

Expression of myosin isoenzymes in cardiac-muscle cells in culture

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Myosin isoenzyme profiles of rat and chicken embryonic cardiac myocytes were studied during differentiation and growth *in vitro* by native-gel electrophoresis and assay of Ca^{2+} -activated ATPase. The electrophoretic pattern of myosin extracted from 18-day-embryonic-rat myocytes after 7 days in culture exhibits three isoenzyme bands, V1, V2 and V3, of which the slow-migrating V3 is predominant. This resembles the isoenzyme profiles from 18–20-day-embryonic ventricles *in vivo*. However, the isoenzyme profile of the 7-day-old culture differs from that of its counterpart *in vivo*, as well as from that of the young and adult rat ventricles, the last two containing the predominant fast-migrating component, V1. When embryonic cardiac myocytes were grown *in vitro* for 7 days in a medium containing a physiological concentration of L-thyroxine (T_4), myosin isoenzyme profiles of these cells shifted to the adult form, with isoenzyme V1 predominating after day 4 of culture. The 7-day-old intact embryonic-chicken ventricles and isolated myocytes showed a single myosin isoenzyme band after 7 days of culture that resembles the pattern seen for the adult chicken. T_4 had no effect on the electrophoretic mobility of this isoenzyme pattern. ATPase activity of isoenzyme V1 in cultured rat myocytes treated with T_4 was comparable with that of V1 in the untreated adult heart. This study demonstrates that ATPase activity of the chicken myosin isoenzyme is significantly lower than that of isoenzyme V1, but is comparable with that of rat V3. This study shows that the expression of myosin isoenzyme profiles in cultured rat cardiac myocytes does not fully represent the situation *in vivo*. Physiological concentrations of T_4 can modulate the predominant foetal-type isoenzyme V3 to the adult type V1 in cultured embryonic-rat cardiac myocytes within a brief period.

Cardiac myosins exist in various forms in some species (Hoh *et al.*, 1977; Sartore *et al.*, 1978, 1981; Lompre *et al.*, 1979, 1981). Different forms of myosin differ from one another in structure and ATPase activities (Hoh *et al.*, 1979; Scheuer & Bhan, 1979; Pope *et al.*, 1980; Lompre *et al.*, 1981). Hoh *et al.* (1977) demonstrated the presence of three myosin isoenzymes in rat ventricle by electrophoresis of native myosin in pyrophosphate gels, and called them V1, V2 and V3 in the order of their decreasing electrophoretic mobility. V1 is predominant in young and adult rat ventricles and V3 in embryonic ventricles. Myosin isoenzyme profiles appear to change in the ventricles of some species under different physiological conditions (Carey *et al.*, 1979; Maughan *et al.*, 1979; Klotz *et al.*, 1981) or after treatment with T_4 (Goodkind *et*

al., 1974; Yazaki & Raben, 1975; Hoh *et al.*, 1977; Flink *et al.*, 1978; Chizzonite *et al.*, 1982).

Since cell culture is a useful system for investigating the mechanisms which control the expression of specific programmes of differentiation, it is important to establish whether the myosin isoenzyme profile observed from heart cells *in vivo* is also seen in isolated single cardiac-muscle cells in culture. Furthermore, does the isoenzyme profile show a shift in its pattern during different stages of development similar to that observed *in vivo*? Studies with cardiac- and skeletal-muscle cell cultures (Breton *et al.*, 1980; Bader *et al.*, 1982) indicated that certain protein profiles of such cells differed from those of their counterparts *in vivo*. Since cell cultures have been used in many studies as model systems for the situation *in vivo*, the present study was undertaken to examine whether cultured myocytes undergoing differentiation exhibit

Abbreviation used: T_4 , thyroxine.

myosin isoenzyme profiles similar to that observed in the heart *in vivo*. Specifically, we have determined the electrophoretic properties of myosin from rat and chicken embryonic cardiac myocytes at different times of culture in a serum-free chemically defined medium during differentiation. Also, we have examined the effects of thyroid hormone on the expression of myosin isoenzyme profiles in these cells.

Materials and methods

Cell culture

Heart cells were isolated from ventricles of 18-day rat embryos and 7-day chicken embryos by a modification of our previous methods (Nag *et al.*, 1981; Nag & Cheng, 1983). Minced ventricular tissue was incubated in a solution consisting of 0.25% trypsin, 0.025% collagenase, 4% chicken serum and 96% Ca^{2+} - and Mg^{2+} -free Tyrode solution. The tissue pieces were then rinsed with a serum-free chemically defined culture medium, containing 99% F-12 (Ham's) nutrient mixture without glutamine, fetuin (0.025%) (Sigma Chemical Co., St. Louis, MO, U.S.A.), bovine serum albumin (1%) (Sigma), ascorbic acid (0.02 mg/ml) (Sigma), endothelial-cell growth supplement (50 $\mu\text{g}/\text{ml}$) (Collaborative Research, Lexington, MA, U.S.A.), epidermal growth factor (10 ng/ml) (Collaborative Research) and insulin/transferrin/selenium mixture (0.1 ml/ml) (Collaborative Research), and subsequently dispersed into a single-cell suspension in the same culture medium. After filtration and resuspension in the above culture medium, cells were plated at a density of 1×10^6 cells/35 mm dish and cultured for 7 days in an incubator in a humidified atmosphere of 5% CO_2 in air. The cell cultures were examined for contractility of the cells routinely with the phase-contrast microscope and terminated at intervals of 1, 4 and 7 days of culture for biochemical studies. For the studies of the effect of thyroid hormone on the myosin isoenzyme profiles in cultured cardiac ventricular myocytes, cells were exposed to 10 nM T_4 in culture media for 7 days. The media were changed every other day, and 12 such experiments were performed.

Electrophoretic analysis of myosin isoenzymes

Preparation of myosin and gels. Ventricular cells were scraped out of the plates with a rubber policeman at selected intervals as mentioned above; myosin extraction was usually conducted immediately after harvest of cells. Sometimes harvested cells were stored in 50% (v/v) glycerol at -20°C for extraction of myosin at a convenient time. We did not observe any differences in results between myosins from freshly harvested cells and from

stored cells. The cells were initially washed in a buffer (pH 7.0) containing 40 mM-NaCl, 5 mM-EGTA, 3 mM- Na_2HPO_4 and 1 mM-phenylmethanesulphonyl fluoride, and homogenized in the same buffer with a Dounce homogenizer. The homogenate was then centrifuged at 3640 g for 10 min, and the pellet was immersed and centrifuged (49000 g, 3 h) in a modified extraction solution (Hoh *et al.*, 1977) containing 100 mM- $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM-EGTA, 15 mM-2-mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride and leupeptin (2 $\mu\text{g}/\text{ml}$) (pH 8.8). The supernatant was collected and used for electrophoresis. Myosin from intact embryonic ventricles was prepared by extracting tissue homogenate with 20 vol. of the above extraction buffer. Protein concentration in solutions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Electrophoresis. Polyacrylamide gels were prepared with 3.88% acrylamide and 0.12% bisacrylamide in a buffer containing 30 mM- $\text{Na}_4\text{P}_2\text{O}_7$ (pH 8.8), 1 mM-EDTA, 2 mM-cysteine and 10% (v/v) glycerol. Myosin samples (1–20 μl) in 50% glycerol were loaded directly on the top of the gels and electrophoresed overnight (17–20 h) with a constant voltage gradient of 11 V/cm. Gels were stained for protein in Coomassie Brilliant Blue R solution. Densitometer tracings of the gels were obtained by using a LKB-2202 Ultrascan laser densitometer equipped with an integrating printer-plotter.

Measurements of Ca^{2+} -activated myosin ATPase in gels

Ca^{2+} -activated myosin ATPase activity of cultured cardiac myocytes, intact embryonic and adult ventricles of rat and chicken was determined in gels containing myosin isoenzymes, by the procedure of Hoh *et al.* (1976, 1977). The absorbance ratio (calcium phosphate:protein) was plotted against incubation time, and the slope of the regression line was taken as a measure of the specific ATPase activity of myosin in the gels and expressed in arbitrary units per minute. Three to five gels were run for the same sample.

ATPase activity was also determined by a modified cytochemical method (Allen & Hyncik, 1963) adapted for use in gels by Hoh (1975) and quantified as in the above method.

Results

Myosins from rat and chicken embryonic cardiac myocytes in culture were examined by native-gel electrophoresis and compared with myosin of embryonic and adult rat and chicken cardiac myocytes *in vivo*. Myosin was also compared in cardiac myocytes at different periods of culture in a serum-

free chemically defined culture medium after treatment with thyroid hormone.

Electrophoretic pattern of native myosin

Rat cardiac myocytes in vitro and in vivo. The electrophoretic patterns of myosin extracted from 18-day intact rat ventricles and isolated cardiac myocytes after 1, 4 and 7 days of culture were alike, exhibiting three myosin isoenzyme bands, V1, V2 and V3, the slow-migrating V3 form being predominant (Figs. 1a and 1b; Table 1). The V2 band was not as clear as those for isoenzymes V3 and V1. However, these isoenzyme profiles of 4- and 7-day cultured myocytes differed from those of their comparable counterparts *in vivo* (myocytes of 1- and 4-day-old hearts *in vivo*) and from young and adult

rat ventricles *in vivo*, which contained a predominant fast-migrating V1 isoenzyme (Fig. 1c). The observations on adult myosin isoenzyme profiles conformed with those by others (Hoh *et al.*, 1977; Lompre *et al.*, 1981; Clark *et al.*, 1982). When embryonic cardiac myocytes in culture were grown in a medium containing a physiological concentration of T₄ (10nM), myosin isoenzyme profiles of those myocytes shifted after 4 days to an adult form, with isoenzyme V1 being predominant (Fig. 2, Table 1). However, myosin extracted from the same culture grown for 7 days showed a more prominent band of V1 than did those grown for 4 days.

Chicken cardiac myocytes in vitro and in vivo. Embryonic (7 day)-chicken ventricles and isolated cardiac myocytes from ventricles of the same age group exhibited a single myosin isoenzyme band

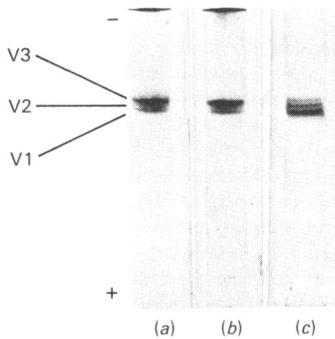


Fig. 1. Myosin isoenzyme profiles and mobility on native-gel electrophoresis in embryonic-rat cardiac myocytes *in vivo* and *in vitro*

Electrophoretic analysis was carried out on myosin obtained from ventricular cardiac myocytes and left ventricle as described in the Materials and methods section. Patterns observed after migration for 24 h at 11V/cm are shown. Lanes: (a) 18-day-embryonic ventricle *in vivo*; (b) 7-day-culture embryonic-rat cardiac myocytes isolated from the same source as above; (c) 1-day-old rat heart.

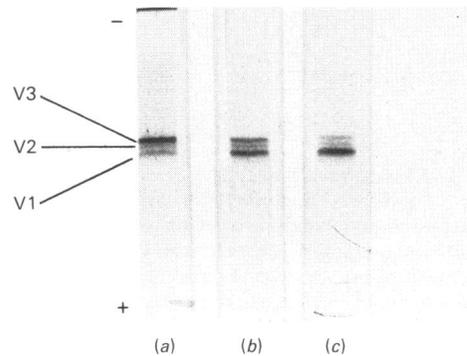


Fig. 2. Changes in myosin isoenzyme profiles in embryonic-rat cardiac myocytes in culture

Electrophoretic analysis of myosin from: (a) 7-day-old control culture without T₄ in the culture medium; (b) 4-day-old culture with 10nM-T₄ in the medium; (c) 7-day-old culture with 10nM-T₄ in the medium.

Table 1. Analysis of myosin isoenzyme contents of embryonic-rat cardiac myocytes in culture and in postnatal hearts. Isoenzymes were measured by graphic resolution of absorbance peaks for protein and measuring the area under the peaks. Isoenzymes V1 and V3 only were measured for cultured cells. Values given are means ± S.D. for seven determinations.

Isoenzyme	Percentage of myosin isoenzyme in total myosin						
	Cell culture				Heart		
	Serum- and T ₄ -free media		Serum-free media with T ₄		1-day neonatal rat	110g rat	450g rat
	4-day	7-day	4-day	7-day			
V3	78 (±5)	79 (±4)	35 (±4)	4 (±1)	6 (±2)	13 (±4)	25 (±4)
V2					29 (±5)	30 (±6)	31 (±5)
V1	19 (±3)	18 (±4)	60 (±9)	95 (±3)	65 (±9)	57 (±8)	44 (±7)

after 1, 4 and 7 days of culture. This pattern was also observed in the adult chicken (Fig. 3). When cardiac myocytes were exposed to a physiological or higher concentration of T_4 in the culture media for 1-3 weeks, the mobility of the myosin isoenzyme did not change. The co-electrophoresis of embryonic-chicken myosin with embryonic-rat myosin as a reference distinguished chicken myosin from that of the rat, because of its faster mobility (Fig. 3). This difference in the mobility of myosin isoenzyme profiles was also observed between adult chicken and rat (Fig. 3).

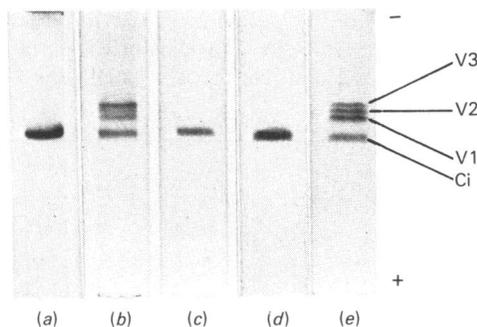


Fig. 3. Electrophoretic analysis of ventricular myosin from embryonic and adult rats and chickens
 Electrophoretic analysis of myosin from: (a) 7-day-embryonic-chicken ventricle *in vivo*; (b) co-electrophoresis of myosin from 7-day-cultured embryonic-rat and -chicken ventricular cardiac myocytes; (c) 7-day-cultured chicken cardiac myocytes; (d) adult chicken left ventricle; (e) co-electrophoresis of myosin from adult rat and chicken left ventricles. Abbreviation: Ci, chicken isoenzyme.

Ca²⁺-activated ATPase activity

The ATPase activity of the isoenzyme V3 of embryonic-rat cardiac myocytes in culture was comparable with that of the embryonic ventricles *in vivo* (Table 2). This ATPase activity was signifi-

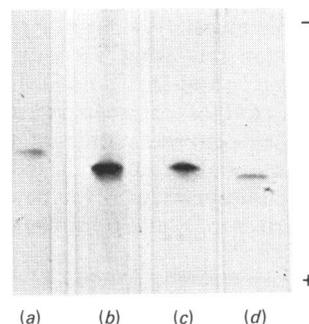


Fig. 4. Cytochemical staining of Ca^{2+} -activated myosin ATPase reaction product on gels
 Gels were incubated in ATPase assay medium for 2h at 37°C; the white bands which appeared were subsequently converted into brownish-black bands by the lead precipitation method as described in the Materials and methods section. Myosins from: (a) 7-day control cultured embryonic-rat cardiac myocytes without T_4 treatment, showing less staining intensity of ATPase reaction product on the V3 band; (b) 7-day-cultured embryonic-rat cardiac myocytes treated with T_4 , exhibiting high staining intensity of the reaction product on the V1 band; (c) 450 g-rat left ventricle, showing high staining intensity of the reaction product on the V1 band; (d) 7-day-cultured embryonic-chicken cardiac myocytes, with less staining intensity of ATPase reaction product. Note highest staining intensity on the V1 band.

Table 2. Ca^{2+} -activated myosin ATPase activities of rat and chicken cardiac myocytes *in vivo* and *in vitro* as measured in gels with and without cytochemical staining (see the text)

ATPase activities are given as means \pm s.d. for six determinations in arbitrary units/min as defined in the Materials and methods section. ATPase activity of chicken isoenzyme is not included under V1 or V3, since this isoenzyme has not been named as it has been in rats.

Isoenzyme	ATPase activity	
	V1	V3
Rat embryonic ventricles		0.19 (\pm 0.05) 0.16 (\pm 0.03)*
Rat embryonic ventricular myocytes in 7-day culture in serum- and T_4 -free media		0.17 (\pm 0.03) 0.14 (\pm 0.03)*
Rat embryonic ventricular myocytes in 7-day culture in serum-free and T_4 -containing media	0.67 (\pm 0.15) 0.64 (\pm 0.13)*	
Adult rat ventricle	0.75 (\pm 0.17) 0.72 (\pm 0.14)*	
Chicken embryonic (<i>in vitro</i> and <i>in vivo</i>) and adult (<i>in vivo</i>) ventricular myocytes		0.21 (\pm 0.07) 0.18 (\pm 0.04)*

* Data from gels with cytochemical staining.

cantly lower ($P < 0.001$) than that of the isoenzyme V1 of the cultured cardiac myocytes treated with T_4 . ATPase activity of this V1 isoenzyme was comparable with that of the adult rat ventricles (Table 2). Although the electrophoretic mobility of the myosin isoenzyme of embryonic (*in vitro* and *in vivo*) and adult (*in vivo*) chicken was faster than that of isoenzyme V1 of the adult rat ventricle *in vivo* and embryonic-rat cardiac myocytes treated with T_4 *in vitro*, the ATPase activity of the adult and embryonic-chicken myosin isoenzyme was lower than that of the V1 of the adult rat ventricle and T_4 -treated cultured embryonic-rat cardiac myocytes (Table 2). This difference in ATPase activity between chicken and rat cardiac isoenzymes is statistically significant ($P < 0.005$). However, the ATPase activity of chicken myosin isoenzyme was comparable with that of V3 of the embryonic-rat cardiac myocytes. The staining of Ca^{2+} -activated-ATPase reaction product, i.e. calcium phosphate, with a cytochemical technique which increased the intensity of the colour reaction of the final reaction product (i.e. lead sulphide) also enabled us to quantify the high and low ATPase activities of the control and T_4 -treated rat cardiac myocytes in culture and of adult rat and chicken myosin isoenzymes. ATPase activity as determined with this technique (Fig. 4) agreed fully with the above findings (Table 2).

Discussion

Electrophoretic analysis and Ca^{2+} -activated-ATPase assay have provided evidence that rat myosin isoenzyme profiles and ATPase activities of cardiac ventricular myocytes in culture differ from those of ventricles *in vivo*. When myosin isoenzyme profiles and ATPase activity of 1-day-cultured cardiac myocytes were compared with those of their counterpart *in vivo*, they conformed well with each other. However, 4- and 7-day-cultured cardiac myocytes did not exhibit similarities to their comparable counterparts *in vivo* with respect to these properties. The isoenzyme profiles and ATPase activity of the comparable cardiac myocytes *in vivo* (1- and 4-day-old) were similar to those of adult myocytes, with V1 being predominant and containing higher ATPase activity than V3. Cardiac myocytes even after 7 days of culture exhibited embryonic isoenzyme profiles and ATPase activity different from those of their comparable counterparts *in vivo*. It is noteworthy that the physiological concentration of T_4 changes the predominant foetal-type myosin isoenzyme V3 to the predominant adult type V1 in rat embryonic cardiac myocytes in culture within a brief period, in contrast with rabbit hearts *in vivo* and hearts from hypophysectomized and hypothyroid rats,

which require a prolonged treatment with a higher dose of the same hormone (Hoh *et al.*, 1977; Banerjee & Morkin, 1977; Clark *et al.*, 1982). It can be argued that this rapid change in the myosin pattern in cultured cells reflects a situation *in vivo* that requires a physiological concentration of T_4 . The evidence that supports this interpretation is the observation of the shift of isoenzyme pattern in the newborn rats, corresponding to the 4-day-cultured cardiac myocytes, which contain predominantly V1 at this early stage of development onward. The synthesis of isoenzyme V1 in newborns is probably triggered by the physiological alterations that occur at birth. Physiological alterations involve hormonal imbalance, including hyperthyroidism (Fischer *et al.*, 1977) in newborns, cardiac supersensitivity to noradrenaline (Friedman, 1972) and other factors. The increase in velocity of shortening of cardiac muscle of the newborns is greatly influenced by these factors and can be correlated well with the synthesis of the isoenzyme V1.

There is a possibility that this change in the isoenzyme pattern could take place earlier than day 4 of culture, since our experimental protocol excluded analyses of 2- and 3-day-old cultures. It was expected that, after exposure of the cardiac myocytes to the thyroid hormone, the shift of their isoenzyme patterns in culture would be reflected in their ATPase activity, since it is well known that Ca^{2+} -activated myosin ATPase activity in V1 (Hoh *et al.*, 1977; Lompre *et al.*, 1981) is the highest for all three isoenzymes. In accordance with the expectation, the findings of the electrophoretic mobility of myosin isoenzymes in control and experimental cardiac myocytes in culture agreed well with their ATPase activities, showing the presence of a lower ATPase activity in the predominant V3 of the control embryonic cardiac myocytes and higher ATPase activity in isoenzyme V1 of the experimental culture. These findings indicate that the monolayer cell culture with a proper concentration of thyroid hormone shows a situation similar to that *in vivo*, where target cells respond quickly to the hormone in circulation.

Chicken myocytes exhibited a single band of myosin isoenzyme. This was the case for all the cultured cells and for both intact embryonic and adult ventricles. Short or long treatment with T_4 did not produce any change in the mobility of this isoenzyme. ATPase activity of embryonic and adult chicken myosin was significantly lower than that of the isoenzyme V1 of the adult rat and T_4 -treated cultured myocytes.

This study demonstrates that, although the electrophoretic mobility of the chicken myosin isoenzyme is faster than that of V1 of the adult rat heart and of T_4 -treated cultured myocytes, its ATPase activity is significantly lower than that of

V1 and comparable with that of the predominant foetal rat isoenzyme V3. Furthermore, a physiological concentration of T_4 modulates the predominant foetal isoenzyme V3 to the predominant adult type V1 in cultured embryonic-rat cardiac myocytes within a brief period. Immunological studies (Clark *et al.*, 1982) revealed that chicken myosin isoenzyme reacted only with the anti-V3 antibody. Nevertheless, our findings suggest that the chicken myosin isoenzyme has an affinity to the type V3 isoenzyme.

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