponding to myo-

fibrillar proteins. Scanning of the gels (Fig. 3) did not reveal either any shift in molecular weight or disappearance of any of the myofibrillar proteins of the myofibrillar fractions from the four different sources. This is in agreement with kinetic and radioimmunoassay studies that we have performed.

For gel electrophoresis of purified myosin, 25 μ l samples containing 50 μ g of protein were separated on SDS/12%-polyacrylamide gels. The gels showed three major bands, corresponding to heavy chain (mol.wt. 200000), light chain 1 (mol.wt. 27000), and light chain 2 (mol.wt. 19000). The SDS/polyacrylamide-disc-gel-electrophoretic patterns of highly purified myosins from adult- and neonatal-rat hearts, and from rat heart cells from younger and older cultures, were quite similar, and scanning of the gels of myosin from all four different

Fig. 3. Densitometric scans of SDS/polyacrylamide gels from the electrophoresis of myofibrillar protein fraction from different sources

Samples (50 μ l) containing 100 μ g of protein was separated on a 4% stacking gel and 12% running gel by using a method described by Maizel (1971). Densitometry of the Coomassie Brilliant Blue R-250-stained gels was performed on a Gilford spectrophotometer (model 240) equipped with a linear-transport gel carrier. Sources were: (a) neonatal-rat hearts; (b) adult-rat hearts; (c) younger cultured cells; (d) older cultured cells. Abbreviations used: HC, heavy chain; A, actin; TN-T, troponin T; TM, tropomyosin; TN-I, troponin I; TN-C, troponin C; LC₁, light chain 1; LC₂, light chain 2; U₁ and U₂, high- and low-molecular-weight intermediates (unknown).

Fig. 4. Densitometric scans of SDS/polyacrylamide gels from the electrophoresis of purified myosin from different sources. Samples (20 μ l) containing 100 μ g protein were placed on each gel. Other conditions were identical with those in Fig. 3. Sources were: (a) adult-rat hearts; (b) neonatal-rat hearts; (c) younger cultured cells; (d) older cultured cells. Abbreviations used: HC, heavy chain; TM, tropomyosin; LC₁, light chain 1; LC₂, light chain 2; U₁ and U₂, high- and low-molecular-weight intermediates (unknown).

sources (Fig. 4) did not reveal either any shift in molecular weights for light chain 1 and light chain 2 or any significant change in light chain 1/light chain 2 ratio or any appearance of a 'light chain 3'. Furthermore, a mixture of myosins from the four different sources in a ratio of (1:1:1:1) gave gels with patterns similar to those of each of the component myosins in the area of light chain 1 and light chain 2 bands. We did not observe multiple forms of rat cardiac myosin, reported by Hoh *et al.* (1978), in our system. However, these results do not preclude the possibility that isoenzyme forms of myosin may be changing as the cultured cells age.

Discussion

Systematic study of the enzymic properties of myosins isolated from adult- and neonatal-rat hearts and rat heart cells from younger and older cultures indicated that the loss in Ca-ATPase activity of

myosin obtained from older cultures was an intrinsic property of the myosin itself, and that there was no ATPase inhibitor involved. This result was supported also by using a radioimmunoassay technique (results not shown). Parallel studies using gel electrophoresis did not show any loss of bands or appearance of new bands in the region of light chains in the gels. The lower Ca-ATPase activity of myosin from older cultures occurred concomitantly with a decrease in the rate and intensity of beating.

Moreover, kinetic studies of myosins from adult- and neonatal-rat heart cells and rat heart cells from younger and older cultures showed that the myosin from cells of old cultures was more sensitive to alkaline denaturation with respect to K-(EDTA)-ATPase activity and more temperature-sensitive with regard to Ca-ATPase activity. These results may indicate either a change in conformation of the myosin molecules at or near the active site

of the enzyme or alternatively a change in light chain 1–light chain 2 and/or light chain–heavy chain interaction in the molecule.

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