BRIEF COMMUNICATIONS

Histochemical Detection of Specific Isozymes of Myosin in Rat Ventricular Cells

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SUMMARY. A histochemical method for distinguishing isozymes of myosin in rat ventricles has been developed. The procedure involves preincubation in pH 10.5, which inhibits Ca-activated ATPase of the V3 isozyme but not the V1 isozyme of myosin. The specificity of the technique has been demonstrated by comparison of results in hearts from young euthyroid and hypothyroid rats, in which the predominant isozymes are, respectively, V1 and V3. The technique is capable of detecting as small a change in the relative amount of V1 as 15% of the total myosin. Isozymes appear to be uniformly distributed within each ventricular cell. There is only a small difference in the content of V1 among the cells in a ventricular chamber of hearts from young euthyroid and hypothyroid rats, but in the period of rapid transition of isozyme content after thyroidectomy, there is considerable heterogeneity of V1 concentration among the cells. The functional implications of the mixture of isozymes is discussed. (Circ Res 51: 802-809, 1982)

VENTRICLES of mammalian hearts can contain more than one type of myosin. In their very important study of rat ventricle, Hoh et al. (1977) found three isozymes of myosin that differed structurally in their heavy chains and functionally in both Ca-activated and actin-activated ATPase activity. These isozymes can be separated on nondissociating pyrophosphate gels (Hoh et al., 1977) or they can be distinguished by immunological techniques (Sartore et al., 1981) or peptide mapping (Hoh et al., 1979; Schwartz et al., 1980). The isozyme that moves most rapidly during electrophoresis on pyrophosphate gels, referred to as V1, has the highest ATPase activity, whereas the slowest, V3, has the lowest ATPase activity (Hoh et al., 1977). Each of these two isozymes is a homodimer consisting of different monomers. The third isozyme of myosin V2 is believed to be a heterodimer formed from the two different monomers. The relative amounts of myosin isozymes differ among mammals. A general correlation exists between heart rate and the amount of V1, so that shorter contraction cycles are associated with higher concentration of the isozyme with the more active ATPase (Carey et al., 1979; Lompre et al., 1981). The relative amounts of the isozymes are also sensitive to the functional state of the heart. Hyperthyroidism increases the concentration of V1 and hypothyroidism, the concentration of V3 (Hoh et al., 1977; Chizzonite et al., 1982; Martin et al., 1982). In cardiac hypertrophy from overload, the quantity of V3 rises (Mercadier et al., 1981).

The existing methods for estimating the relative amounts of V1 and V3 isozymes require a minimum amount of cardiac tissue and are incapable of giving resolution at the level of the individual myocardial cell. This study was directed toward developing a histochemical technique that would detect the presence of V1 and provide at least a semi-quantitative indication of the relative amount of the isozyme in a single cell. Biochemical data in the literature indicate that there are at least three different conditions—pH, ionic strength, and exposure to sulfhydryl reagents—to which the V1 and V3 isozymes respond differently (Yazagi et al., 1974, 1979; Syrovy, 1975; Morkin et al., 1977). On the basis of the published data, response to different pH, which has been successfully used in the histochemical distinction of fast from slow skeletal muscle (Padykula and Herman, 1955; Brooke and Kaiser, 1970), seemed the most promising method for distinguishing the cardiac isozymes, and a protocol using pH to distinguish fast from slow cardiac myosin has been developed.

Methods

Histochemistry

Hearts were removed from young 100 to 150-g rats (4-6 weeks old) after sacrifice and immersed in two changes of oxygenated cold modified Krebs solution until the blood was washed away (no more than 3 minutes). While still immersed in cold Krebs solution, the hearts were divided under a stereo microscope into right and left ventricles and atria. Each piece of tissue was blotted and embedded with one exposed side in a small amount of 8% Gum Tragacanth which has been already placed on a piece of styrofoam. The tissue that is attached to the styrofoam is picked up by long forceps and quickly immersed for 15 seconds in isopentane that had been cooled in liquid nitrogen, and then stored in
the liquid nitrogen until it is sectioned on a Damon/IEC Minotome at −20°C. Sections are 6 μm thick.

The frozen sections were picked up on coverslips that had been coated with a gelatin solution and allowed to dry at room temperature for 1 hour before initiation of the ATPase reaction. It is good practice to assay ATPase activity the day of sectioning, but if the coverslips are stored at 4°C overnight, they can be used the following day without loss of reaction. The ATPase incubation solutions must be prepared and the pH measured just before use.

The ATPase procedure is a modification of the techniques of Padykula and Herman (1955). Small Koplen staining jars were filled with the successive preincubation, staining, and washing solutions. The coverslips with the sections were preincubated for 15 minutes at room temperature in 0.02 M CaCl₂ solution buffered with 0.006 M barbital at pH 10.5, and then 30 seconds at room temperature in a solution of 0.02 M Na barbital, 0.018 M CaCl₂, 5 mM NaN₃, 0.2 mM ouabain buffered at pH 10.5. This was followed by incubation for 10 minutes at 37°C in a solution containing 0.02 M Na barbital, 0.018 M CaCl₂, 5 mM ATP, 5 mM NaN₃ and 0.2 mM ouabain. The remainder of the procedure was conducted at room temperature. The coverslips were washed four times for 3 minutes each in 1% CaCl₂; the first wash solution contained ouabain and azide. This was followed by incubation in 2% CoCl₂ for 1 minute. After 3–5 minutes in running water, the coverslips were dehydrated by briefly dipping the coverslips in two changes each of 80% and 90% ethanol, three changes in 100% ethanol, and three changes of xylene. Coverslips were then mounted onto glass microscope slides with Permount.

Sections that were stained at acidic pH were preincubated according to the method of Brooke and Kaiser (1970) in 0.2 M barbital acetate buffer for 5 minutes at room temperature then washed in 0.02 M Na barbital, 0.018 M CaCl₂ solution, pH 10.5, for 30 seconds, and incubated in a solution containing 0.02 M Na barbital, 0.018 M CaCl₂, 5 mM ATP, pH 10.5, for 15 minutes at room temperature. All solutions after preincubcation contained 5 mM NaN₃, and 0.2 mM ouabain. The washing and dehydration procedure is the same as the alkaline staining procedure.

Sections were viewed with a Zeiss Photomicroscope II using bright field illumination. Photographs were taken on Kodak Panatomic X film, which was then developed with Microdol X.

For the studies of hypothyroid rats, animals that had already been thyroidectomized were obtained from Charles River Breeding Laboratories. Rats of a similar age and the same strain were used for controls.

**Gel Electrophoresis**

Some tissue from the hearts of normal and thyroidec- tomized rats was used for examining the electrophoretic pattern on nondissociating pyrophosphate gels according to the technique of Hoh et al. (1977). The buffer contained 20 mM sodium pyrophosphate and 10% glycerol at pH 8.8. Temperature was carefully controlled at 1-2°C. Voltage gradient was 14 V/cm and the concentration of polyacrylamide was 3.96%. The relative amount of each isozyme was estimated by comparing the heights of each peak on a densitometric scan of the myosin bands on each gel and assuming that the areas representing each isozyme were similar triangles. This estimation was checked with a Hewlett-Packard Reporting Integrator 3390A.

### Results

**ATPase Technique**

In order to evaluate the ability of a histochemical technique to distinguish the two major isozymes of myosin V₁ and V₃, we first studied tissues in which cells contain primarily one of the two isoforms. Since it has been well established that right ventricles of young euthyroid rats contain only V₁, and myosin from right ventricles of rats 5 weeks after thyroidec- tomy is almost entirely V₃ (Hoh et al., 1977), hearts from young euthyroid and hypothyroid rats were used for evaluating the specificity of the histochemical procedures (Fig. 1). The standard Gomori technique (Gomori, 1941, 1949) of calcium precipitation of inorganic phosphate liberated from the hydrolysis of ATP followed by cobalt substitution for calcium and then sulfide substitution for phosphate was used to detect the amount of ATP hydrolyzed in a fixed period of time. Sodium azide (5 mM) and ouabain (0.2 mM) were included in the reaction medium to inhibit mitochondrial and sarcolemmal ATPase. The high concentration of calcium ions that was used to activate myosin ATPase inhibits the ATPase in the sarcoplasmic reticulum. The results were no different when azide and ouabain were not present, but they were routinely included as a precaution.

According to the behavior of the myosins in iso- lated protein studies, alkaline preincubation should inhibit the Ca-activated myosin ATPase of V₃ with little effect on V₁, whereas the response to acid prein- cubation should be very similar for both isoforms. Frozen, dried sections of the heart were preincubated in solutions containing CaCl₂ and sodium barbital at pH from 4.0 to 11.0 to see if pH sensitivity could be used as the basis of histochemical ATPase assay for distinguishing the myosin isoforms. After 15 minutes of preincubation at pH 4.0 or 4.1, very little reaction product was detectable in sections from either normal or hypothyroid rats, but the reaction product, cobalt sulfide, became quite prominent at pH 4.2 and essentially maximal at pH 4.3. Further increase of pH in the preincubation buffer to 4.8 caused no increase in the density of reaction product. Within this pH range for preincubation, there was no difference between sections from the normal and hypothyroid hearts.

Preincubation in an alkaline solution reduced the amount of reaction product in sections from hypo- thyroid hearts. After preincubation in pH anywhere between 9.0 and 11.0, sections of hearts from euthyroid rats were always much more heavily stained than those from hypothyroid rats. At pH above 10.6, however, the intensity of staining began to decrease in hearts from euthyroid rats. Staining of the normal heart was maximal after the tissue sections had been preincubated in pH from 10.4 to 10.6, and there was practically no staining of the sections from hypothyroid hearts (Figs. 2 and 3) after preincubation in pH anywhere between 10.4 and 10.6. The smooth muscle in the walls of arterioles stained equally well regardless of whether they came from normal or hypothyroid.
Electrophoresis of myosin on polyacrylamide gels using pyrophosphate medium to prevent dissociation. Panel A: left tube consists of myosin from right ventricle of a 10-week euthyroid rat. Middle and right tubes consist of myosin from right ventricles of two young rats that had been thyroidectomized 5 weeks earlier. Note the more rapid migration of the dark band of myosin from euthyroid rat. Panel B: Electrophoresis of combination of myosin from euthyroid and hypothyroid rat hearts. From left right: (1) hypothyroid (2) mixture in ratio of hypothyroid to euthyroid of 5 to 1 (3) and (4) mixture ratio of 1 to 1.

This staining provided an indication that the histochemical assay was working normally, as well as a convenient internal standard against which the staining of the myocardial cells could be compared (Fig. 3). In view of these results, the pH of 10.5 for the preincubation solution was selected since minor variations at this point did not produce significant changes in the degree of staining of either euthyroid or hypothyroid hearts.

As another check that a significant relation existed between the degree of staining after preincubation at pH 10.5 and the concentrations of V1 myosin isozyme, hearts from old rats, in which the relative concentration of V1 is much lower, were examined. Degree of staining and the amount of V1 were closely correlated (Fig. 4). Of interest as well was the uniform staining of cells in all but the very old rats in spite of the mixture of myosin isozymes that is present.

The temperature at which the tissue section was exposed to the solution containing ATP was varied from 25 to 40°C, and the duration of the exposure from 5 to 15 minutes. Although the degree of staining was enhanced by raising the temperature and by increasing the period of ATP hydrolysis, the difference between the responses of the primarily V1 containing euthyroid hearts and the primarily V3 containing hypothyroid hearts after preincubation in pH of 10.5 was not sensitive to these changes. The physiological temperature of 37°C and the duration of 10 minutes were chosen in order to produce substantial but not maximal staining. The influence of section thickness on the results of the assay was examined by cutting serial sections of increasing thickness from 1 to 15 μm from the right and left ventricles of a heart from a euthyroid rat. There was some non-uniformity of staining in 1- to 2-μm and 10- to 15-μm sections resulting from the difficulty in cutting uniformly thin sections of 1-2 μm and unevenness, particularly in the center of the 10- to 15-μm sections. Within the range of 3-9 μm, the density of the stain was uniform and increased with thickness (Fig. 5). The relation between density of stain and thickness appeared to be linear, but it was not measured photometrically. A thickness of 6 μm in the middle of the satisfactory range of 3-9 μm was chosen for standard use.

Examination of the stained sections of ventricles from normal hearts with the light microscope at high resolution revealed that the final produce of the ATPase histochemical reaction, that is cobalt sulfide, was present exclusively in the myofibrils (Fig. 2). No stain was ever seen in the mitochondria or in the spaces between the myofibrils. The location of reaction product using standard light optics was compared with the transverse striation pattern formed with phase contrast optics. When a localization within the myofibril could be made, the cobalt sulfide was deposited almost exclusively in the A bands. Staining was uniform among all of the myofibrils within a cell. Very careful examination of several hundred cells in photographic negatives was made with densitometry, and in no case was a significant difference seen in staining of myofibrils within a single cell. This was
true not only for young, euthyroid hearts, in which there is a single predominant isozyme of myosin, but also for both older euthyroid rats that have a significant mixture of isozymes of myosin and rats in which the isozyme pattern is changing following thyroidectomy (see below).

The uniformity of staining among the cardiac cells from young euthyroid rats was measured with densitometry. Although some variation existed among several hundred cells that were examined, the range of variation was very small, and resembled a normal distribution skewed somewhat toward the lower density. (Fig. 6) The skewing is almost certainly the result of the method of scanning that was used. Thirty-five millimeter negatives of high magnification photomicrographs were scanned with a spot approximately 100 \( \mu \text{m} \) in diameter, and the density measured in a matrix of 256 by 256 points. At any spot, the densitometer saw either exclusively myofibril or myofibril with some intermyofibrillar space. Since the latter was free of stain, the density of some points was contaminated by a variable fraction of unstained intracellular material. There was no significant difference in the distribution of densities of cells in consecutive sections from the same tissues.

Additional studies were conducted to see whether the histochemical procedure could be used on tissue that had already been used for physiological studies. After trabeculae from the endocardial surface of the right ventricle had been made hyperpermeable by an overnight soak in a solution containing 10 mM EGTA (McClellan and Winegrad, 1978), they were sus-
Within the sensitivity of the ATPase method, the distribution of myosin isozyme in the young euthyroid heart is homogenous (Fig. 6). Cells in the right and left ventricles and in the septum stain uniformly, and there is no detectable difference in the concentration of reaction product between right and left ventricles. Ten days after thyroidectomy, a small but definite decrease in the staining of the ventricular cell could be detected, compared with cells from a normal, euthyroid rat of the same age (Figs. 6 and 7). Sections from both normal and thyroidectomized hearts were processed in the same solution at the same time to enhance the validity of the comparison. In spite of the decline in reaction product in sections from thyroidectomized rats, all cells were almost equally stained 10 days after thyroidectomy. Seventeen days after thyroidectomy, the degree of staining is less than after 10 days, and some variability of the relative intensity of staining among cells could be seen. Within a single cell, however, staining remained uniform and restricted to the myofibrils.

Three and a half weeks after thyroidectomy, further
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Decreasing density

FIGURE 6. Histograms of the relative density of staining (panel in A) young euthyroid hearts, and at 10 days (panel B), 24 days (panel C) and 35 days (panel D) after thyroidectomy. Each histogram represents 65,536 separate measurements of density (a 256 X 256 matrix) made over 650 cells that were present in a single section of ventricular tissue. The ordinate indicates relative frequency with which a given density appeared. The abscissa is the same for all four histograms. Note the clear separation of each distribution from the others and the narrowness of the distributions except in the tissue 24 days after thyroidectomy.

At this stage in the transition of the myosin content of the ventricular cells from V1 to V3, when the range of intensity of staining among the cells was the greatest, the distribution of intensity of staining within a large population of cells was measured with densitometry. The relative intensity of staining of cells as measured by densitometry fell into a normal distribution with a small degree of skewing toward the lower densities (Fig. 6). The range of staining was very much greater than in the young, euthyroid heart, and the skewing was again almost certainly due to the presence of unstained spaces between the myofibrils. Within a single cell, there was no detectable non-uniformity in staining of the myofibrils.

Five weeks after thyroidectomy, all cells are very lightly stained (Fig. 2). Although some cells are slightly more darkly stained than others, the difference is small. There is no clear heterogeneity within individual cells.

Sensitivity of the Methods

For maximum use of the information generated by the histochemical technique, one would like to know the size of the smallest change in the concentration of V1 that can be detected. To assess sensitivity of the procedure, the relative amount of the myosin isozymes was determined electrophoretically in ventricles from young euthyroid rats in which V1 is the predominant isozyme, and from rats of the same age that had been thyroidectomized 10 days earlier. This

FIGURE 7. Photomicrographs of results of ATPase staining in young rat 10 days after thyroidectomy. Intensity of staining is less than in euthyroid rat and slight non-uniformity of staining among cells exists. Preincubation in pH 10.5.

comparison was chosen because the decrease in staining within this period was the smallest that could be unequivocally detected with the alkaline preincubation method for inhibiting V₃ myosin. In the first 10 days after thyroidectomy, there was a relative decline according to densitometrical analysis of the electrophoretic pattern of the myosin of approximately 15% in V₁ as the concentration of V₃ began to rise. Therefore, this histochemical technique is capable of detecting changes as small as 15%.

Discussion

Since the slowest isozyme of cardiac myosin, V₃, is inhibited by alkaline pH (Yazaki et al., 1974, 1979; Syrový, 1975) it has been possible to use alkaline preincubation to suppress the ATPase activity of V₃ in frozen sections of rat heart. This allows the histochemical examination of the activity of the fast isozyme of myosin, V₁, in cells that also contain a significant amount of V₃. Possible interference from mitochondrial, sarcolemmal, or sarcoplasmic reticular ATPase has been eliminated by the inclusion of azide, ouabain, and a concentration of Ca²⁺ high enough to inhibit the Ca pump in the sarcoplasmic reticulum. In normal young rats, in which myosin is almost entirely V₁ (Hoh et al., 1977; Lompre et al., 1981), the staining of the ventricles is altered only slightly by preincubation in solutions with pH between 4.3 and 10.6. The staining of sections of ventricle from hypothyroid rats, in which cardiac myosin is almost all V₃ (Hoh et al., 1977), is the same as in normal heart when the pH of the preincubation solution is between 4.3 and 4.6. Preincubation at pH between 10.0 and 10.6, however, almost completely eliminates staining in sections from hypothyroid rats, and the same correlation between loss of staining after preincubation at pH 10.5 and concentration of V₃ exists as rats age. Since V₃ is inhibited by high pH, alkaline preincubation can be used for specific examination of the activity of V₁ in the presence of V₃. A difference in intensity of staining between hearts from normal young rats and another tissue after preincubation of both at pH 10.5 indicates the existence of a significant amount of V₃ in the latter.

The technique is semi-quantitative. Ten days after thyroidectomy, the concentration of V₁ has decreased by about 15% and, after 17 days, there is another 10-15% decline as measured by electrophoresis on non-dissociating gels. A decrease in ATPase activity with alkaline preincubation is detectable histochimically in each case, indicating the capability of the technique for measuring a change in the concentration of V₁ that is as small as 15% of the total myosin content of the cells.

In the hearts from young euthyroid and the young hypothyroid rats, there is uniformity of staining among the cells, but in each of these cases the cells contain predominantly one type of myosin isozyme. In view of the sensitivity of the histochemical technique, this means that all cells contain within 15% of same concentrations of V₁ myosin. During the period after thyroidectomy when the rate of change in the concentration of isozymes is greatest and there are large fractions of both V₁ and V₃, considerable heterogeneity in the staining of individual cells was observed. The staining of some cells resembles the dark level achieved by normal young rat heart, and the staining of others the pale level of the end stage hypothyroid heart. The switch from primarily V₁ to primarily V₃ seems to occur relatively rapidly in individual cells compared with the time required for the change to be completed in the whole ventricle. This raises the possibility that transcription within a cell changes abruptly from one RNA to the other. There does not appear to be any special pattern of distribution of the differently stained cells either in regard to position within the ventricular wall or proximity to blood supply. The heterogeneity may simply be the consequence of temporal randomization within the population of myocardial cells. Heterogeneity may be restricted to periods of rapid transition of the isozyme content of cells in view of the uniformity of staining of hearts from older euthyroid rats in which the concentration of both V₁ and V₃ myosins is substantial, but relatively stable. Uniformity has not always been found in heart cells when labeled antibodies have been used to detect the presence of V₁ and V₃ myosins (Sartore et al., 1981). The explanation for any discrepancy between the results of the immunological and histochemical approaches probably lies with a relative difference in their sensitivities. The much more sensitive antibody technique is more
likely to detect the presence of small amounts of an isozyme or small differences when the concentrations involved are low. In the absence of double antibody staining of the same cell, it is difficult to be sure how much of the total myosin has been labeled by a single antibody. This problem is considerably less when the histochemical assay of myosin V1 ATPase activity is used with tissue from a young euthyroid rat as a control.

Although the histochemical technique does not have sufficient resolution for one to infer that individual myosin filaments are the same in their isozyme content or that heterogeneity within a small part of a myofibril does not exist, the uniformity of staining of myofibrils within each cell regardless of the amounts of the myosin isozymes indicates that the isozymes are relatively evenly distributed throughout the cell. Different regions of the cell should not contract with different velocities of shortening. The implications of a mixture of two populations of myosin with different kinetics of cross-bridge cycling are important even when the mixture is uniform. The slower myosins should form cross-bridges that are more likely to act as a drag on force, as the filaments slide past each other and the myosin links to the thin filament are repeatedly broken. The consequence of this mixture would be a slower velocity (Huxley, 1957). The velocity at any given load and degree of activation would vary with the mixture of fast and slow myosins. Control of the relative amounts of fast and slow myosin or V1 and V3 that are activated in a given contraction without change in the total active content or that heterogeneity within a small part of a myofibril does not exist, the uniformity of staining of myofibrils within each cell regardless of the relative amounts of the myosin isozymes indicates that the isozymes are relatively evenly distributed throughout the cell. Different regions of the cell should not contract with different velocities of shortening. The implications of a mixture of two populations of myosin with different kinetics of cross-bridge cycling are important even when the mixture is uniform. The slower myosins should form cross-bridges that are more likely to act as a drag on force, as the filaments slide past each other and the myosin links to the thin filament are repeatedly broken. The consequence of this mixture would be a slower velocity (Huxley, 1957). The velocity at any given load and degree of activation would vary with the mixture of fast and slow myosins. Control of the relative amounts of fast and slow myosin or V1 and V3 that are activated in a given contraction without change in the total active myosin would allow change of velocity without change of load at a constant level of activation. There are indications that maximum velocity is a continuous function of the relative amounts of V1 and V3 in intact rat heart cells (Schwartz et al., 1981) and that the cell can select which isozyme of myosin is activated by Ca (Winegrad et al., unpublished data).

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