Shortening Velocity and Myosin and Myofibrillar ATPase Activity Related to Myosin Isoenzyme Composition During Postnatal Development in Rat Myocardium

V. Cappelli, R. Bottinelli, C. Poggesi, R. Moggio, and C. Reggiani

The relation between functional properties of the contractile apparatus, such as shortening velocity and ATPase activity, and myosin isoenzyme composition was studied in ventricular myocardium of adult (60-90-day-old) rats and of newborn (3-day-old) and young (10- and 20-day-old) rats. In adult animals, variations of isomyosin pattern were produced by reducing food intake and by changing the thyroid state. Hyperthyroidism was induced with triiodothyronine daily injection for 15 days; hypothyroidism was induced with iodine-free diet and KC1O4 in drinking water for 50-60 days. The following parameters were studied: 1) calcium-magnesium-activated and magnesium-activated ATPase activity of washed and purified myofibrils, 2) calcium-activated ATPase activity of purified myosin, 3) isomyosin composition and relative content of α-myosin heavy chains (α-MHCs), and 4) force-velocity curve of left and right ventricle papillary muscles. To take into account the difference in excitation-contraction coupling between newborn and adult myocardium, the determination of the force-velocity curve was repeated in Krebs' solution with normal [CaCl2] (2.5 mM) and in Krebs' solution with high [CaCl2] (10 mM). During postnatal growth, the relative content of α-MHC increased and reached a maximum at about 20 days. Pronounced increases of myofibrillar and myosin ATPase activity and in shortening velocity occurred during the same period. In adult hyperthyroid rats, α-MHC content as well as enzymatic activity and shortening velocity were higher than in control adult animals. Hypothyroidism and food deprivation caused a decrease of α-MHC content and a reduction of both enzymatic activities and shortening velocity. The study of the relations between α-MHC relative content and functional parameters showed that 1) in ventricular myocardium of adult rats a linear relation existed between α-MHC content and shortening velocity, 2) in newborn and young rat ventricular myocardium, both enzymatic activities and shortening velocity were lower than would have been expected on the basis of the linear relation described above. This latter observation could be accounted for by a variation in specific activity of myosin during postnatal development or by the presence of peculiar isomyosins that cannot be detected with usual electrophoretic techniques. (Circulation Research 1989;65:446-457)

Two distinct isoforms of sarcomeric myosin heavy chains (MHCs) are expressed in the ventricular myocardium.1,2 They are indicated α-MHC and β-MHC and, in association with equal proportions of ventricular light chains, give origin to three different isomyosins: V1, composed of two α-MHCs; V2, composed of two β-MHCs; and V3, which appears to be an α-β heterodimer. Several factors such as thyroid state, hemodynamic load, and substrate use modulate the phenotypic expression of the MHC genes and, therefore, the isomyosin pattern.3,4 It has been shown that functional properties of the contractile apparatus are influenced by the isomyosin pattern. A linear relation between the relative content of α-MHC and shortening velocity5,7 and myosin or myofibrillar ATPase activity8-10 has been reported.

A remarkable variation of the isomyosin pattern occurs during cardiac development. The predominance of β-MHC in fetal myocardium is followed by an increase of α-MHC (which in some species, e.g., rat, can reach almost 100%11,12) and later by a new progressive increase of β-MHC that is associ-
ated with maturation and aging. Developmental changes of the biochemical and mechanical properties of the contractile apparatus have been described\textsuperscript{13,15}; however, their relation with the variations of isomyosin distribution is not completely clear. A dissociation between the rates of increase of V\textsubscript{1} content and calcium-activated myosin ATPase activity was found by Watras.\textsuperscript{14} A myofibrillar ATPase activity lower than expected was observed by Nakanishi et al\textsuperscript{16} in newborn rabbit ventricular myocardium. Very recently Horowitz and Winegrad\textsuperscript{17} demonstrated a developmental increase in the specific V\textsubscript{1} myosin activity as determined with quantitative histochemical and immunohistochemical methods on rat ventricular cryosections.

This study represents an attempt to reinvestigate the relation between myocardial contractile function expressed by shortening velocity and myofibrillar and myosin ATPase activities and changing levels of \(\alpha\)-MHC and \(\beta\)-MHC relative content. This relation was studied in two separate experimental situations. In the first, myocardial tissue containing different \(\alpha\)- and \(\beta\)-MHC proportions was obtained from newborn, young, and adult rats, whereas in the second, variations in MHC relative content were induced by 3,5,3'-triiodothyronine administration, antithyroid treatment, and food deprivation in adult rats.

Some of the results have been published previously in preliminary form.\textsuperscript{18}

### Materials and Methods

#### Animals

The experiments were performed on hearts removed from male Wistar rats of the following groups: 1) 3-, 10-, and 20-day-old newborn and young rats; 2) adult rats with hypothyroidism that was induced by 6-8 weeks of iodine-deficient diet and 1% KClO\textsubscript{3} in drinking water; 3) adult rats with hyperthyroidism induced by daily injection of 20 \(\mu\)g/100 g body wt s.c. triiodothyronine for 2 weeks; 4) adult food-deprived rats with a progressively reduced caloric intake that reached 20% of the control animal intake at the end of the 5-week treatment period; 5) adult (60-90-day-old) control rats. This last group of animals was obtained as a pool of three different control groups, each corresponding to one of the above listed treated groups. The general characteristics of these animals are reported in Table 1. All the animals were born and maintained at the facilities of the Institute of Human Physiology, University of Pavia. The number of animals per litter was kept at eight, and young animals were separated from their mothers at the age of 4 weeks. Before the experiments were performed, the animals were anesthetized with ethyllic ether, the chest was opened, and the heart was quickly removed for use in biochemical or mechanical studies.

### Determination of the Myofibrillar ATPase Activity

Freshly excised hearts were immersed in cool (0-4°C) saline (0.9% NaCl) solution, atria and blood vessels were trimmed, and ventricular walls and septum were quickly cut in little pieces with fine scissors and homogenized for 1 minute in cold imidazole buffer (0.3 M sucrose, 10 mM imidazole, pH 7). The resulting homogenate was then placed in a centrifuge (model J2-21, Beckman Instrs., Fullerton, California) for 15 minutes at 17,300 g at 4°C. From this preparation, washed and purified myofibrils were obtained according to a procedure derived from that proposed by Solaro et al\textsuperscript{19} and described in detail elsewhere.\textsuperscript{20}

The ATPase activity of the myofibrillar preparation was determined from the rate of release of inorganic phosphate in an incubation medium of the following composition (mM): KCl 50, MgCl\textsubscript{2} 2, NaATP 2, imidazole 20, CaCl\textsubscript{2} 0-1.5, EGTA 0-1.6, pH 7, at 27°C. The concentrations of CaCl\textsubscript{2} and EGTA were varied in order to obtain pCa values from 4.3 to 8. The pCa value in the incubation medium was calculated by means of a computer program similar to that described by Fabiato and Fabiato.\textsuperscript{21} The calcium-EGTA affinity constant was assumed to be 3.96 x 10\textsuperscript{4}. In each myofibrillar preparation, the enzymatic activity was assayed in several samples under two different conditions: 1) magnesium-activated ATPase activity at pCa values higher than 7 and 2) calcium-magnesium-activated ATPase activity at pCa values of 4.7 and 5. This latter condition yielded the highest activity that could be elicited from each preparation. Several intermediate pCa values were also explored in separate experiments on preparations from 10-, 20-,

### TABLE 1. Body Weight and Heart Weight of Experimental Wistar Rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Body weight (BW) (g)</th>
<th>Heart weight (mg)</th>
<th>(Heart wt/body wt)x100</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-day-old</td>
<td>13.9±0.2</td>
<td>47.2±2.9</td>
<td>0.339±0.006</td>
<td>11</td>
</tr>
<tr>
<td>10-day-old</td>
<td>26.8±0.7</td>
<td>96.7±3.0</td>
<td>0.360±0.005</td>
<td>17</td>
</tr>
<tr>
<td>20-day-old</td>
<td>51.3±2.2</td>
<td>210.1±12.5</td>
<td>0.407±0.026</td>
<td>16</td>
</tr>
<tr>
<td>60-90-day-old control</td>
<td>301.4±7.2</td>
<td>868.6±28.7</td>
<td>0.297±0.010</td>
<td>21</td>
</tr>
<tr>
<td>90-day-old hyperthyroid</td>
<td>314.5±6.3</td>
<td>1,225.3±41.2</td>
<td>0.390±0.011</td>
<td>10</td>
</tr>
<tr>
<td>90-day-old hypothyroid</td>
<td>298.4±9.3</td>
<td>599.7±14.1</td>
<td>0.201±0.004</td>
<td>12</td>
</tr>
<tr>
<td>60-day-old food-deprived</td>
<td>104.1±3.4</td>
<td>367.8±7.5</td>
<td>0.354±0.013</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Heart weight was determined after removal of atria.
and 60–90-day-old control animals to determine the complete pCa-ATPase activity curve.

In all cases, the reaction was started by adding myofibrillar proteins (final dilution 1 mg/ml) to the medium and was stopped after 2 minutes by adding 10% trichloroacetic acid. The amount of inorganic phosphate released was determined with the Fiske-Subbarow method and expressed in μmol/mg protein/min. The protein content was determined by the Lowry method with bovine serum albumin as a standard.

Native Myosin Electrophoresis and Determination of Myosin ATPase Activity

The ventricular samples to be used for myosin extraction were cleansed of blood in cool saline, cut in little pieces, and stored in a buffer solution containing 50% glycerol, 0.2 μg/ml peptatin, and 0.2 μg/ml phenylmethylsulfonyl fluoride at −30° C. Myosin was then extracted and purified by the procedure described by Barany. All steps were carried out at low temperatures and in the presence of the above mentioned antitryptic factors at the concentration of 0.2 μg/ml. The resulting preparation could be stored for a long time at −30° C in 1 M KCl with 50% glycerol. In the case of small samples (1–4 mg), such as papillary muscles, an alternative method was used for myosin extraction. Samples were washed for 4 hours at 4° C in Eppendorf test tubes containing 50% glycerol, 10 mM Tris HCl, 1 mM EGTA, and 0.1 mM DTT, pH 7.5. Then myosin was extracted overnight in a solution of the following composition: 50% glycerol, 50 mM NaP2O7, and 1% β-mercaptoethanol, pH 8.8, at 4° C.

Pyrophosphate gel electrophoresis of the purified myosin preparation was carried out by the method of Hoh et al. After a prerun of 30 minutes at 50 V, myosin samples (5–30 μl) in 50% glycerol were loaded on the top of freshly prepared polyacrylamide gels, and electrophoresis was performed at a constant voltage of 55 V for 30 hours. A refrigerated chamber (1° C), equipped with a pump that recirculated the electrophoretic buffer (20 mM NaP2O7, 1 mM EDTA, 0.025% 2-mercaptoethanol, and 10% [vol/vol] glycerol, pH 8.5) at the rate of 1 l/min, was used. Gels were stained for 3 hours at 25° C in Coomassie brilliant blue G250 (0.4 g/l) and 70% perchloric acid (42 ml/l) and destained in 7% acetic acid. The destained gels were scanned with a densitometer equipped with a chart recorder (model 2202, LKB a.b., Bromma, Sweden). From densitometric tracings, the quantification of the myosin isoenzyme distribution was carried out by measuring the areas under Vis, V2, and V3 peaks following a procedure similar to that already used by other authors. V1 and V2 areas were calculated assuming that V1 and V2 peaks were symmetric and that their outer halves were not contaminated by V2. V2 area was obtained as the difference from total area. Then the relative content of α-MHC was calculated adding V1 area to one half of V2 area. To test the reliability of the quantification procedure, four samples were analyzed several times by repeating the complete procedure from electrophoretic separation to α-MHC relative content calculation. The standard deviation of the repeated measurements was 2.52–12.2% of the mean values (five measurements).

The myosin preparations that were obtained according to Barany’s method were also used for the determination of the calcium-activated ATPase activity. The purified protein was incubated in 2 ml of the following medium: 0.3 M KCl, 50 mM Tris-Cl, 10 mM CaCl2, and 2 mM sodium ATP, pH 7, at 27° C. The reaction was started by adding 50–70 μg myosin and was stopped 10 minutes later by adding 10% trichloroacetic acid. The amount of inorganic phosphate released was determined with the Fiske-Subbarow method and expressed in μmol/mg protein/min. The protein content was determined by Lowry’s method with bovine serum albumin as a standard.

Evaluation of the Contractile Performance

The hearts to be used for mechanical studies were quickly rinsed in warm (30° C) oxygenated Krebs’ bicarbonate solution and then placed in a dissection chamber filled with the same solution. The millimolar composition of this medium was: Na+ 145.2, K+ 3.6, Ca2+ 2.5, Mg2+ 1.2, Cl− 128.4, HCO3− 24.8, H2PO4− 1.2, SO42− 1.2, and glucose 5.6. In the dissection chamber, under a stereomicroscope, the left, or alternatively the right, ventricle was opened, and a papillary muscle was carefully dissected. The isolated papillary muscle was transferred to the testing chamber, which was filled with the same medium, bubbled with 95% O2-5% CO2 at a pH of 7.4, and kept at constant temperature (27° C) by an Endocal thermostat (Neslab Instruments, Newington, New Hampshire).

In the testing chamber, the preparation was mounted horizontally between the hook of a semiconductor strain gauge transducer (model, Sensonor a.s., AE 801 Horten, Norway) and the shaft protruding from an electromagnetic puller (model 101 vibrator, Ling Dynamic System, Royston, UK), which was provided with an inductance position transducer. The preparation was firmly connected to the transducer and to the puller by means of thread rings; it was then slightly stretched and aligned in order to reduce sideways movements and torsions during contraction. The preparation was stimulated with supramaximal (approximately 30% above the threshold) rectangular 3-msec pulses delivered through platinum multifire electrodes placed 3 mm apart on both sides of the muscle, parallel to its long axis.

The electromagnetic puller was driven by a feedback circuit whose input could be represented either by the output of the position transducer (length control mode) or by the output of the force transducer (load control mode). In the former mode, the length of the preparation could be kept constant,
and isometric responses could be produced; in the second mode, the load applied could be controlled, and by switching at appropriate times from the length control to the load control, isotonic contractions with different loads could be obtained.

The outputs of the force transducer and of the position transducer were displayed on a storage oscilloscope (model 5113, Tektronix, Beaverton, Oregon) and fed through an A/D converter to a digital computer (model MCZ 20, Zilog, Cupertino, California). The sampling interval was 0.5 msec, and the digitizing resolution was 12 bits. Data from experiments were recorded on floppy disks for off-line analysis.

Once mounted in the testing apparatus, the papillary muscles were allowed to equilibrate for 2 hours in isometric conditions at a constant stimulation rate of 3/min. At the end of the stabilization period, the length of the preparation was gradually increased to reach \( L_{\text{max}} \) (the length at which developed tension is maximum), and the threshold for stimulation was checked. Then isometric responses and isotonic contractions with different loads were recorded at a stimulation rate of 3/min. In several experiments, the bicarbonate Krebs' solution described above was replaced with another with the same composition but higher calcium chloride concentration (10 mM, high calcium solution), and a new series of isometric twitches and isotonic contractions with different loads was recorded. At the end of the experiment, length and three diameters of the preparation were measured in a stereomicroscope (magnification, ×40). Cross-sectional area was calculated from the mean of the three diameters assuming a circular shape of the preparation. In this study, cross-sectional area ranged from 0.167±0.22 mm\(^2\) (3-day-old rats) to 0.394±0.37 mm\(^2\) (hyperthyroid rats).

Isometric responses were characterized by the measurement of the resting tension, the peak total tension, and the time from the stimulus to the peak tension. Isotonic contractions generated a force-velocity curve. In each muscle and in each condition (normal calcium, high calcium), 10-14 isotonic contractions with different loads ranging from 5% to 80% of peak total tension were recorded. For each contraction, the highest value of shortening velocity against that given load was calculated by means of a linear regression on a selected 20-msec interval. Shortening velocity was expressed in length per second, and the corresponding load was expressed as a fraction of peak isometric total load (relative load). A hyperbolic equation was fitted to the data points between 0.05 and 0.7 relative load. From this hyperbolic equation, equally spaced points (with the interval of 0.1 relative load) could be calculated to produce mean values of shortening velocity for each experimental group.

**Statistical Analyses**

Data were expressed as means and standard errors. The correlation between functional parameters (enzymatic activity or shortening velocity) and relative content of \( \alpha \)-MHC was studied by means of the linear regression analysis. Since it is known that percent data deviate from normal distribution, the reliability of the correlation coefficient (Pearson's \( r \) coefficient) was tested by recalculation after an arcsine transformation and by calculation of the rank order Spearman's coefficient. All three procedures yielded high and consistent values of the correlation coefficient. The comparison between the regression of ATPase activity (or shortening velocity) upon \( \alpha \)-MHC relative content that was determined in adult myocardium and the corresponding regression that was determined during postnatal maturation was carried out by comparing first the residual variances, then the slopes, and lastly the elevations. A \( p \) value of 0.05 or less was considered to be statistically significant.

**Results**

**Variations of Myosin Isoenzyme Distribution**

Typical densitometric tracings of myosin isoenzymes obtained from ventricular samples of rats of different ages are shown in Figure 1. The corresponding mean values of the percentage distribution of V\(_1\), V\(_2\), and V\(_1\) isomyosins and of \( \alpha \)-MHC are reported in Table 2. Our observations confirm what has already been demonstrated in other studies. During postnatal maturation, the percentage of \( \alpha \)-MHC increased, reached a maximum at about 20 days, and became slightly lower at about 60-90 days.\(^{1,11} \) When hypothyroidism was induced by an iodine-free diet and KClO\(_3\) in the drinking water, a homogeneous \( \beta \)-MHC composition appeared; the opposite, that is, a homogeneous \( \alpha \)-MHC composition, was obtained with a daily 3,5,3'-triiodothyronine administration.\(^{20} \) Finally, progressive food deprivation caused a reduction in the percentage of \( \alpha \)-MHC as already observed by Dillmann et al.\(^{27} \) The left side of Figure 1 shows examples of the moderate heterogeneity that was found in adult (60-90-day-old) rat ventricle. Mean values of the relative content of \( \alpha \)-MHC in the left and right ventricular papillary muscles are reported in Table 2.

**Variations of Myofibrillar ATPase Activity**

An increase of myofibrillar ATPase activity occurred during postnatal ventricular growth in the rat. As can be seen in Table 2, the calcium-magnesium-activated ATPase activity (determined at pCa values of 4.7-5) increased from 0.183 \( \mu \)mol/mg/min (one determination performed on a pool of 20 hearts) in 3-day-old rats to 0.251 \( \mu \)mol/mg/min in 20-day-old animals. A very close value was also found in 60-90-day-old control animals. A corresponding increase of magnesium-activated myofibrillar ATPase activity was observed.

These marked variations of ATPase activity seem to be the consequence of genuine changes in the properties of the actomyosin interaction; this is supported by the fact that only minor variations in
FIGURE 1. Examples of electrophoretic patterns and densitometric tracings of purified myosin obtained from Wistar rats of different ages. a: 3-day-old rat; b: 10-day-old rat; c: 20-day-old rat; d: right ventricular papillary muscle of adult rat; e: left ventricular papillary muscle of adult rat; and f: pooled ventricular myocardium of adult rat. The direction of migration of electrophoretic patterns is from top to bottom.

TABLE 2. Myosin Isoform Distribution, Calcium-Magnesium-Activated and Magnesium-Activated Myofibrillar ATPase Activity, and Calcium-Activated Myosin ATPase Activity in Rat Ventricular Myocardium

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Isomyosin distribution</th>
<th>Myofibrillar ATPase activity</th>
<th>Myosin ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_1) (%)</td>
<td>(V_2) (%)</td>
<td>(V_3) (%)</td>
</tr>
<tr>
<td>3-day-old</td>
<td>47.8±5.1</td>
<td>25.6±4.2</td>
<td>26.5±4.8</td>
</tr>
<tr>
<td>10-day-old</td>
<td>76.2±4.8</td>
<td>1.1±3.6</td>
<td>22.5±4.4</td>
</tr>
<tr>
<td>20-day-old</td>
<td>84.2±4.0</td>
<td>9.8±4.2</td>
<td>6.1±3.5</td>
</tr>
<tr>
<td>60-90-day-old</td>
<td>71.3±2.7</td>
<td>8.2±3.1</td>
<td>19.4±2.1</td>
</tr>
<tr>
<td>control</td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>90-day-old</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hyperthyroid</td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>90-day-old</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>hypothyroid</td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>60-day-old</td>
<td>36.8±7.8</td>
<td>25.7±4.6</td>
<td>37.2±6.8</td>
</tr>
<tr>
<td>food-deprived</td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MHC, myosin heavy chain; \(V_1\), isomyosin composed of two \(\alpha\)-MHCs; \(V_2\), isomyosin composed of \(\alpha\)-MHC and \(\beta\)-MHC; \(V_3\), isomyosin composed of two \(\beta\)-MHCs.

* Determined at pCa 4.7-5.
† Determined at pCa higher than 7.
‡ \% \(\alpha\)-MHC relative content in right ventricular papillary muscles, 77.9±4.7 (n=5); in left ventricular papillary muscles, 73.5±1.8 (n=6).
myofibrillar preparations.

ATPase activity determined at pCa values higher than 7 was subtracted, and the remaining calcium-activated activity was normalized to the maximum (reached between 4.7 and 5). The equation $y=100/[1+(10^{pCa})^n]$, where $Q=(10^{pCa})$, was fitted to the experimental data by a nonlinear regression program contained in the Graphpad package (ISI 1987). The resulting curves are shown in the figure. Calculated Hill’s coefficients ($n$) are 1.565±0.255 (10-day-old rats), 1.558±0.184 (20-day-old rats), 1.201±0.162 (60-90-day-old rats). Calculated pCa values for 50% activity (pCa 50%) are 6.045±0.049 (10-day-old rats), 6.012±0.036 (20-day-old rats), and 5.970±0.055 (60-90-day-old rats). Each point on the diagram is given by the mean of five determinations on different myofibrillar preparations.

the sensitivity to free calcium occurred (at least between 10-day-old and 60-90-day-old animals) as demonstrated by the pCa-ATPase activity curves reported in Figure 2.

Table 2 also shows the variations of myofibrillar ATPase activity of adult ventricular myocardium. Compared with control values, myofibrillar ATPase activity was higher in hyperthyroid and much lower in hypothyroid rats. A decrease of activity was also present in food-deprived animals.

Variations in Shortening Velocity

A study of the influence of isomyosin composition on shortening velocity of intact cardiac preparations obtained from animals of different ages or exposed to different experimental treatments is made difficult by the possible interference of other factors, such as activation degree. It is well known that myocardial excitation-contraction coupling undergoes an extensive reorganization during the first weeks after birth.\textsuperscript{28,29} In conditions in which adult myocardium reached a quasi optimal mechanical performance “in vitro” (low stimulation rate, 2.5 mM [Ca\textsuperscript{2+}]), and $L_{max}$ at 27\textdegree C), neonatal rat myocardium could only develop a rather weak contractile response. On the other hand, appropriate inotropic interventions such as the increase of [Ca\textsuperscript{2+}] from 2.5 to 10 mM could induce a fourfold increase in the peak isometric tension developed by a neonatal papillary muscle, whereas no increase could be elicited from an adult (60-90-day-old) papillary muscle (see Table 3).

The force-velocity curve was, therefore, determined in two different experimental conditions: normal calcium solution (2.5 mM [Ca\textsuperscript{2+}]) and high calcium solution (10 mM [Ca\textsuperscript{2+}]). The former represented an optimal condition for adult myocardium, the latter for neonatal myocardium. Table 3 shows the values of tension output and shortening velocity obtained in the different groups of animals. The shortening velocity at 0.1 relative load was selected for comparison purposes. The value at 0 load was less reliable due to the uncertainty connected with the extrapolation procedure of the hyperbolic equation fitted to the experimental data. From the inspection of Table 3, it can be easily observed that 1) the effect of the inotropic intervention (expressed as percentage in the lower part of the table) on force and shortening velocity was comparatively lower in the older age groups and 2) shortening velocity, in both inotropic conditions, increased with maturation reaching at 20 days, in high calcium medium, a value similar to that of 60-90-day-old control animals.

Table 3 also shows the values of shortening velocity at 0.1 relative load determined in papillary muscles isolated from adult rats in different experimental conditions. Hyperthyroid papillary muscles exhibited the fastest shortening velocity, whereas the hypothyroid preparations exhibited the slowest. In control papillary muscles, a small (15%), significant difference was present between right ventricle papillary muscles, which were faster, and left ventricle papillary muscles. No difference could be observed between left and right papillary muscles in hyperthyroid and hypothyroid rats; therefore, the muscles from both ventricles were pooled together.

Correlation Between Shortening Velocity and Calcium-Magnesium-Activated Myofibrillar ATPase Activity

The correlation between shortening velocity and myofibrillar ATPase activity is shown in Figure 3. Each point on the diagram is given by the mean of ATPase activity measured on pooled left and right ventricular samples (abscissa) and the mean shortening velocity determined on the right or on the left papillary muscles (ordinate) of hearts of the same animal group. The range of regulation of shortening speed and ATPase activity in young adult (60-90-day-old) myocardium is represented by the line extending from the point indicated as TX (measurements done on hypothyroid animals) to the point indicated as T3 (measurements performed on hyper-
TABLE 3. Shorening Velocity and Other Mechanical Parameters of Rat Papillary Muscles

<table>
<thead>
<tr>
<th>Age</th>
<th>3-day-old LVP</th>
<th>10-day-old LVP</th>
<th>20-day-old LVP</th>
<th>60–90-day-old control LVP</th>
<th>60–90-day-old Hyperthyroid LVP</th>
<th>60–90-day-old Food-deprived LVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium medium (2.5 mM CaCl₂)</td>
<td>1.29±0.57</td>
<td>1.85±0.61</td>
<td>2.30±0.66</td>
<td>4.19±2.07</td>
<td>4.19±2.07</td>
<td>4.19±2.07</td>
</tr>
<tr>
<td>Resting tension (mN/mm²)</td>
<td>171±6</td>
<td>179±10</td>
<td>166±8</td>
<td>155±4</td>
<td>155±4</td>
<td>155±4</td>
</tr>
<tr>
<td>Time to peak tension (s)</td>
<td>0.92±0.14</td>
<td>1.38±0.12</td>
<td>1.38±0.13</td>
<td>1.38±0.12</td>
<td>1.38±0.12</td>
<td>1.38±0.12</td>
</tr>
<tr>
<td>Shortening velocity at 0.1 relative load (myofibrillar ATPase activity)</td>
<td>2.11±0.24</td>
<td>2.15±0.25</td>
<td>2.15±0.25</td>
<td>2.15±0.25</td>
<td>2.15±0.25</td>
<td>2.15±0.25</td>
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</tbody>
</table>

FIGURE 3. Plot of relation between myofibrillar ATPase activity and shortening velocity at 0.1 relative load. Each point represents mean and standard error (horizontal bars) of ATPase activity and mean and standard error (vertical bars) of shortening velocity determined in preparations belonging to same age or treatment group. The relation obtained in adult (60–90-day-old) animals is represented by the continuous line extending from TX (hypothyroid rats) to T3 (hyperthyroid rats) passing through FD (food-deprived) and CTRL (control rats). A linear regression analysis yields the equation y = -2.0602 + 18.6016x (n=36, r=0.9237, p<0.001). Arrows connect the points obtained at different stages during postnatal growth: 3d (3 days), 10d (10 days), and 20d (20 days). Continuous arrows and filled circles refer to the measurements of shortening velocity carried out in normal calcium solution, whereas dotted lines and triangles refer to the measurements carried out in high calcium medium. L/S, length per second.

thyroid animals). The points indicated as FD (food-deprived animals) and CTRL (mean of all measurements done in seven right and six left ventricular papillary muscles of control 60–90-day-old animals and reported separately in Table 3) lie along this line. The points corresponding to newborn and young hearts lie below the above described line when shortening velocity is determined in normal calcium solution. When shortening velocity is determined in high calcium solution, the points lie on the line (20d) or even above it (3d and 10d).

Relation Between Myofibrillar ATPase Activity, Shortening Velocity, and Myosin Isoenzyme Pattern

Figure 4A shows the relation between calcium-magnesium-activated myofibrillar ATPase activity and the relative α-MHC content. Both parameters were determined on pooled samples of left and right ventricular myocardium. In adult rat (60–90-day-old) heart, the range of regulation of myofibrillar ATPase activity based on variations of α-MHC relative content was 30% of the highest
value, in accordance with the figure reported by Rupp. A high correlation (p<0.001) was found to exist between enzymatic activity and percentage of α-MHC obtained in hypothyroid, hyperthyroid, food-deprived, and control (60–90-day-old) animals. The points obtained from determinations performed in maturing hearts lie below this correlation line. The linear regression of myofibrillar ATPase activity on α-MHC relative content determined in newborn and young cardiac muscle is significantly different from that determined in adult myocardium (see legend of Figure 4).

Figure 4B shows the correlation between shortening velocity at 0.1 relative load and α-MHC relative content. In adult rat myocardium, the range of variation of shortening velocity based on variations of isomyosin content was 41% of the highest value (T3 animals); this is consistent with the range of variation observed by Ebrecht et al in rat skinned ventricular preparations. A highly significant correlation was found to exist between shortening velocity and α-MHC relative content in all adult rat experimental groups. On the contrary, the points obtained in developing heart samples lie below the line representing the regulation of shortening velocity based on isomyosin expression in adult myocardium. The difference is very pronounced when shortening velocity is measured in normal calcium solution, but it is still present and statistically significant when measurements are performed in high calcium medium.

**Myosin ATPase Activity and Myosin Isoenzyme Pattern**

Calcium-activated ATPase activity of myosin preparations was determined in all experimental groups except in 10-day-old rats. Values obtained are reported in Table 2. Enzymatic activity increased more than two times during postnatal development from 3 days to 60–90 days. Moreover, as expected in accordance with other studies in adult (60–90-day-old) animals, the activity was about three times higher in hyperthyroid than in hypothyroid hearts. Figure 5A shows the relation between

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**Figure 4.** Graphs of relations between calcium-magnesium-activated myofibrillar ATPase activity (panel A) or shortening velocity (panel B) and relative content of α-myosin heavy chain (MHC). Continuous lines represent the linear regressions of myofibrillar ATPase activity or shortening velocity upon relative content of MHC in adult rat myocardium [hypothyroid rats (TX), food-deprived rats (FD), control rats (CTRL), and rats with hyperthyroidism (T3)]. The corresponding equations are A = 0.1924 + 0.00082(% α-MHC) (n = 33, r = 0.7965, p<0.001) for myofibrillar ATPase activity and V = 1.5264 + 0.0151(% α-MHC) (n = 36, r = 0.9194, p<0.001) for shortening velocity. Arrows connect the points obtained in myocardium at different stages during development: 3d (3 days), 10d (10 days), and 20d (20 days). In panel B, filled circles and continuous arrows refer to shortening velocity determined in normal calcium medium, and triangles and dotted arrows refer to measurements carried out in high calcium medium. A regression analysis of the data obtained from determination of myofibrillar ATPase activity during maturation yields the following equation A = -0.0304 + 0.00313(% α-MHC) (n = 19, r = 0.5497, p<0.01). When compared with the equation obtained for myofibrillar ATPase activity in adult preparations, a significant difference was found both in slope (p<0.02) and in elevation (p<0.001). Regression analyses were carried out also on the relations between shortening velocity and α-MHC relative content obtained during maturation. For shortening velocity determined in normal Krebs’ solution ([CaCl2, 2.5 mM], the equation V = 1.3442 + 0.0365(% α-MHC) (n = 18, r = 0.7774, p<0.001) was obtained. A significant difference in slope (p<0.01) and in elevation (p<0.001) can be found when this equation is compared with the equation obtained in adult myocardium. The equation for shortening velocity in high CaCl2 Krebs’ solution is V = 1.1655 + 0.01569 (% α-MHC) (n = 18, r = 0.5156, p<0.02); when compared with the equation obtained in adult preparation, a significant difference in elevation (p<0.001) but not in slope can be found.
calcium-activated myosin ATPase activity and the relative content of α-MHC. As in Figure 4A, the line connecting the hyperthyroid animal group with the hypothyroid one and passing through control and food-deprived groups represents the range of regulation in adult animals. The points obtained during development in 3- and 20-day-old animals lie below that line. A statistically significant difference was found between the linear regression of myosin ATPase activity on α-MHC relative content determined respectively in adult cardiac preparations and in maturing heart preparations. The similarity with the pattern of variation of myofibrillar ATPase activity (see Figure 4A) is very evident. This parallel behavior can be further confirmed by plotting myosin ATPase activity versus myofibrillar ATPase activity (Figure 5B). A linear regression analysis demonstrates a highly significant correlation between the two variables.

Discussion

In this study, we examined the relation between functional properties of the cardiac contractile system (such as shortening velocity and myosin and myofibrillar ATPase activity) and myosin isoenzyme composition. It was found that the relation present during the maturation of rat ventricular myocardium was different from that present in adult rat myocardium in which MHC composition was altered by changing the thyroid or the nutritional state.

The possibility that such a difference is due to some methodological problems must be taken into consideration. In the first place, the lower ATPase activity of developing myocardium might be attributed to a higher degree of contamination of the preparations of myosin or myofibrils. The comparable quality of the myofibrillar preparation in all the age groups is supported by the fact that the ratio between magnesium-activated and calcium-magnesium-activated activities, although higher than in other studies, is very consistent (between 0.34 and 0.39, see Table 2) and that a significant correlation can be demonstrated between myofibrillar and myosin ATPase activity (see Figure 5B). This latter observation is especially relevant. Since myosin and myofibrils are prepared with two completely independent procedures, it is very unlikely that a contaminating factor can affect both preparations in the same way.

A different sensitivity to activator calcium can also be excluded. A neonatal troponin isoform, likely associated with a higher calcium affinity, has been demonstrated in fetal rat heart. In agreement with the observation that this isoform disappears within 1 week after birth, the curves of calcium activation of myofibrillar ATPase activity obtained in 10-day-old, 20-day-old, and adult animals (see Figure 2) do not show any significant difference.

Further difficulties arise when parameters characterizing mechanical activity are to be correlated with biochemical parameters such as ATPase activity or isomyosin distribution. A first problem is caused by the fact that in most cases biochemical studies could not be carried out on the same prep-
arations that were used for mechanical measurements. Exceptions to this are represented by the papillary muscles of adult rats in which isomyosin composition and shortening velocity could be determined on the same preparation by the myosin extraction method suitable for small samples. On the contrary, myofibrillar and myosin ATPase activity were determined on pooled samples of septum and left and right ventricular walls and in young and newborn animals on many complete ventricles pooled together. The heterogeneity of ventricular myocardium reported here (see difference between left and right papillary muscles) and elsewhere\textsuperscript{24,33} casts some doubt upon the reliability of the correlation between mechanical properties of papillary muscles and averaged biochemical properties of ventricular myocardium.

A second problem is represented by the selection of a mechanical parameter that can be used for correlation studies. In skeletal muscle, shortening velocity at 0 load has proved to be suitable for correlation with ATPase activity and isomyosin composition\textsuperscript{24,35} due to its insensitivity to sarcomere length and activation degree and to the lack of internal load; however, in cardiac muscle, shortening velocity at 0 or low load is influenced by the level of activation, by the time of measurement, and by the sarcomere length.\textsuperscript{36,37} In this study, we measured shortening velocity in an interval in the middle part of the rising phase of the contractile cycle; we removed the influence of maximal contractile activation by scaling each load on the peak total tension. We preferred a velocity measured at 0.1 relative load to the value at 0 load because the former is an interpolated value whereas the latter is obtained by extrapolation on the steepest part of the force-velocity curve. In adult rat papillary muscles, the good quality of the correlation between biochemical parameters and shortening velocity together with the little sensitivity of this latter to variations in \([\text{CaCl}_2]\) seems to confirm that our measurements of speed of shortening are a reliable evaluation of the kinetic properties of the contractile system. On the contrary, in newborn and young rat myocardium, remarkable variations in both developed tension and shortening velocity can be induced by increasing stimulation rate\textsuperscript{38} or external calcium concentration. The increase of shortening velocity obtained by increasing \([\text{CaCl}_2]\) in newborn rat papillary muscles was not sufficient to reach the values of shortening velocity expected on the basis of the relation between shortening velocity and isomyosin composition in adult animals (see Figure 4B), but it was big enough to exceed the values predicted by the relation between myofibrillar ATPase activity and shortening velocity (see Figure 3). In other words, a newborn rat preparation that contained about 50% \(\alpha\)-MHC could reach a shortening velocity in high calcium slightly higher than a hypothyroid rat preparation that exhibited a similar myofibrillar ATPase activity but was free of \(\alpha\)-MHC. A possible explanation is that some factor, other than the feature of the actomyosin interaction, can influence shortening velocity. A lower internal load in newborn than in adult muscles\textsuperscript{38,39} could be an example of such a factor. Alternatively, hypothyroid papillary muscles might have a rather low level of activation, as suggested by the low-developed tension, in the presence of a long-lasting contractile cycle.\textsuperscript{39} Attempts to increase activation in hypothyroid papillary muscles by increasing calcium concentration were performed, but in most cases a contracture occurred. Contractures are not uncommon in these muscles at normal calcium concentration\textsuperscript{39} and can be attributed to difficulties in controlling intracellular calcium levels. When contracture did not develop, only a moderate increase (+5.8%, mean of two experiments) of shortening velocity could be elicited by shifting from normal to high calcium solutions.

The results reported in the present study contribute to the description of the developmental variations of the ventricular contractile proteins in the rat. The myosin isoform transition from a dominant \(\beta\)-MHC type during fetal life to a dominant \(\alpha\)-MHC type soon after birth and followed by a further increase in \(\beta\)-MHC has been first described by Hoh et al\textsuperscript{1} and by Lompré et al.\textsuperscript{11} It has recently been confirmed using complementary DNA techniques by Mahdavi et al.\textsuperscript{2} The essential role played in these transitions by thyroid hormones has been demonstrated by Chizzonite and Zak.\textsuperscript{12}

Myosin ATPase activity at different stages during development was studied by Watras\textsuperscript{14} and by Horowitz and Wingrad.\textsuperscript{17} In spite of the different techniques, both these studies showed that the increase of ATPase activity was higher than expected from variations of isomyosin pattern. Our results confirm and extend these previous observations and show that not only myosin but also myofibrillar ATPase activity for a given isomyosin pattern is lower in a developing heart than in an adult heart subjected to its regulatory processes. Shortening velocity, although influenced by simultaneous variations in excitation-contraction coupling processes, exhibits a pattern of variations similar to that of the ATPase activities.

The interpretation of these results could follow two different lines. On one hand, the results seem to indicate that myosin increases its specific activity during postnatal development. There is evidence that functional properties of \(V_t\), isomyosin can be regulated by the activation of \(\beta\)-adrenergic receptors, as shown by Winograd et al,\textsuperscript{40} either through phosphorylation\textsuperscript{41} or through the release of a soluble factor.\textsuperscript{42} Postnatal maturation coincides with the period of development of \(\beta\)-adrenergic system in rat cardiac muscle.\textsuperscript{43} A modulation of functional properties of myosin by developmental variations of thin filament composition could be also taken into account. This latter explanation is contradicted by the fact that the isoform transitions involving actin\textsuperscript{34,35}
and troponins occur earlier than the age (10–20 days) when a clear difference in respect to adult myocardium is still present. Furthermore, during maturation not only myofibrillar ATPase activity but also myosin ATPase activity determined at high ionic strength is lower than expected from α-MHC relative content.

On the other hand, Watras hypothesized that other forms of myosin that were enzymatically different from V1, V2, and V3 but not distinguishable with the usual electrophoretic technique could appear during postnatal growth; thus, discrepancies between ATPase activity and isomyosin pattern were produced. Observations that suggest the existence of a second type of α-MHC or a second type of β-MHC have been reported. Recently a skeletal MHC isoform has been demonstrated in developing chicken heart. The present results cannot provide final evidence in favor of one or the other of these interpretations.

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Shortening velocity and myosin and myofibrillar ATPase activity related to myosin isoenzyme composition during postnatal development in rat myocardium.

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