Isolation and Characterization of Myosin Heavy Chain Isozymes of the Bovine Conduction System

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To determine the characteristics of cardiac myosin in the conduction system, a pure Purkinje fiber preparation, consisting of atrioventricular nodes and the ventricular conduction system, was obtained from bovine hearts. Two types of myosin heavy chain isozymes, α-type and β-type, were fractionated by affinity chromatography using monoclonal antibodies CMA19 and HMC50, which are specific for the α-type heavy chain and β-type heavy chain, respectively. Competitive enzyme-linked immunosorbent assay demonstrated that the content of β-type in the atrioventricular node (30-40%) was higher than that in atrial ordinary myocardium (10-20%) and that of the α-type was 30-40% in the ventricular conduction system, which was much higher than that in the ventricular ordinary myocardium (less than 10%). By one- and two-dimensional electrophoresis of the peptides produced by partial and complete digestion, the peptide compositions of α-type and β-type in the conduction system were shown to be very similar to those of α-type and β-type in ordinary myocardium, respectively. The Ca²⁺-activated ATPase activity of myosin of the atrioventricular nodes was lower than that of ordinary atrial myosin (0.46 ± 0.03 versus 0.58 ± 0.02 μmol Pi/mg/min, mean ± SEM, p<0.05) and in contrast, that of ventricular specialized myocardium was higher than that of myosin in the ventricular ordinary working myocardium (0.32 ± 0.03 versus 0.22 ± 0.01 μmol Pi/mg/min, p<0.05). This was in good agreement with the relative proportion of myosin isozymes. We concluded that there were two distinctive myosin heavy chain isozymes in the conduction system and that they were closely related to heart α and β of the ordinary working myocardium. (Circulation Research 1987;61:859-865)

Myosin is the major contractile protein and is composed of two heavy chains (HCs) and two pairs of low molecular weight light chains (LCs). Many studies have recently demonstrated the heterogeneity of cardiac myosin. There are two types of heavy chain isozymes (αHC and βHC) in cardiac myosin, and the differences between them have been demonstrated by peptide mapping, by immunologic properties, by measurement of ATPase activities, and by sequence analysis of cDNA. The isomyosins V1, V2, and V3, identified from their electrophoretic mobility in nondenaturing gels, correspond to homodimers of αHC, heterodimers of αHC and βHC, and homodimers of βHC, respectively. V1 has a higher ATPase activity, and the change from V1 to V3 is correlated with a decrease in the speed of muscle contraction and an increase in the economy of force generation. Isoforms of cardiac myosin HC are differentially expressed according to species.

Materials and Methods

Myosin Preparation

Using a dilution technique as previously described, myosin was extracted from bovine left atrial and ventricular free wall, atrioventricular (AV) nodes, and
the ventricular conduction system, consisting of the bundle of His, the right and left bundle branches, and false tendons. The LCs (I and II) were isolated from the myosin molecule by guanidine denaturation. 20

**Hybridoma Procedures and Screening Assay for Antimyosin Antibodies**

Monoclonal antibodies were obtained from cloned hybridomas as previously described, 24,29 essentially by the protocol of Köhler and Milstein. 31 Antimyosin activity in a medium from hybridoma colonies was screened quantitatively by enzyme-linked immunosorbent assay (ELISA), essentially by the method of Guesdon et al. 32 Antibodies from 2 hybridoma lines, CMA19 and HMC50, specific for αHC and βHC, respectively, were used in this study.

**Competitive Enzyme-Linked Immunosorbent Assay**

Competitive ELISA was performed according to the method of Engvall. 19 In brief, bovine atrial αHC or ventricular βHC, isolated as described below (0.1 μg/ml in 0.1 M carbonate buffer, pH 9.6), was bound to each well of a 96-well microtiter plate, and 0.1 μg of CMA19 or HMC50 and 10^−10−30 μg bovine myosin of ordinary myocardium and specialized myocardium, atrial αHC, and ventricular βHC were incubated with bound antibodies for 1 hour at 37° C. After 3 successive washings with phosphate buffer saline (PBS), biotinylated goat anti-mouse immunoglobulin was added to each well and incubated for 1 hour. The wells were washed 3 times with PBS containing 0.5%Tween-20, avidin D peroxidase was added with H2O2, and after washings with PBS, biotinylated goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad, Richmond, Calif.) with H2O2 and N-chloronaphthol.

**Preparative Electrophoretic Elution and Proteolytic Digestion of Myosin Heavy Chain**

Myosin isozymes were electrophoretically separated on preparative gels. The bands of myosin HC were cut from the gel, and HC was then eluted by electrophoresis. Limited proteolytic cleavage of myosin HC with α-chymotrypsin and Staphylococcus aureus V8 protease was performed according to the method of Cleveland et al. 36 In brief, myosin (100 μg/ml) was cleaved with α-chymotrypsin and V8 protease in the presence of SDS at a myosin-to-protease ratio of 10:1 for 30 minutes at 37° C. The reaction was stopped by boiling for 3 minutes in the presence of 2% SDS and 6% mercaptoethanol. The samples were electrophoretically separated on 14% acrylamide slab gels, using the Laemmli’s buffer system at 10 mA constant for 12 hours. The polypeptides were stained with silver according to the method of Morrissey. 37 For cyanogen bromide (CNBr) cleavage, myosin was dissolved in 70% formic acid and treated with solid CNBr in amounts 200 times greater than methionine residues for 18 hours at 25° C. The peptides were diluted with distilled water and lyophilized. Amino acid analysis indicated a 90–95% completion after the digestion. Two-dimensional gels were run according to the method of O’Farrell. 38

**Determination of ATPase Activities**

ATPase activities were assayed as previously described. 3 In brief, Ca2+-activated ATPase activities were assayed in 10 mM CaCl2, 5 mM ATP, and 0.05 M Tris-HCl (pH 7.5), and K+-EDTA-activated ATPase activities were assayed in 0.6 M KCl, 1 mM EDTA, 5 mM ATP, and 0.05 M Tris-HCl (pH 7.5). Myosin (100 μg) in 0.1 ml column buffer was added to 1.9 ml of the above reaction mixture and incubated for 5 minutes at 25° C. The reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid, and the precipitated protein was removed by centrifugation at 4° C. ATPase activity was determined by measuring the liberation of inorganic phosphate according to the method of Youngberg and Youngberg. 39 Protein was determined by the method of Lowry et al. 40 using crystalline bovine serum albumin as a standard.

**Results**

Figure 1 shows the competitive ELISA analysis. The reactivity of the myosin of ordinary ventricle with CMA19 was one order of magnitude less than that of the ordinary atrium, and those of the AV nodes and ventricular conduction system were intermediate between those of the ventricle and atrium. The apparent affinity of HMC50 decreased in the order of ordinary ventricular myosin, myosin of ventricular conduction system, myosin of AV nodes, and atrial myosin. The relative affinity for each of these myosins was quantified from the concentration required to inhibit 50%...
Competing Myosin

FIGURE 1. Competitive enzyme-linked immunosorbent assay analysis of bovine cardiac myosins with anti-aHC (A) and anti-βHC (B) monoclonal antibodies. Competitive enzyme-linked immunosorbent assay was conducted using four different preparations of cardiac myosin from atrium (•), AV node (△), ventricular conduction system (×), ordinary ventricle (○), and affinity-purified αHC (■) and βHC (□). All points represent the average of four analyses.

binding of the atrial αHC to CMA19 or the ventricular βHC to HMC50. As shown in Figure 1 and Table 1, these differences in reactivity with CMA19 and HMC50 between the myosin of ordinary myocardium and that of the specialized myocardium indicated that ventricular specialized myocardium contained much more αHC than ventricular ordinary myocardium, and that βHC was more highly expressed in the AV nodes than in the atrial ordinary myocardium.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that affinity-purified myosin isozymes were very pure and did not contain any actin or regulatory proteins (Figure 2A). Since the isozymes were eluted from the affinity column with 4 M guanidine, some LCs were dissociated, but 3 LCs were expressed in the conduction system. The LC1', which is known to be identical with atrial LC1, was present in both the αHC and βHC of the conduction system but not in the myosin of ordinary ventricular myocardium, while ventricular LC1 existed in both αHC and βHC of the AV nodes as well as in the ventricular conduction system.

Immunoblot analyses were performed to confirm the purity of affinity-fractionated isozymes. αHC of both ordinary myocardium and specialized myocardium purified by the affinity chromatocolumn coupled with CMA19 reacted with CMA19 but not with HMC50, while βHC purified by affinity chromatography using HMC50 reacted only with HMC50 (Figure 2B). In the affinity chromatography, all myosin bound to either CMA19 affinity column or HMC50 affinity column (data not shown). Based on the reactivity of the isozymes with CMA19 and HMC50, there were at least two immunologically distinctive isozymes in the myosin of the conduction system.

To elucidate the peptide composition of the myosin HC of the specialized myocardium, we isolated HC by preparative electrophoretic elution and compared the polypeptide fragments produced by proteolytic digestion. Figure 3 demonstrates the representative one-dimensional peptide maps of the αHC and βHC of the AV nodes, ventricular conduction system, and ordinary ventricle produced by digestion with α-chymotrypsin and Staphylococcus aureus V8 protease. Although there was a marked difference between the αHC and βHC maps, the peptide maps of the αHC of ordinary myocardium and specialized myocardium were indistinguishable, as was the βHC of these mycardia. In the two-dimensional analysis of the peptides produced by complete CNBr digestion, the peptide maps of αHC showed a great difference from those of βHC. On the contrary, although several peptides varied in intensity or identity, the αHC and βHC of the ventricular conduction system were very similar to the αHC and βHC of ventricular ordinary myocardium, respectively (Figure 4).

To determine the enzymatic properties of the myosin of the conduction system, we assayed the Ca2+- and K+-EDTA-activated ATPase activities (Table 2). The Ca2+-activated ATPase activity was highest in the atrial myosin (0.58 ± 0.02 μmol Pi/mg/min, mean ± SEM) and lowest in the ventricular ordinary myosin (0.22 ± 0.01 μmol Pi/mg/min). The activity of the myosin of the AV nodes and that of the ventricular conduction system were intermediate (0.46 ± 0.03 and 0.32 ± 0.03 μmol Pi/mg/min, respectively). This was in good agreement with the proportion of myosin isozymes in each myocardium.

Discussion

As previously reported from our laboratory, there are two distinctive myosin isozymes, α-type and β-type, in the conduction system. We purified the two types of HC isozymes from the bovine conduction

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>CMA19 (anti-αHC)</th>
<th>HMC50 (anti-βHC)</th>
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</thead>
<tbody>
<tr>
<td>Tissue type</td>
<td>(μg)</td>
<td></td>
</tr>
<tr>
<td>Atrial αHC</td>
<td>1.6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ordinary atrium</td>
<td>2.0</td>
<td>9.0</td>
</tr>
<tr>
<td>AV node</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Specialized ventricle</td>
<td>4.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Ordinary ventricle</td>
<td>16</td>
<td>1.2</td>
</tr>
<tr>
<td>Ventricular βHC</td>
<td>&gt;100</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The values given are micrograms of myosin required to inhibit 50% of antibody binding to atrial αHC or ventricular βHC as calculated from competitive enzyme-linked immunosorbent assay. The average SEM for all measurements was less than 5%.
FIGURE 2. Analysis of purified myosin isozymes by SDS-polyacrylamide gel electrophoresis (A) and immunological characterization of myosin isozymes by immunoblot technique (B). A: About 5 μg of each myosin isozyme, affinity-purified ventricular αHC (a), βHC (b), αHC (c), and βHC (d) of AV node and αHC (e) and βHC (f) of ventricular conduction system, was applied on a 10% slab gel, electrophoresed at a 10 mA constant current for 8 hours, and stained with silver. The positions of the heavy chain subunits (HC) and of light chain subunits of ventricular myosin (VLC1 and VLC2) and of light chain subunit of myosin of conduction system (LC1) are indicated. B: Affinity-purified myosin isozymes, ventricular αHC (a) and βHC (b), αHC (c) and βHC (d) of AV node, and αHC (e) and βHC (f) of ventricular conduction system, were electrophoresed, transferred from the polyacrylamide gels to nitrocellulose sheets, and stained immunologically with monoclonal antibodies (CMA19 and HMC50).

System by affinity chromatography using monoclonal antibodies and characterized these isozymes. By competitive ELISA, β-type HC was expressed in the AV node more than in the ordinary atrial myocardium, and α-type HC was much more abundant in the ventricular conduction system than the ordinary ventricular myocardium. Peptide composition was investigated by one- and two-dimensional peptide mapping produced by partial and complete digestion. Primary structures of the two isozymes, α-type and β-type, in the conduction system were very similar to the two isozymes, αHC and βHC, respectively, in the ordinary myocardium. In the assay of Ca²⁺-activated ATPase activity, it was higher in the myosin of the ventricular conduction system than in the ordinary ventricular myosin. This was in good agreement with the higher proportion of α-type myosin in the conduction system. Thus, we concluded that in the conduction system, there were also two distinctive myosin HC isozymes (α-type and β-type), which were closely related to αHC and βHC in the ordinary working myocardium, respectively, and that the proportion of the two isozymes in the conduction system was markedly different from that in the ordinary myocardium.

A variety of biochemical, structural, and immunological studies have shown that muscle cells of the Purkinje fiber system contain special features distinct from ordinary working myocytes. With the exception of LCs, there were only limited studies concerning the myosin in the conduction system. Sartore et al and Gonzalez-Sanchez and Bader demonstrated in chicken that the myosin of Purkinje fibers differs from that of ordinary working myocytes. The assay solutions for Ca²⁺- and K⁺-EDTA-activated ATPase activities are those described in "Materials and Methods." Values are mean ± SEM for 4 experiments.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Ca²⁺-ATPase (μmol Pi/mg/min)</th>
<th>K⁺-EDTA (μmol Pi/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrium</td>
<td>0.58 ± 0.02</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>AV node</td>
<td>0.46 ± 0.03</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>Specialized ventricle</td>
<td>0.32 ± 0.03</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>Ordinary ventricle</td>
<td>0.22 ± 0.01</td>
<td>0.65 ± 0.03</td>
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</tbody>
</table>

The assay solutions for Ca²⁺- and K⁺-EDTA-activated ATPase activities are those described in "Materials and Methods." Values are mean ± SEM for 4 experiments.
gene. In the two-dimensional peptide maps, however, there were some variations in intensity or identity between isozymes of the conduction system and those of the ordinary myocardium. These differences might indicate some conduction system–specific peptides. In either case, compared with the distinctive differences between the maps of αHC and those of βHC, of which homology at nucleotide sequence level was very high (more than 90%), the differences between isoforms of the conduction system and those of the ordinary myocardium were so little that α-type and β-type HC in the conduction system should be closely related to αHC and βHC in the ordinary myocardium, respectively. Schiaffino et al. stated that nodal-myosin HC appears to be antigenically similar but not identical to the myosin HCs present in fetal skeletal muscle. Whether a distinctive myosin gene is expressed in nodal tissue needs further investigation.

The Ca²⁺-activated ATPase activity of the myosin of the ventricular conduction system was higher than that of ventricular ordinary myocardium, which is consistent with the observation of Tamura et al. Saito et al suggested the existence of a new type of cardiac myosin isoform in specialized myocardium because of the different ATPase activities of myosins between ordinary myocardium and specialized myocardium. But there was also a different proportion of the two isoforms. αHC was more highly expressed in the ventricular conduction system than in ventricular ordinary myocardium. Considering the ATPase activity of the myosin of the conduction system and the relative proportion of the myosin isoforms, the ATPase activity of each isoform in the conduction system was thought to be almost the same as that of the ordinary myocardium.

The myosin from bovine ventricular conduction tissue gave 3 bands of light chains in SDS-PAGE, which is consistent with the finding of Whalen et al. and Saito et al. Although the AV node exists in the atrium,
ventricular LC1 was present in the AV nodal myosin. These findings indicate that in the conduction system, two types of LC1 are expressed in the atrium and the ventricle. Since αHC was predominant in the atrium, it is likely that LC1', which is identical with atrial LC1, expressed with the HCa in the conduction system, but in this study, LC1' was expressed not only with αHC but also with βHC (Figure 2A). This suggests that LC1' was expressed independently of αHC and βHC as well as other LCs.34-49-50 In crossreinnervation studies of skeletal muscle, the light and heavy chains of skeletal myosin always appear to be matched, i.e., the synthesis of fast light chains is accompanied by the synthesis of fast heavy chains, while slow light chains and slow heavy chains also appear synchronously.51,52 Of great interest is that in the cardiac myosin, the genes of HCs and LCs must be regulated independently, though the HCs and LCs construct the subunit of the myosin molecule and function together.

We have demonstrated the presence of two types of HC isoymes in the conduction system as observed in ordinary myocardium. Furthermore, the peptide maps and enzymatic activities of the individual isoymes in the conduction system were very similar to those in the ordinary myocardium. We speculate that the same genes coding for HCs and LCs are expressed in both specialized and ordinary myocardium, though the gene coding for αHC is more highly expressed in the ventricular conduction system, and that the genes coding for atrial and ventricular LC1 are expressed together in the specialized myocardium.

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