Developmental Changes in Contractile Protein Adenosine 5′-Triphosphatase in the Rabbit Heart

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SUMMARY. This study was designed to investigate developmental changes in contractile protein adenosine 5′-triphosphatase in the rabbit heart. Myofibrils and myosin were isolated from ventricular muscles from the fetal, newborn, and adult rabbits. Actin and troponin-tropomyosin complex were isolated from the adult skeletal muscle. Myofibrillar (actomyosin) adenosine 5′-triphosphatase measured at low ionic strength increased with development. In contrast, myofibrillar calcium adenosine 5′-triphosphatase at high ionic strength was the greatest in the newborn and the lowest in the adult. Myosin calcium adenosine 5′-triphosphatase and actin-activated myosin adenosine 5′-triphosphatase were also the greatest in the newborn and the lowest in the adult. The relative proportion of myosin isozyme V_1 was the greatest in the newborn and the lowest in the adult. The addition of troponin-tropomyosin complex stimulated myosin adenosine 5′-triphosphatase in the presence of calcium in the adult, but not in the newborn and fetus. As a result, actin-activated myosin adenosine 5′-triphosphatase in the presence of troponin-tropomyosin complex was the greatest in the adult, followed by the newborn and fetus. These data suggest that the low myofibrillar adenosine 5′-triphosphatase activity at low ionic strength in the premature heart may be due to the age-related difference in the interaction of myosin with troponin-tropomyosin. Developmental change in myosin calcium adenosine 5′-triphosphatase (which is determined by the relative proportion of isomyosin V_1 and V_3) may not be directionally identical to that of the physiologically important myofibrillar adenosine 5′-triphosphatase. **(*Circ Res 58: 890–895, 1986*)**

RECENT studies suggest that isomyosin composition regulates myosin adenosine 5′-triphosphatase (ATPase) activity (Hoh et al., 1978), and this in turn controls myocardial contractile function (Pagani and Julian, 1984; Schwartz et al., 1981; Ebrecht et al., 1982). Lompre et al. (1981) and Everett et al. (1983) have shown that there is increased isomyosin V_1 (which has a high enzyme activity) in newborn rabbit ventricle. Although, from these data, one might expect to find increased myosin ATPase activity in the newborn, Nakanishi and Jarmakani (1984) showed that there was, in fact, depressed myofibrillar ATPase activity in the newborn and fetal rabbit. Furthermore, Park et al. (1980) and Nakanishi and Jarmakani (1984) have demonstrated decreased contractile function in the isolated heart of the newborn rabbit. Thus, the relationships between myosin isozyme, its ATPase activity, and cardiac function in the developing myocardium remain unclear. The present study was designed to investigate developmental changes in the contractile protein ATPase in the rabbit heart.

Methods

Myofibrillar and Myosin Isolation

The experiments utilized the fetus (28th day of gestation), newborn (7-day-old), and adult (6–12 months old) New Zealand White rabbits. After the doe had been killed by a sharp blow to the head, the fetuses were delivered by cesarean section and were used within 10 minutes after delivery. One adult and approximately 25 fetal and 10 newborn rabbits were killed by a head blow, and their hearts were excised from the chest cavities. Atria and connective tissues were trimmed away, and only ventricular muscles were used for myofibrillar or myosin isolation.

The myofibrillar fraction was isolated by a modification of the method of Solaro et al. (1971) as described previously (Nakanishi and Jarmakani, 1948). Myosin was prepared by the method of Shiverick et al. (1975).

Actin and Troponin-Tropomyosin Complex Isolation

Actin and troponin-tropomyosin (TN-TM) complex were isolated from the back and leg muscles of the adult rabbit, according to the method of Spudich and Watt (1971). Actin and TN-TM complex were separated by SDS-gel electrophoresis (Fig. 4) in a manner similar to that of Spudich and Watt (1971).

ATPase Assay

All measurements of ATPase activity were performed at 37°C. Myofibrillar (actomyosin) ATPase and actin-activated myosin ATPase were measured at a low ionic strength (μ = 0.0758) and at various concentrations of calcium. A desired calcium concentration in the medium was obtained by using the EGTA buffer system as described previously (Nakanishi et al., 1982, 1985, Nakanishi and Jarmakani, 1984). The medium contained 30 mM *N*-tris(hydroxymethyl)-2-aminoethane-sulfonic acid

(TES), 1 mM EGTA, 3 58 mM adenosine 3',5'-triphosphate (ATP), 6.7 mM $MgCl_2$, 36 mM KCl, 5 mM NaN_3, and various amount of $CaCl_2$ (pH 7.1 with KOH) In some experiments, the pH of the reaction medium was adjusted to 6.5, 7.5, or 8.0 with KOH. The reaction mixture (final volume, 1 ml) contained either 0.2 mg of myofibrillar protein or 0.3 mg of myosin. For measurement of actin-activated myosin ATPase, actin concentration was varied from 0.08 to 1.8 mg/ml (1.9 to 43 μM). In some experiments, the effect of protein kinase on actin-activated myosin ATPase was examined. In these experiments, actin concentration was 1 8 mg/ml and the protein kinase [catalytic subunit, cyclic adenosine 3',5'-monophosphate (cAMP) independent, Sigma] concentration was varied from 10 U/ml to 100 U/ml. For determination of actin-activated myosin ATPase in the presence of TN-TM complex, the actin concentration was 1.8 mg/ml and the TN-TM complex concentration was varied from 0.3 to 1 0 mg/ml. Calcium-activated ATPase and potassium-EDTA ATPase in the myofibrillar and myosin preparations were measured at a high ionic strength (Martin et al., 1982). The medium contained 450 mM KCl, 30 mM TES, 5 mM ATP, 5 mM NaN_3, 5 mM ouabain, and 10 mM $CaCl_2$ (or 10 mM EDTA) The pH of the medium was adjusted to 6 5, 7 1, 7 5, or 8 0 with KOH The reaction was started by the addition of ATP and stopped by the addition of 0.5 ml of 20% trichloroacetic acid Inorganic phosphate in the reaction medium was measured by the method of Fiske and Subbarow (1952) Protein was measured by the method of Lowry et al (1951), using bovine serum albumin as a standard.

Gel Electrophoresis

Sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Acrylamide concentration was 12.5%. The SDS-gels were calibrated for molecular weight determinations with standards (Bio-Rad). For electrophoresis of myofibrils, samples of myosin, actin, tropomyosin, and troponin components were also run as markers Myosin isozyme components were determined by pyrophosphate polyacrylamide gel electrophoresis, according to the method of Hoh et al. (1978).

Statistics

The results are expressed as means ± SE. One-way analysis of variance was used for multigroup components. Statistical significance of differences between group means was determined by modified t-test (Wallenstein et al., 1980). The probability was considered to be significant if the P value was less than 0 05

Results

Myofibrillar (actomyosin) ATPase in the adult rabbit at pH 7.1 (170 ± 14 nmol/mg per min, n = 6) was significantly greater than the newborn (127 ± 16, n = 6) and fetal values (93 + 8, n = 6). Since developmental change in the pH dependence of contractile protein ATPase has not been determined, which might explain the age-related difference in the ATPase, the ATPase activity was measured at different pH values. Although the ATPase activity was slightly depressed at pH 6.5, it was relatively stable at pH from 7.1 to 8.0 in the three age groups (Fig. 1).

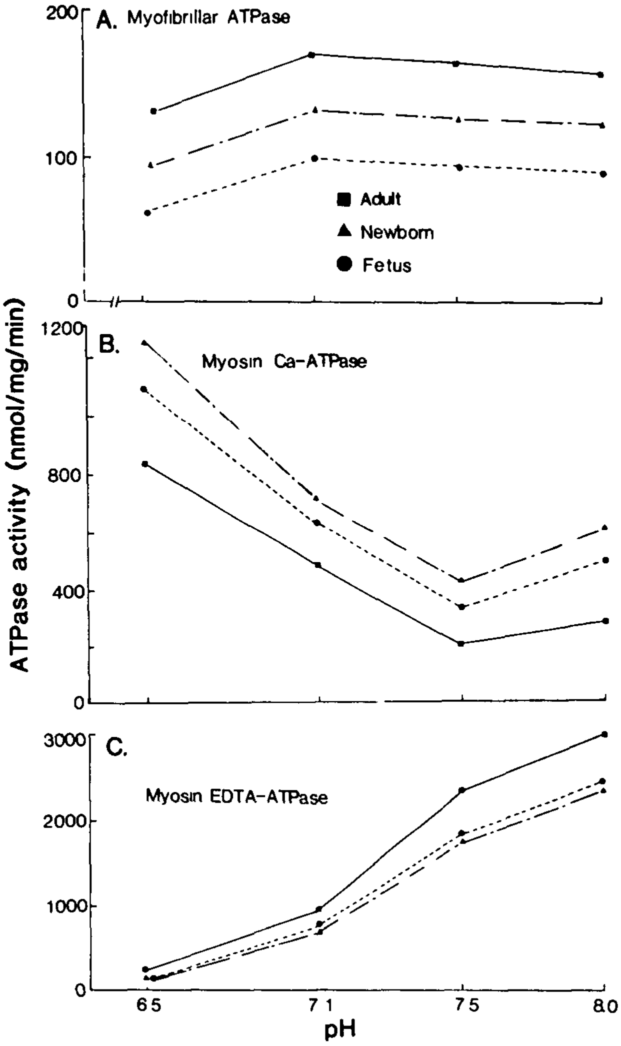

FIGURE 1. *Effect of pH on myofibrillar (actomyosin) ATPase (panel A), myosin calcium-ATPase (panel B), and myosin potassium-EDTA ATPase (panel C)*

Myosin calcium-ATPase and potassium-EDTA ATPase activities were measured at high ionic strength. In contrast to the myofibrillar (actomyosin) ATPase, both calcium- and potassium-EDTA ATPase levels were highly dependent on the pH, and the pH dependence was directionally identical in the three age groups (Fig. 1). At all values of pH studied, myosin calcium-ATPase activity in the newborn was the greatest, followed by fetal and adult values, and the potassium-EDTA ATPase activity in the adult was greater than the values in the newborn and fetus (Table 1). Since myosin does not interact with other contractile elements at high ionic strength, myosin calcium-ATPase and potassium-EDTA ATPase were also measured in the myofibrillar preparation according to the method of Martin et al. (1982). This experiment was performed to determine whether the lower myofibrillar (actomyosin) ATPase in the premature heart resulted from the greater contamination of the myofibrillar preparation. Both calcium- and potassium-EDTA

TABLE 1
Calcium- and Potassium-EDTA ATPase in the Myosin and Myofibrillar Preparations

		Myosin preparation		Myofibrillar preparation	
	n	Calcium	Potassium-EDTA	Calcium	Potassium-EDTA
Adult	6	509 ± 24	894 ± 25	209 ± 8 (41%)	376 ± 23 (42%)
Newborn	6	750 ± 30	720 ± 65	300 ± 11 (40%)	284 ± 13 (39%)
Fetus	6	585 ± 29	699 ± 38	246 ± 7 (42%)	288 ± 26 (41%)
P values					
Adult vs newborn		<0 001	<0 05	<0 001	<0 01
Newborn vs fetus		<0 01	NS	<0 01	NS
Adult vs fetus		NS	<0.01	<0 05	<0.05

Values are means ± SE in nmol/mg protein per min Percent values in parentheses were calculated as ATPase activity in the myofibrillar preparation/ATPase activity in the myosin preparation ATPase activity was measured at a high ionic strength in the medium containing 10 mM calcium or 10 mM EDTA

ATPase levels in the myofibrillar preparation were about 40% of those in the myosin preparation in the three age groups (Table 1), suggesting that the amount of proteins other than myosin in the myofibrillar preparation was similar in the three age groups.

In an attempt to delineate the mechanisms for the low myofibrillar ATPase activity in the premature heart, we added actin and TN-TM complex to the myosin preparation. As shown in Figure 2, actin-activated myosin ATPase was the greatest in the newborn, followed by the fetal and adult values. Addition of TN-TM complex further enhanced the actin-activated myosin ATPase activity only in the adult, and as a result, the enzyme activity was the greatest in the adult and the lowest in the fetus (Fig. 3). In the presence of TN-TM complex, the ATPase activity became calcium dependent. Actin-activated myosin ATPase in the presence of TN-TM complex at various calcium concentrations is shown in Table 2. Adult values were significantly greater than the fetal and newborn values at pCa 6 and 5.

Since TN-TM complex might contain endogenous protein kinase (Reddy, 1976), and this might activate the ATPase activity in the adult, the effect of protein kinase per se on the actin-activated myosin ATPase was studied. Protein kinase (100 U/ml) increased the ATPase activity from 168 ± 8 to 262 ± 9 nmol/mg protein per min (n = 5) in the adult, from 210 ± 9 to 294 ± 10 in the newborn (n = 5), and from 190 ± 7 to 282 ± 9 in the fetus (n = 5). There was no significant difference in the protein kinase-activated myosin ATPase between the three age groups.

SDS-gel electrophoresis of myofibrils showed that there were no marked differences in the amounts of myosin heavy chain, actin, light chains, troponin T, and tropomyosin in the three age groups (Fig 4). However, the amount of troponin I (molecular weight 29,000) in the fetus was less than in other groups, and the protein with molecular weight 33,000 and myosin light chain with molecular weight 28,00 were observed only in the fetus and

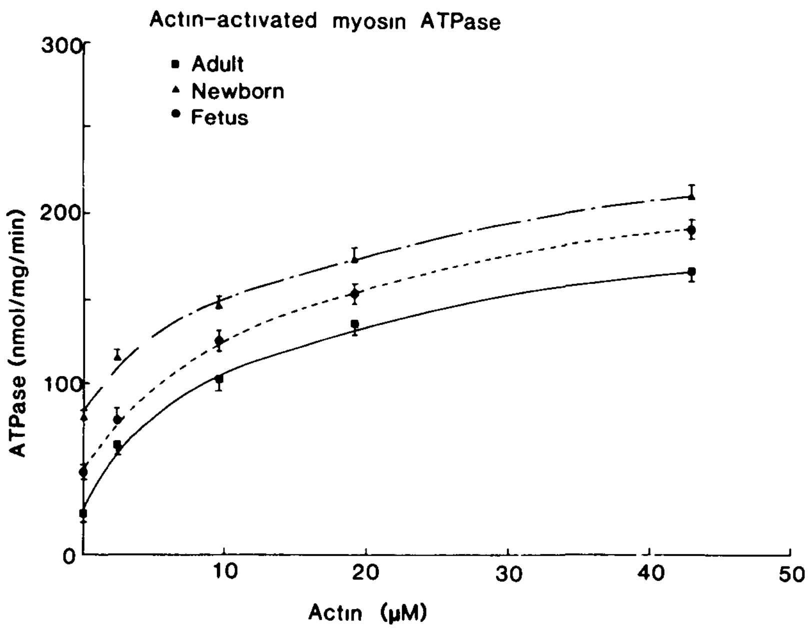

FIGURE 2. *Actin-activation of myosin ATPase. Assay conditions are described in Methods*

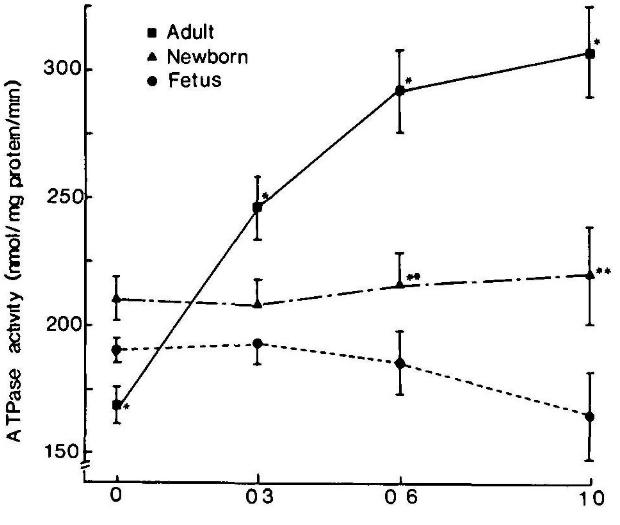

FIGURE 3. *Effect of TN-TM complex on the actin-activated myosin ATPase activity. ATPase activity was measured at a low ionic strength in the medium containing calcium (10^{-5} M) and actin (1 8 mg/ml) Other assay conditions are described in Methods. * Significantly (P < 0 05) different from other two age groups, ** Significantly (P < 0 05) greater than the fetus*

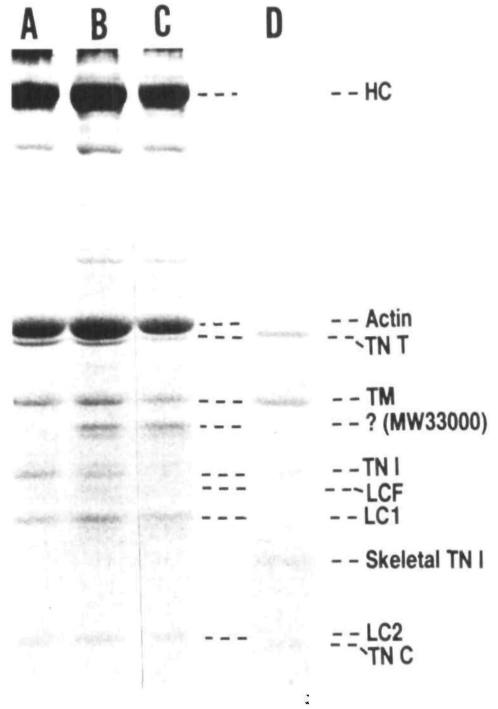

FIGURE 4. *Typical examples of sodium dodecyl sulfate polyacrylamide (12 5%) gel electrophoresis Panel A, adult cardiac myofibril; panel B, newborn cardiac myofibril; panel C, fetal cardiac myofibril, panel D, adult skeletal troponin-tropomyosin complex. HC, myosin heavy chain, TNT, troponin T, TM, tropomyosin, TNI, troponin I, LCF, fetal type myosin light chain; LC1, light chain 1, LC2, light chain 2 The proteins were identified by the following mobilities. myosin heavy chain (mol wt 200,000), actin (mol wt 41,000), troponin T (mol wt 40,000), tropomyosin (mol wt 35,000), troponin I (mol wt 29,000 and 23,000), light chains (mol wt 27,000 and 20,000), and troponin C (mol wt 19,500) (Leger et al, 1975, Wilkinson et al, 1972). There were no marked differences in the amounts of myosin heavy chain, actin, light chains, troponin T, and tropomyosin in the three age groups. Fetal-type light chain (mol wt 28,000) and protein with mol wt 33,000 were observed only in the fetus and newborn, and troponin I (mol wt 29,000) was observed only in the adult and newborn*

newborn (Fig. 4). The presence of fetal-type myosin light chain in the newborn and fetus has been shown previously (Watras, 1981). The exact nature of the protein with molecular weight 33,000 remained unclear.

Electrophoresis of myosin on polyacrylamide gels under nondissociating conditions showed that the proportion of isomyosin V_1 was the greatest in the newborn and the least in the adult (data not shown).

Discussion

The present study was designed to determine developmental changes in the contractile protein ATPase in the rabbit heart. Previous experiments showed that changes in myosin calcium-ATPase and myofibrillar (actomyosin) ATPase activity were directionally identical; both ATPase activities were depressed in the diabetic rat (Pierce and Dhalla, 1981; Malhotra et al, 1981), and both were increased in the thyrotoxic rabbit (Martin et al., 1982). The relationship between myosin calcium-ATPase and myofibrillar ATPase has not been studied in the developing heart, and the present study demonstrates the discrepancy between the two ATPase activities. The present study also confirmed the findings of Everett et al. (1983) and Lompre et al. (1981)

TABLE 2
Actin-Activated Myosin ATPase in the Presence of TN-TM Complex

	n	pCa9	pCa7	pCa6	pCa5
Adult	9	46 ± 12	82 ± 14	279 ± 17	305 ± 18
Newborn	9	63 ± 15	89 ± 10	203 ± 12	218 ± 18
Fetus	9	48 ± 16	85 ± 11	154 ± 13	164 ± 17
P values					
Adult vs newborn		NS	NS	<0.001	<0 01
Newborn vs fetus		NS	NS	<0 001	<0 05
Adult vs. fetus		NS	NS	<0 001	<0 001

Values are means ± SE in nmol/mg protein per min Actin and troponin-tropomyosin (TN-TM) complex concentrations were 1 8 mg/ml and 1 0 mg/ml, respectively ATPase activity was measured at a low ionic strength. Other assay conditions are described in Methods that isozyme V_1 was increased in the newborn rabbit. The study of Hoh et al. (1977) suggests that myosin calcium-ATPase activity is dependent on the relative amount of isomyosin V_1 and V_3. From these data, it is most likely that the increased myosin calcium-ATPase activity in the newborn is due to the greater proportion of V_1 isozyme.

Park et al. (1980) and Nakanishi and Jarmakani (1981) showed that, in the isolated heart preparation, myocardial force and the rate of tension development in the newborn rabbit were less than in the adult. Since myofibrillar ATPase activity increased with age, this ATPase may be more important physiologically.

The mechanisms for the depressed myofibrillar (actomyosin) ATPase in the developing myocardium remain unclear. One may argue that the greater contamination of the myofibrillar preparation in the newborn and fetus might depress the myofibrillar ATPase activity. However, since the ratio of the ATPase activity in the myosin preparation and that in the myofibrillar preparation were similar in the three age groups (Table 1), the discrepancy between myosin calcium-ATPase and myofibrillar (actomyosin) ATPase cannot be explained by the myofibrillar contamination.

Lower myofibrillar ATPase in the developing heart may be due to age-related changes in (1) the composition of contractile proteins and/or (2) the interaction of myosin with other contractile proteins. In the present study, there were no marked differences in the amounts of myosin heavy chain, actin, light chains, and tropomyosin in the three groups. Although the amounts of fetal light chain, troponin I, and the protein with molecular weight 33,000 changed with age (Fig. 4), the physiological significance is not clear. From these data, it is unlikely that developmental changes in the contractile proteins other than myosin explain the low myofibrillar ATPase activity in the fetus and newborn. Furthermore, Nakanishi and Jarmakani (1984) showed that the sensitivity of myofibrillar ATPase to calcium did not change with development, suggesting that the calcium sensitivity of troponin does not change with age. Of course, since SDS-gel electrophoresis might not detect small changes in the regulatory proteins, more precise studies regarding developmental changes in the regulatory proteins are required.

Previous studies have shown that, in the cardiac muscle of the thyrotoxic rabbit, isomyosin V_1 was increased and actin-activated myosin ATPase activity was greater than control (Martin et al , 1982; Banerjee, 1983). In agreement with these studies, actin-activated myosin ATPase activity in the newborn was the greatest, followed by that in the fetus (Fig. 2). Therefore, it is unlikely that actin activation is less in myofibrils of the fetus and newborn.

One may argue that actin and TN-TM extracted from skeletal muscle might not be completely identical to those from cardiac muscle In the present study, actin and TN-TM complex were isolated from the adult skeletal muscle because it was difficult to obtain adequate amounts of these proteins from rabbit cardiac muscle. However, Katz et al. (1966a, 1966b) and Barany et al. (1971) have shown that actin and troponin-tropomyosin complex extracted from cardiac and skeletal muscles had similar effects on cardiac myosin ATPase activity. Therefore, it is likely that the present experiments indeed showed the age-related difference in the myosin interaction with other contractile proteins.

In the presence of calcium, addition of the TN-TM complex activated the ATPase activity in the adult (Fig. 3), and this finding was in agreement with those of previous studies (Reddy, 1976; Banerjee and Morkin, 1977). Banerjee and Morkin (1977) showed that, in the thyrotoxic rabbit, actin-activated myosin ATPase in the presence of TN-TM complex was greater than control. Because this enzyme activity in the newborn was less than that in the normal adult in the present study, myosin from the newborn rabbit and that from the thyrotoxic adult rabbit may be different (although both have an increased amount of V_1 isozyme).

Since protein kinase stimulated the actin-activated ATPase similarly in the three age groups, age-related differences in the effect of TN-TM complex cannot be explained by the endogenous protein kinase which might exist in the TN-TM complex (Spudich and Watt, 1971; Reddy, 1976). Although the reasons for the depressed activation by TN-TM complex in the fetus and newborn remain unclear, the data suggest that interaction of myosin with TN-TM complex differs with develoment. Since actin-activated myosin ATPase in the presence of TN-TM complex increased with development, this, at least in part, may explain the lower myofibrillar ATPase in the fetus and newborn.

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