Transmural Distribution of Isomyosin in Rabbit Ventricle during Maturation Examined by Immunofluorescence and Staining for Calcium-Activated Adenosine Triphosphatase

Brenda R. Eisenberg, Joy A. Edwards, and Radovan Zak
From the Department of Physiology, Rush Medical College, and Department of Medicine, University of Chicago, Chicago, Illinois

SUMMARY. Mammalian ventricle contains two major isomyosins, V_1 and V_3, which differ in the primary structure of their heavy chains (HC_{\alpha\alpha} and HC_{\beta\beta}, respectively) and in their adenosine triphosphatase activity. The distribution of the HC_\alpha isomyosin in the left ventricle of the rabbit was followed as a function of age and transmural location. HC_\alpha was detected with a monoclonal antibody found to be specific for the hinge region of V_1 myosin molecules when viewed in the electron microscope after low-angle rotary shadowing. Frozen sections were observed with indirect immunofluorescence developed to this anti-HC_\alpha hinge antibody. Serial sections were observed with the histochemical assay for calcium-activated myosin adenosine triphosphatase, using preincubation at various pH levels. Results show that all the ventricular myocytes in baby rabbits (2 weeks) are stained by the HC_\alpha-antibody from the epicardium to endocardium. The isomyosin content of myocytes varies through the epicardium to endocardium of the right ventricular wall of the adult (1-year-old) rabbit, with the HC_\alpha form predominating in the outer epicardial third of the wall and the lowest amount of HC_\alpha in the middle third of the wall. A mixture of stained and unstained myocytes is seen in the endo- and subendocardial regions. The spatial distribution of HC_\alpha in 4-month-old rabbits varies between that of the baby and adult. There is good agreement between myocyte classifications made by histochemical and antibody staining methods. A rabbit ventricular myocyte with HC_\alpha detected by antibody has high adenosine triphosphatase activity, and a myocyte without HC_\alpha has a low adenosine triphosphatase activity. Myocytes with similar staining properties appear in large clusters, long strands, and major anatomical regions. However, heterogeneous myocytes are also seen connected by an intercalated disc. (Circ Res 56: 548-555, 1985)

MYOSIN in cross-striated muscles belongs to a multigene family composed of approximately eight variants which differ in their structure and enzymatic properties. The divergence among isomyosins is reflected both in their heavy chains (HC) and light chains (reviewed in Zak and Galhotra, 1983). In the heart, two subclasses of myosin can be demonstrated; atrial and ventricular (Hoh et al., 1978). In the ventricle, the \( \alpha \) and \( \beta \) HC associate in the cell as two homodimers of HC_{\alpha\alpha} and HC_{\beta\beta}, and one heterodimer HC_{\alpha\beta}, called V_1, V_3, and V_2, respectively (Hoh et al., 1978; Everett et al., 1983a, 1983b). The HC_\alpha is very similar, if not identical, to the one present in atria, and the HC_\beta is similar to the form present in slow skeletal muscle (Sinha et al., 1984).

Ventricular isomyosins have been detected in all mammalian species examined. Their relative content in the ventricle differs depending on animal species (Lompre et al., 1981; Clark et al., 1982). Thus, in hearts of small animals, such as a mouse, the V_1 isoform predominates, but in large animals, e.g., human, the V_2 variant is most common. The proportions of the ventricular isomyosins vary depending on the age of the animal (Lompre et al., 1981; Clark et al., 1982; Martin et al., 1983), hemodynamic load (Lompre et al., 1979; Gorza et al., 1981; Litten et al., 1982), and a variety of hormones and nutritional factors, of which the thyroid hormone is the most potent (Morkin et al., 1983).

At the cellular level, the isomyosin composition of a ventricle has been analyzed by the immunofluorescence technique (Samuel et al., 1983). In those hearts that have been found to contain all three isomyosins, e.g., the 8-week-old rat, the isomyosin distribution within the population of myocytes was found to be heterogeneous. Some cells react only with anti V_1 or V_3 antibodies, whereas others react with both. Of particular interest is the fact that myocytes of different isomyosin content were found in cells directly connected by an intercalated disc (Sartore et al., 1981). Considering that neighboring cells in the myocardium have similar electrical and mechanical properties, the heterogeneity in isomyosin content of individual cells poses a great challenge as far as the identification of factors determining cardiac phenotype is concerned.

In this paper, we report a comparison of two histological techniques which localize the V_1 and V_3
isomyosins. First, we use a monoclonal antibody specific for HCa. One drawback of the immunofluorescence technique is that, although it is very sensitive to small amounts of antigen, it is not very quantitative. Progress has been made recently in differentiation between the V1 and V2 isoforms by a second histochemical method which is based on differential pH lability of ATPase activity of individual isomyosins, and which may suffer less from the problem of the threshold phenomenon. We have examined the effect of age and transmural location on the presence of the HCa in rabbit cardiac tissue so that the work being done may be related to the kind of myosin being expressed.

**Methods**

Three New Zealand white rabbits were used at each selected stage of maturity: baby animals weighing under 500 g (about 2 weeks old), young animals of 2.5-3 kg (approximately 4 months), and adults of 4-5 kg (approximately 1 year). All animals were anesthetized with sodium pentobarbital, the thoracic cavity opened, and the heart trimmed free. Tissue was excised from the region midway between the apex and the base of the ventricle, and segments were carefully oriented so that a transmural surface was in the sectioning plane. The segments were attached to a slice of cork with embedding medium and frozen in isopentane slush that had been precooled in liquid nitrogen. Serial sections (6 μm thick) were cut on an Amers Cryostat (Miles) at −20°C. Some sections were stained with periodic acid Schiff's stain (PAS) for glycogen, or NADH tetrazolium reductase for mitochondrial enzymes. In addition, alternate sections were stained for immunofluorescence or ATPase, as described below. All sections were stored at −20°C and were used within 24 hours.

The monoclonal antibody mcAb 37 reacts specifically with rabbit myosin HCo with the purity specified by radioimmunoassay (RIA) (Chizzonite et al., 1982). Myosin preparation consisting of pure heterodimers of HCa or HB was obtained from hyperthyroid or PTU-treated rabbits. Antibody (60 μg/ml) and myosin (144 μg/ml) were mixed in 33% glycerol and 0.3 M ammonium formate buffer at pH 7.3 (Claviez et al., 1983). The tagged molecules were immediately sprayed onto freshly cleaved mica at room temperature, and, after rotary shadowing with platinum/palladium at an angle of 70° and coating with carbon, were observed in the electron microscope (Pullman, 1978). Micrographs of at least 300 molecules were printed at 80,000 X, and each molecule was classified as having two, one, or zero antibodies bound to it. The location of the antibody on the myosin was noted.

Indirect immunofluorescence to the HCo antibody was performed on sections picked up on coverslips coated with poly-L-lysine at pH 8.5 and air-dried to prevent detachment (Wolosowick and DeMey, 1982); these sections then were washed in 0.01 M phosphate-buffered saline (PBS) containing 0.02% NaN3 and 2% heat-inactivated fetal calf serum (Gibco) to block nonspecific antibody binding. The sections were incubated for 45-60 minutes at room temperature with the final dilution of monoclonal antibody (mc Ab 37) of 1:1000 which was selected empirically for maximal signal with minimal nonspecific background binding. Sections were rinsed with phosphate-buffered saline (PBS) and stained for 1 hour with fluorescein-labeled rabbit anti-rat IgG (Cappel Laboratories, Inc.). Aliquots of antibody were refrigerated, and aliquots of labeled IgG solutions were frozen to ensure standardized immunostaining. Sections were again rinsed with PBS and mounted on slides with glycerol/PBS (9:1) containing 0.5% phenylenediamine to protect against fading (Johnson and Araujo, 1981). A control was designed to give a positive reaction by using an incubation with rat serum to replace the first incubation of fetal calf serum. Other controls were run, omitting the antibody or the fluorescein label, and were always negative. Fluorescein-stained slides were stored in the dark at 4°C until viewed within 3 days with a Nikon microscope equipped with epifluorescent illumination (excitation filter of B2, wavelength 460-490, barrier filter 515).

Frozen sections alternating with those used for the antibody localization were stained for ATPase (myosin adenosine triphosphatase, Ca++-activated, EC 3.6.1.3.) activity (p32 in Dubowitz and Brooke, 1973). The acidic preincubation solution consisted of 50 mM sodium acetate and 30 mM sodium barbital, with a pH of 4.2, 4.4, or 4.6, adjusted with 1 M HCl. Basic preincubation solutions consisted of 100 mM 2-amino-2-methyl-1-propanol and 18 mM CaCl2, 2H2O, with a pH of 10.1, 10.2, 10.3, or 10.4, adjusted with 1 M NaOH (Thornell and Forsgren, 1982). Sections were viewed and photographed with a Zeiss Photomicroscope III using bright field illumination.

The proportion of the ventricular wall occupied by myocytes containing HCa was estimated from slides stained by the ATPase reactions at pH 10.2, which always correlated with the bright immunofluorescence. The stereological method of linear integration was used where a length ratio estimates a volume fraction (Eq. 2.23, Weibel, 1979). The ratio of length of the test line lying over the cells dark with the ATPase stain to that over light cells, Ld/Ll, is equal to the ratio of the volume of HCa- to HB-containing cells. A micrometer was used in the eyepiece of the light microscope at a magnification of 5.5 to measure lengths in two noncontiguous frozen sections. The lengths Ld and Ll were measured at five different locations with sufficient number of fields to cover the entire ventricular wall in each section. In addition, the transmural width and the position of the dark (HCo) cells from the endocardial surface was measured so that the gradient of cell types from the epicardium to endocardium could be determined.

**Results**

**Specificity of the Antibody**

We assign differential ATPase staining to a specific myosin by comparing adjacent frozen sections, one preincubated at alkaline pH and reacted for ATPase, and the other identified by a fluorescent tag to the anti-HCa antibody. The specificity of the monoclonal antibody (Chizzonite et al., 1982) was confirmed by immuno-electronmicroscopy by direct observation of myosin molecules of known heavy chain composition (Fig. 1). Of the 300 HCa myosin molecules counted, 23% had a pair of antibodies bound at the hinge in the tail, 7% had a single antibody bound at the hinge, and 67% had no bound antibody (Fig. 1, upper panels). Only 3% had bind-
FIGURE 1. Upper panels show V_myosin molecules of the heterodimer HCa from rabbits tagged with monoclonal antibody mcAb 37. The location of the bound antibody is at the hinge region in the tail, as seen in the upper right panel where one tagged myosin lies next to another bent at the hinge. Lower panels show untagged heterodimers of HCB/V3 myosin molecules. Bar length = 0.1 μm.

FIGURE 2. Frozen sections in the mid-wall region of a 4-month-old rabbit. In Figure 2A, the A-band is stained dark with ATPase reaction at pH 10.2, and in Figure 2B, the A-band is bright with immunofluorescence staining of myocytes with anti-HCa. Myocytes containing HCa and high ATPase are seen directly coupled to nonstaining myocytes. Bar = 10 μm.

In frozen sections stained by immunofluorescence (Fig. 2A), the A-band appears bright when reacted with anti-HCa antibody. However, nonspecific binding is not seen elsewhere in the tissue.

ATPase Activity

Myocytes from rat ventricle have different intensities of ATPase staining, depending upon the duration, temperature, and pH of the preincubation solutions (Weisberg et al., 1982; Carlsson et al., 1982). In the rat ventricle, incubation at various pH levels presumably inhibits the V1, V2, and V3 iso-myosins differentially in both the alkaline and acid range, making it possible to estimate the isomyosin composition of the myocyte. The A-band appears dark when staining reveals a high level of Ca**+-activated ATPase (Fig. 2B). The staining reaction seen in the baby rabbits confirmed that the ATPase activity was correctly assigned to HCa. Acid preincubation affected all the isomyosins equally, inactivating enzymes below pH 4.6 (data not shown). However, in the range, pH 10.1–10.3, differential staining of HCa was possible by the ATPase histochemical method (Fig. 3). At a given pH preincubation, the appearance of the stain varied from one animal to another. Thus, in one animal, the sharpest division into dark and light cells occurred at pH 10.2, and in another it was at pH 10.3. In all animals there were several alkaline preincubations that gave grey, intermediate stained cells (see Fig. 3C). It is possible that these represent the ATPase activity derived from the V2 heterodimer, HCaβ. However, the grey level could also have arisen from numerous other technical sources. We did not take special precautions to control such factors as section thickness, substrate levels, temperature, and so on, which makes it awkward to utilize the histochemical method to determine ATPase activity, quantitatively (see discussion by Weisberg et al., 1982). It therefore is difficult to make absolute estimates of ATPase activity with this assay.

HCa Myosin Distribution with Age

Baby (2-week-old) rabbits contain 50% HCa, as determined by native state electrophoresis, and by the age of 1 year, contain over 95% HCB (Everett et al., 1983b). This shift in isomyosin content should be reflected in the staining patterns obtained with both ATPase activity and immunofluorescent reactions in serial sections. In the baby, the ventricle appears uniformly bright with the fluorescent tag to anti-HCa and uniformly dark when stained by the Ca**+-activated ATPase after an alkaline preincuba-
Eisenberg et al. / Ventricular Isomyosin Distribution

**FIGURE 3.** Frozen sections through the mid-wall region of the left ventricular wall of an adult rabbit (wt 5 kg) stained with the ATPase reaction run after preincubations at different pH levels [at pH 10.3 (panel A), pH 10.2 (panel B), and pH 10.1 (panel C)]. In the alkaline preincubation (panels A–C), there is differential staining between myocytes. All myocytes were dark at pH 9.4, and all myocytes were light at pH 4.2, except for smooth muscle in the wall of the blood vessels, which was still stained dark at pH 4.2 (not shown). Bar = 100 μm.

Transmural Distribution of Ventricular Isomyosins

Transmural sections were obtained from oriented blocks of tissue, permitting the study of this anatomical variable. Histochemical stains for glycogen and mitochondrial content showed uniform distribution across the ventricular wall, in all animals. Baby rabbits stain uniformly bright with anti-HCa immunofluorescence (Fig. 5A), and darkly with the alkaline-preincubated ATPase reaction (Fig. 5D), as expected, since their isomyosin content is almost entirely HCa. In the adult animals weighing over 4 kg, there is a marked transmural gradient with both immunofluorescence (Fig. 5C) and ATPase staining (Fig. 5F), with an epicardial zone comprised of HCa myocytes. The endocardial region is predominantly unstained and is, therefore, HCB. In the mid-wall region, there are some myocytes of each kind. Groups, strands, or clusters of similar stained cells are often contiguous. However, myocytes with different staining patterns are also seen directly connected (Fig. 2), as has been reported by Sartore et al. (1981). The 4-month-old (2-kg) rabbits have a marked predominance of HCa myocytes in the epicardial region, but the mid- and endocardial zones have large patches of tissue that stain for Vi with both the fluorescent anti-HCa and dark, alkaline ATPase reactions.

The morphometric method of linear integration was used to determine the volume of the ventricular wall occupied by HCa-containing myocytes which stain dark with the ATPase reaction after the alkaline preincubation. This analysis was not repeated on the immunofluorescent sections because of problems with variance in the absolute intensity of the stain when fresh, compounded by long-term fading during storage. In the baby animals (2 weeks), 100% of the tissue volume is occupied by myocytes expressing HCa (n = 3 rabbits). In the young rabbits (4 months), 61 ± 7% of the tissue volume has HCa myocytes (mean ± SD, n = 3 rabbits). In adults, 49 ± 7% of the tissue volume has HCa myocytes (n = 3 rabbits). In all cases, the HCa-staining myocytes were found in the entire epicardial zone (defined as the outer third of the transmural wall thickness). Both HCa and HCB myocytes are dispersed throughout the mid-wall and endocardium of young and adult rabbits. In some cases, we observed a concentration of HCa myocytes just away from the walls of larger blood vessels (Fig. 4, C and D), although we did not attempt a quantitative estimation.

**Discussion**

We have evaluated transmural distribution of HCa myosin in ventricles of rabbits of different ages by immunofluorescence and histochemical assessment of ATPase. Both techniques gave similar results. All ventricular myocytes of baby rabbits contain HCa myosin, in agreement with native state electrophoretic analysis done in our laboratory (Everett et al., 1983a). As the animal ages, HCa myosin is eliminated from some cells, with resulting cellular heterogeneity and a transmural pattern of isomyosin distribution. In the epicardial region of adult animals, most of the myocytes stain positively for HCa.
myosin, while the midportion and the endocardium contain a substantial number of cells which do not.

The trend that we detected toward loss of HC\textsubscript{a} myosin with maturity agrees, in general, with previous immunofluorescence study (Sartore et al., 1981) and myosin analysis by native gel electrophoresis (Hoh et al., 1978; Everett et al., 1983a). However, our data indicate consistently more heterogeneity in the hearts of adult animals than would be predicted from densitometric scans of electrophoretically separated isomyosins. This lack of correlation between histochemical and electrophoretic analysis has also been seen in isolated rat myocytes, in which only traces of V\textsubscript{2} and V\textsubscript{3} isomyosin were detected electrophoretically, whereas about half of the isolated cells stained positively with anti-V\textsubscript{3} antibody (Samuel et al., 1983). These combined data indicate that the observed quantitative discrepancies are not a result of sampling error (the native state electrophoresis is usually performed with 10-mg samples of tissue), but, rather, results from method sensitivity.

The biological significance of regional distribution and of cellular heterogeneity of isomyosin distribution in the heart is presently unknown. This is not surprising, since the regional stresses and strains within the heart have not been described; the mechanical properties of the V\textsubscript{1} and V\textsubscript{3} isomyosin have recently been reported (Paganini and Julian, 1984). It has been established, however, that the V\textsubscript{1} isomyosin, which is dimer of heavy chains \( \alpha \), has about three times higher ATPase activity (Pope et al., 1980) than the HC\textsubscript{b} dimer in V\textsubscript{3}. The correlation between the shortening velocity and ATPase activity in the heart is less clear-cut than in studies of skeletal muscles (Barany, 1967). Nevertheless, the maximum velocity of shortening of the ventricular myocardium varies between animal species (Schwartz et al., 1981), as well as in the heart of a single animal (Hamrell and Low, 1978), in parallel with the Ca\textsuperscript{2+}-activated myosin ATPase.

Whether any correlation between shortening velocity and isomyosin pattern applies to the regional heterogeneity seen in our study remains to be established. It is clear that the heterogeneity is in some cases small, such as in the case of human ventricle (Gorza et al., 1984), or it is absent altogether, such as in the hearts of young rabbits or hyperthyroid animals which contain solely V\textsubscript{1} isomyosin, and in hearts in the hypothyroid state, which lose all V\textsubscript{1} at
Eisenberg et al. /Ventricular Isomyosin Distribution

FIGURE 5. Typical transmural sections through the left ventricle of the rabbit midway between the apex and the base. Photomicrographs from the endocardium (left) to the epicardium (right) showing the transmural distribution of the HCa myosin (panels A, B, and C) and high ATPase activity at pH 10.2 (panels D, E, and F). In the ventricle of baby (2-week-old) rabbits (panels A and D), all the myocytes are HCa and high ATPase. In the ventricle of the 4-month-old rabbits (panels B and E), there are large regions of both myosins and levels of ATPase activity present. In the ventricle of adult rabbits (panels C and F), there is a transmural gradient, with the epicardium being predominantly of the HCa myocyte type, the mid- to subendocardium being predominantly HCβ, and the endocardium being mixed. Sections from animals of the same age are not necessarily from serial sections. Bar = 1 mm.

the expense of Vβ isomyosin (Sartore et al., 1981). Also, because the immunofluorescence technique is not a quantitative one and is sufficiently sensitive to detect minute amounts of reactive material, the amount of myosin contributing to the apparent heterogeneity might be quite small. Moreover our method does not allow us to determine whether there is co-expression of HCβ in HCa cells. This could account for some of the apparent discrepancies between the isomyosin content determined from tissue extracts (Everett et al., 1983a) and those determined in the present study.

Isomyosin type is regulated by mechanical demands and a variety of humoral factors. If we understand this epigenetic regulation, we might also understand why regions of the heart are anatomically and functionally different. The heart has a complex architecture, with myocyte columns spiralling upward and outward from the apex to the base (Stree tor, 1979). Thus, the myocyte orientation varies with anatomical location from apex to base and from endo- to epicardial surface, the same route followed by a depolarizing wave initiated via the Purkinje conduction system. The longest-lasting action potentials are found in the endocardial myocytes and the shortest are found in the epicardial myocytes, which give rise to the inversion of the T-wave seen in the electrocardiogram (Cohen et al., 1976). The mechanical result is that force is first generated in the endocardial myocytes, and that the epicardial layers have the briefest contraction. The endocardial layer is thought to do more work in a normal cycle, but when the ventricles are stretched, the epicardial layers are recruited, and may contribute to the increased tension described by Starling (Ford and Forman, 1974). The sarcomere length also varies with anatomical location, the greatest length being found in the midwall and the shortest near the epicardium (Yoran et al., 1973; Grimm et al., 1980).

Although regional specialization of function exists, myocyte diversity is not readily seen with conventional anatomical techniques. Electron micro-
scopic studies using quantitative ultrastructural analysis have not shown differences in cell size and subcellular contents within the rabbit ventricle (Anversa et al., 1971). However, stresses induced in the rat by renal hypertension which produce hypertrophy increase cell size more in the epicardial layers, and resulting transmural gradients become evident in mitochondrial-to-myofibrillar ratio and in the T-system surface density (Anversa et al., 1978). Regional regulation of electrical properties in the epicardial myocytes has also been seen in the rat during hypertrophy (Keung and Aronson, 1981).

Regional work loads might be assessed by regional oxygen consumption and metabolic profiles. Recent studies based on the transmural pH profile have suggested that, normally, the metabolism and energy balance is uniform, and that only during ischemia when the balance between supply and demand are perturbed do transmural gradients occur (Watson et al., 1984), and result in the greatest ischemic damage in the subendocardial zone (Moir, 1972). Metabolic gradients were suggested by earlier histochemical reports of a gradient in the glycogen content transmurally (Jideikin, 1964), but have not been confirmed by our histochemical stains for glycogen and for mitochondrial content.

Cellular heterogeneity might also be the result of a noncoordinated change of cellular phenotype during maturation. In this case, heterogeneity between two adjacent cells expressing different amounts of an isomyosin might depend on a stage of genetic reprogramming reflecting the different lineage of each myocyte, rather than on microenvironmental signals.

We thank Darlene Bruner for her excellent assistance with the microscopy.

Research was supported by grants from the American Heart Association (R.E.), by a Basic Research Science Grant (J.A.E.), by U.S. Public Health Service Grants HL 16627 and HL 20592 from the National Heart, Lung, and Blood Institute, and by a grant from the Muscular Dystrophy Association of America (R.E.).

Address for reprints: Dr. Brenda R. Eisenberg, Physiology Department, Rush Medical College, 1750 West Harrison Street, Chicago, Illinois 60612.

Received September 4, 1984; accepted for publication January 18, 1985.

References

Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50: 197–216
Everett AW, Chizzonite RA, Clark WA, Zak R (1983a) Relationship of changes in molecular forms of myosin heavy chain to endogenous levels of thyroid hormone during postnatal growth. Perspect Cardiovasc Res 8: 83–92
cardiac myosin isoenzymes. FEBS Lett 118: 205–208


INDEX TERMS: Isomyosin • ATPase activity • Transmural gradients • Left ventricle
Transmural distribution of isomyosin in rabbit ventricle during maturation examined by immunofluorescence and staining for calcium-activated adenosine triphosphatase.

B R Eisenberg, J A Edwards and R Zak

Circ Res. 1985;56:548-555
doi: 10.1161/01.RES.56.4.548

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1985 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/56/4/548