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 heavy chain 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 VI, 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. VI 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, and fiber types, age, hormonal state, and cardiac overload. Although specialized myocardium is known to exhibit characteristic morphological features and differs from the contractile myocardium in several respects, including cell form and size, intercellular contacts, and the absence of T tubules, there are quite a few reports concerning the characteristics of the myosin HC in specialized myocardium. We have recently demonstrated the existence of the two types of myosin heavy chain isozymes, α-type and β-type, in the conduction system and a marked difference in their distribution between specialized and ordinary working myocardium by immunohistochemical study using monoclonal antibodies, but it is not known whether the two isozymes in the conduction system are the same as those in the ordinary myocardium. In this study, we isolated the two HC isozymes from specialized myocardium of bovine heart by affinity chromatography, using the monoclonal antibodies CMA19 and HMC50, specific for αHC and βHC, respectively, and determined their peptide composition and enzymatic activities with reference to those of ordinary working myocardium.

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.30

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. 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.32 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 10–1000 μg bovine myosin of ordinary myocardium and specialized myocardium, atrial αHC, and ventricular βHC were incubated with bound antigens 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, 4-amino antipyrin, and phenol as substrate.

Preparation of Affinity Columns and Purification of Myosin by Affinity Chromatography

Preparation of affinity columns and isolation of myosin isozymes were performed as previously reported.24 In brief, monoclonal antibodies (CMA19 and HMC50) in a coupling buffer (0.1 M NaHCO3, pH 8.3, containing 0.5 M NaCl) were added to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). To exclude the heterodimer, 1 mg of myosin was first applied to the column in 1 ml of column buffer (20 mM Tris- HCl containing 0.5 M KCl, pH 7.5) and recycled 10 times over the column for 6 hours at 4° C. The unbound myosin was collected by washing with column buffer according to the procedure of Everett et al29 and reapplied to another affinity column. The column was washed with column buffer, and the bound myosin was eluted with 4 M guanidine HCl in a column buffer. The fractions were collected until the absorbance at 280 nm was less than 0.05. Atrial HCa and ventricular βHC for competitive ELISA were fractionated as nonadenaturing myosins, which were not bound to the HMC50 and CMA19 columns, respectively. Immunoblot analysis was performed as previously described.24 Briefly, the purified HC isozyme was electrophoresed in the presence of sodium dodecyl sulfate (SDS), transferred from the polyacrylamide gels to nitrocellulose sheets, incubated with the antibodies, and stained by 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 lyophylized. 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.39 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 al40 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%
Competitive enzyme-linked immunosorbent assay analysis of bovine cardiac myosin with anti-aHC (A) and anti-/3HC (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 aHC (●) and /3HC (●). All points represent the average of four analyses.

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 LCI', which is known to be identical with atrial LCI, was present in both the aHC and /3HC of the conduction system but not in the myosin of ordinary ventricular myocardium, while ventricular LCI existed in both aHC and /3HC of the AV nodes as well as in the ventricular conduction system.

Immunoblot analyses were performed to confirm the purity of affinity-fractionated isozymes. aHC of both ordinary myocardium and specialized myocardium purified by the affinity chromatocolumn coupled with CMA19 reacted with CMA19 but not with HMC50, while /3HC 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 /3HC maps, the peptide maps of the αHC of ordinary myocardium and specialized myocardium were indistinguishable, as was the /3HC of these myocardia. 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 /3HC. On the contrary, although several peptides varied in intensity or identity, the αHC and /3HC of the ventricular conduction system were very similar to the αHC and /3HC 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 isoforms in each myocardium.

**Discussion**

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

**Table 1. Relative Specificity of Anti-Myosin Monoclonal Antibodies for Myosins of Ordinary Myocardia and of Specialized Myocardia**

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>CMA19 (anti-αHC)</th>
<th>HMC50 (anti-βHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial aHC</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 /3HC</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 aHC or ventricular /3HC 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 (LCl') 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).

Table 2. Comparison of ATPase Activities of Myosin of Ordinary Myocardia and of Specialized Myocardia

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Ca²⁺&lt;sup&gt;+&lt;/sup&gt; ATPase</th>
<th>K⁺-EDTA ATPase</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>
</tr>
</tbody>
</table>

The assay solutions for Ca²⁺<sup>+</sup>- 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 isozymes 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 isozyme 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 isozymes. 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 isozymes, the ATPase activity of each isozyme 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 Hcka 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 isozymes in the conduction system as observed in ordinary myocardium. Furthermore, the peptide maps and enzymatic activities of the individual isozymes 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 ventricle. Since αHC was predominant in the atrium, it correlates with speed of myocardial contraction. J Mol Cell Cardiol 1981;13:1071–1075

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