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In Vitro Motility Assay of Atrial and Ventricular Myosin From Pig

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Abstract The role of myosin isoforms in determining contractile filament velocity in the atrium and ventricle of the pig heart was studied by measuring the motion of fluorescently labeled actin over myosin (in vitro motility assay). A rapid and relatively simple method for purification of myosin from small tissue samples was used. The relative extent of light chain-2 phosphorylation was about 30% in both atrial and ventricular myosin extracts. Although the extracted myosin was not free from contaminating proteins, mainly actin, the mean velocity at optimal pH and 32°C of both atrial (3.3 μm/s) and ventricular (2.3 μm/s) myosin were similar to those obtained using extensively purified myosin. The filament sliding velocities using isolated myosin and actin are lower than those estimated from previously published experiments on skinned fiber preparations, which might reflect an influence on sliding velocity by the filament organization or regulatory proteins in the muscle fiber. However, the ratio between velocities of atrial and ventricular myosin was similar in the motility assay (1.5) and muscle fiber experiments (1.6), which might suggest that these two methods reflect the same fundamental processes in cardiac contraction and that the difference in filament sliding velocity between the atrium and ventricle of the pig heart is determined my their myosin isoforms. J. Cell. Biochem. 67:241–247, 1997.

Key words: motility assay; myosin; atrium; ventricle; pig; cardiac; isoforms

Myosin is the main motor protein in the heart, and its enzymatic properties determine many of the functional characteristics of cardiac contraction. Cardiac myosin consists of two heavy chains (Mw approximately 200 kD), two light chains type 1 (LC-1, 27 kD), and two light chains type 2 (LC-2, 18 kD) [Delcayre and Swynghedauw, 1975; Morano et al., 1988]. It is well known that mechanical and enzymatic properties in different cardiac muscles can be associated with the type of their predominating myosin isoform [e.g., Pope et al., 1980; Schwartz et al., 1981; Morano et al., 1988]. Expression of different myosin isoforms can alter the mechanical properties of the cardiac muscle in one species (e.g., in response to altered functional demands or hormonal changes) [e.g., Schwartz et al., 1981]. Recent genetic analysis has linked some cardiomyopathies to specific mutations in the β-myosin heavy chain gene [e.g., Geisterfer-Lowrance et al., 1990; Elstein et al., 1992].

Several different methods have been utilized to assay the function of contractile proteins from cardiac muscles. The use of isolated muscle fiber preparations enables direct assay of important mechanical parameters (e.g., maximal shortening velocity (V_{max}), force, or stiffness). However, the mechanical properties can be influenced by changes in several contractile and regulatory proteins and tissue structure, which makes a specific coupling of mechanics to protein isoform changes difficult in many cases. On the other hand, measurements of actomyosin ATPase using isolated contractile proteins require highly purified proteins and do not give information on mechanical properties.

In in vitro motility assays, the motion of myosin-coated beads over actin cables or of fluorescently labeled actin over a myosin surface is directly measured [Scheetz and Spudich, 1983; Yanagida et al., 1984; Kron and Spudich, 1986]. This enables the actin-myosin interaction to be studied without possible influence of

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regulatory proteins and myofilament arrangement. The translation velocity, at least under some experimental conditions, is considered to reflect the same events as those determining the shortening velocity of muscle fibers [Scheetz and Spudich, 1983; Homsher et al., 1992]. The in vitro motility assays have been applied to cardiac muscle to study the correlation between sliding velocity and myosin isoform distribution in rat and rabbit hearts from animals with altered thyroid hormone levels [Yamashita et al., 1992; Sata et al., 1993]. An increase in the relative amount of the α-myosin heavy chain (reflected in an increase in V_1 and a decrease in V_3 isoforms of the native myosin) was associated with an increased ATPase rate and an increased sliding velocity in these studies. In cardiomyopathic hamsters, a decrease in sliding velocity was correlated with a change in myosin isoforms towards more of the V_3 form [Yamashita et al., 1993]. More recent in vitro studies have determined active force and found that the slower cardiac (V_3) myosin from hypothyroid rabbits have higher force per cross-bridge than the V_1 isoform [VanBuren et al., 1995].

In man and pig, the cardiac myosin is mainly of the slow V_3 type in the ventricle and mainly of the faster V_2 type in the atrium [Morano et al., 1988]. Alterations in the mechanical performance have in these species been associated with changes in the amino acid composition of the myosin heavy chain, in the myosin light chain isoforms, or in myosin light chain phosphorylation [Hirzel et al., 1985; Morano et al., 1995a; Lankford et al., 1995; Morano et al., 1996]. During recent years, the properties of the pig heart have become increasingly interesting due to the possibilities for using this organ for transplantation to man [e.g., Hoopes and Platt, 1996]. To our knowledge, motility assay analysis of pig cardiac myosin is not available at present, and studies on human biopsy material are very limited. In a study by Cuda et al. [1993] on muscle samples from patients with hypertrophic cardiomyopathy coupled with a mutation leading to an amino acid substitution in the β-heavy chain, the actin filament sliding over mutated β-myosin was slow. In this study, β-myosin was in addition purified from slow skeletal muscle, using a specific antibody, revealing similar changes in motility as in cardiac β-myosin. In a paper by Nguyen et al. [1996], myosin was purified from human biopsy samples weighing approximately 2 mg. It was shown that the actomyosin ATPase and in vitro motility were unaltered in cardiac samples from patients with heart failure due to mitral regurgitation.

The purpose of the present investigation was to obtain basic data from pig cardiac myosin. Comparisons were made between atrial and ventricular samples from the pig heart, and we demonstrate a 45% higher translocation velocity of atrial myosin in this species. We used a relatively simple myosin extraction method utilizing a high-speed centrifuge which gave a myosin preparation containing small amounts of mainly thin filament–associated proteins. The measurements on more extensively purified myosin, obtained using ultracentrifugation, gave identical results. This shows that the contaminating proteins in the myosin obtained using the rapid purification did not influence the velocity.

METHODS

Extraction and Purification of Proteins

Small biopsies from the atrium and ventricle weighing 100–150 mg were obtained from fresh adult pig hearts at a local slaughterhouse. The samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Myosin was extracted for motility assay experiments using pyrophosphate extraction and two high-speed centrifugation steps (rapid purification). After thawing, the samples were homogenized in a Guba-Straub extraction buffer (3.4 ml/g tissue) containing (mM) NaCl 300, NaH_2PO_4 100, Na_2HPO_4 50, $MgCl_2$ 1, $Na_4P_2O_7$ 10, EDTA (ethylenediaminetetraacetic acid) 10, and DTT (dithiothreitol) 1. NaN_3 (sodium azide), 0.1%, and leupeptin, 10 μg/ml, were added. The pH was adjusted to 6.5 with NaOH. The samples were extracted in this solution on ice for 20 min with gentle stirring and thereafter centrifuged at 4°C for 10 min at 30,000*g* using a MSE Hi-Spin centrifuge (Crawley, England). The supernatant was collected and diluted 1:12 with a low salt solution containing 1 mM EDTA at pH 7.0 adjusted with Tris, gently stirred for 30 min, and then allowed to stand for another 30 min, followed by a new centrifugation as above. The pellet was dissolved in the same volume as before centrifugation in a low salt ATP solution containing (mM) EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid) 1, $MgCl_2$ 2, ATP 1, and DTT 1 at pH 7.0 (adjusted

with Tris) and left on ice for 30 min before a new centrifugation. The pellet containing myosin was dissolved in 500 µl of storage solution (mM): NaCl 500, Tris 10, EDTA 10, and DTT 1 at pH 7.0 (adjusted with HCl). The myosin (approximately 0.3 mg/ml) was stored on ice and used within 1–2 days. In one series of experiments, we used myosin purified according to the more extended protocol described in Sata et al. [1993] which involved an ultracentrifugation step (extended purification). The starting tissue weight was approximately 20 g in this case. Rabbit skeletal muscle heavy meromyosin (HMM), used for comparison in some experiments, was prepared as described by Okamoto and Sekine [1985].

Actin for motility assays was purified from rabbit skeletal muscle according to Pardee and Spudich [1982]. The actin was labeled with rhodamine-phalloidin (R-415; Molecular Probes Inc., Eugene, OR) as described by Kron et al. [1991].

In Vitro Motility Assay

The assay was performed at 32°C essentially as described by Kron and Spudich [1986] using flow cells coated with nitrocellulose (2,4,6-Collidine (Sigma Chemical Co., St. Louis, MO) diluted to 0.85% with Amyl acetate). The assay solutions contained (mM) $MgCl_2$ 4, EGTA 1, KCl 25, and Imidazol 25 with pH 7.4. The extracted myosin was diluted to approximately 0.05 mg/ml in the assay solution with high salt ([KCl] 300 mM) to allow the myosin to disassemble into monomers. The freshly diluted myosin was applied to the cuvette and incubated for 2 min. The surface was then rinsed with buffer including 1 mM ATP and 125 mM KCl to remove contaminating actin, followed by a rinse with pure assay solution. The rhodamine-phalloidin–labeled actin was infused twice and allowed to bind to the myosin for 30 s each time. The motion was initiated by changing to an assay solution with 1 mM ATP, 80 mM KCl, 5 mM DTT, and 0.6% metylcellulose. All solutions were degassed, and the final ATP containing solution was supplemented with an oxygen-scavenging system consisting of glucose oxidase (0.1 mg/ml), catalase (0.018 mg/ml), and glucose (3 mg/ml) to reduce photobleaching. In one series of experiments the pH dependence of the motility was determined using the protocol above with pH adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in the final ATP solution.

The flow cells were placed on an inverted microscope (Nikon TMD, Tokyo, Japan) equipped for epifluorescence and with a temperature-regulated oil immersion objective (Nikon ×100, NA 1.4). The image was observed using a CCD camera and an image intensifier system (C-2400-80; Hamamatsu, Hamamatsu-City, Japan), processed with an Argus-10 image processing system (Hamamatsu), and recorded on videotape. During analysis, the tape was replayed through the Argus-10 system, and the position of moving filaments was recorded at 1 s intervals. For each preparation one to two flow cells were analyzed, and approximately ten filaments showing regular and straight motion were tracked. The standard deviation in this group was small (between 10–15% of the mean), and the average of their velocity (in micrometers per second) was taken as representative for the myosin sample.

Analysis of Protein Composition and Phosphorylation Measurements

The protein concentrations were determined using the Bradford method [Bradford, 1976] with bovine serum albumin as standard. The protein composition of the extracts was analyzed using SDS–polyacrylamide gel electrophoresis [Laemmli, 1970] on 7–18% acrylamide gradient gels. The gels were stained with Coomassie blue R-250 and scanned to determine the relative contents of myosin and actin. Myosin light chain phosphorylation levels of atrial and ventricular myosin preparations were investigated by two-dimensional gel electrophoresis using a mini gel system (Bio-Rad, Richmond, CA), as described by Morano et al. [1989]. The gels were stained and scanned, and the phosphorylation was calculated as the area under the intensity peaks. Phosphorylation is expressed as the percentage of the total area of the respective light chain peak. All chemicals were obtained from Sigma.

Statistics

Results are given as mean values ± SE. The n values represent the number of myosin preparations.

RESULTS

Figure 1 shows SDS gel separations of proteins from the atrial and ventricular extracts used for motility assays. Myosin is the dominat-

ing protein after extraction, although there are some remains of actin and thin filament–associated proteins in the extracts obtained using the rapid purification (Fig. 1, lanes 1,2). We have not established the identity of all these extra proteins, but the major nonmyosin proteins were actin and thin filament–associated proteins (tropomyosin and troponin subunits). The ratio of actin/myosin in the rapid myosin extract was approximately 0.60. The myosin purified according to Sata et al. [1993] was almost free of contaminating proteins (Fig. 1, lanes 3,4).

The myosin preparations were introduced into the flow cell in a high salt buffer. Thus, the myosin was predominantly in the monomeric form. Since we primarily were interested in minimizing preparation time and sample weights, we did not in this investigation attempt measurements on HMM fragments of cardiac myosin which would have required additional purification steps. Filament motion could be recorded for at least 5 min for each cuvette, and approximately 30 s was used per location. The analysis was based on tracking filaments showing regular motion for 5–10 s. In general, 50–75% of the filaments per view were found to move. For each preparation, ten filaments were analyzed, and their average velocity was taken as representative for that preparation. The data in panels A and B of Figure 2 are the mean of eight and three myosin preparations prepared according to the rapid and more extensive methods, respectively. The velocity of the atrial myosin was 45% higher than that of the ventricular using the rapidly purified proteins. The more extensively purified myosin gave similar results. For comparison we measured the velocity of actin over skeletal muscle HMM. This velocity under our assay conditions was 4.6 $\pm$ 0.2 (n = 3) μm/s, which is approximately two times faster than that of the cardiac ventricular myosin.

The measurements described above were all performed at pH 7.4 in the assay buffer. In order to exclude that the differences in velocity between atrial and ventricular myosin were due to differences in pH optima for actin motion over these myosins, we performed motility assays (rapid purification) in solutions with pH

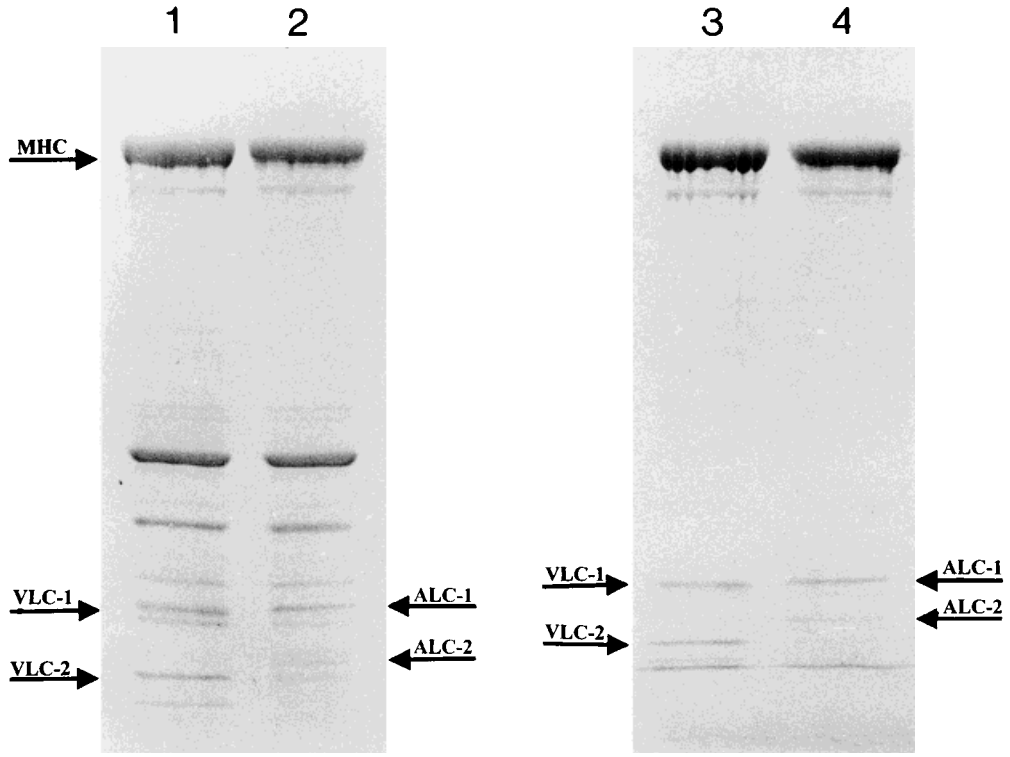

Fig. 1. Photograph of SDS-polyacrylamide gels (gradient 7–18%) with separations of myosin extracts. **Lanes 1,2:** Extracts from the rapid preparation (lane 1, ventricle; lane 2, atrium). The myosin heavy chains (MHC), ventricular (VLC-1, VLC-2), and atrial (ALC-1, ALC-2) light chains are indicated. Some remains of other muscle proteins mainly actin are observed. **Lanes 3,4:** Extracts from extensively purified myosin (lane 3, ventricle; lane 4, atrium).

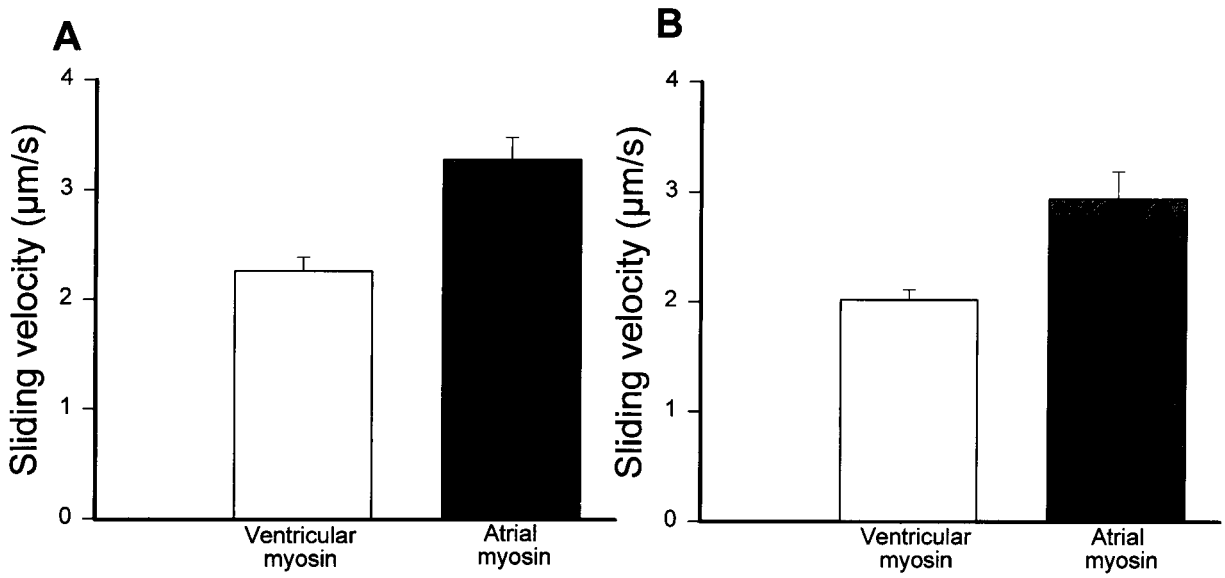

Fig. 2. Mean velocity of actin sliding over myosin purified according to the rapid (**A**) (n = 8 in each group) and more extensive (**B**) (n = 3 in each group) methods.

from 6.5–8.0. Optimal pH for motility was in the range 7.0–8.0 for both the atrium and ventricle myosin (atrium pH 6.0, 0; pH 6.5, 0; pH 7.0, 2.2; pH 7.5, 3.3; pH 8.0, 3.5; pH 8.5, 3.0 µm/s, n = 1; ventricle pH 6.0, 0; pH 6.5, 0; pH 7.0, 2.1; pH 7.5, 2.5; pH 8.0, 3.2; pH 8.5, 2.5 µm/s, n = 2). This material was small but shows that the difference in velocity between atrial and ventricular myosin, measured at pH 7.4, was not due to a difference in pH optimum for velocity of these two myosins.

Since myosin light chain phosphorylation can influence the behavior of cardiac muscle fibers, we considered it important to determine the extent of myosin light chain phosphorylation in the preparations. The myosin light chain-2 phosphorylation in the atrial and ventricular preparations (rapid method) is shown in Table I. The ventricular myosin has two forms of regulatory light chains (VLC-2 and VLC-2*), both of which can be phosphorylated. The corresponding atrial light chain (ALC-2) can be both mono- and double-phosphorylated [Morano et al., 1989]. The relative phosphorylation levels were 25–30% for each light chain.

TABLE I. Relative Light Chain Phosphorylation in Myosin Extracts From Atrium and Ventricle*

Atrium				
Light chain	ALC-2U	ALC-2P	ALC-2PP	
%	75 ± 8	20 ± 6	5 ± 2	
Ventricle				
Light chain	VLC-2U	VLC-2P	VLC-2*U	VLC-2*P
%	87 ± 5	13 ± 5	86 ± 6	14 ± 6

Separations were as described by Morano et al. [1989]. For the atrium, the unphosphorylated (U), the phosphorylated (P) and the double-phosphorylated (PP) variants of the light chain 2 (ALC-2) were separated. In the ventricle, the unphosphorylated (U) and phosphorylated (P) forms of the two light chain isoforms (VLC-2, VLC-2) were separated. The values are expressed in percentage of the total content of the respective light chain (n = 8).

DISCUSSION

We find that the comparatively rapid method to obtain a crude myosin extract from cardiac muscle samples enables reproducible measurements of filament motion in the motility assay. Since the motility data using more extensively purified myosin [Sata et al., 1993] gave similar velocities, we conclude that the contaminating proteins in the crude extract do not influence filament sliding. Regulatory proteins may influence the filament velocity when added to actin in the assay buffer [Fraser and Marston, 1995]. In our experiments, the actin added was pure, and the contaminating proteins in the myosin solution are therefore most likely bound to the glass surface without interfering with labeled actin or myosin function or washed away during the procedure. The comparatively high actin contamination is most likely either washed away by the ATP solution prior to the addition of labeled actin or binds and blocks damaged

myosin heads. The latter effect of unlabeled actin might actually be beneficial and can be used in these assays to remove nonfunctional myosin heads [Sellers et al., 1993]. Pure myosin for ATPase and in vitro motility has been obtained from small biopsy samples using ultracentrifugation [Nguyen et al., 1996], but other methods (e.g., purification from muscle fiber preparations) might give myosin preparations with contaminating proteins. The present investigation shows that the in vitro motility assay is comparatively insensitive to contaminating proteins in the myosin extract. The rapid method is potentially useful for applied studies of cardiac contractile protein function.

In many aspects the myosin composition of the pig heart resembles that of man [Morano et al., 1988], where the ventricular myosin is mainly of β-type. Pig heart has also been proposed as a potential source for transplantation to man, which makes a characterization of pig cardiac myosins of importance. To our knowledge, we present here the first in vitro motility assay data from atrial and ventricular myosin from pig. The sliding velocities at 32°C of the ventricular and atrial myosins were approximately 2 μm and 3 μm per second, respectively. The measurements were performed at optimal pH, and the ratio between the sliding velocity of atrial and ventricular myosin was 1.5.

The filament velocity measured in the motility assay of skeletal myosin is considered to reflect similar cross-bridge reactions as those occurring during unloaded shortening of muscle fibers, although some differences exist and interpretation might be difficult [cf. Homsher et al., 1992]. The maximal (unloaded) shortening velocities of skinned atrial and ventricular muscle fiber preparations from pig at 20°C have been reported to be 4 and 2.5 muscle lengths per second [Morano et al., 1988]. When a sarcomere length of 2 μm was used, these mechanical data would correspond to filament velocities of 4 and 2.5 μm/s in the atrium and ventricle, respectively. When the difference in temperature is considered, these values are higher than those measured using the motility assay, which might be due to several differences in the experimental conditions (e.g., in the composition of thin filaments or in the organization of the myosin molecules). However, the ratio between the velocity of atrial and ventricular myosin in our in vitro motility assay data (1.5) was similar to the ratio between unloaded shortening velocity of atrial and ventricular skinned fibers (1.6 [Morano et al., 1988]), which suggests that these two measurements reflect similar reactions and that the myosin in the atrium is 50% faster than that in the ventricle. On the other hand, the difference between atrium and ventricle appears to be higher when the rate of tension development from rigor after photolytic release of ATP in the presence of Ca^{2+} is compared (atrium is more than sixfold faster [Morano et al., 1995a]). This might reflect that the myosin properties have larger effects on the cross-bridge reactions determining the rate of force development in cardiac muscle. We cannot at present, based on experiments on the skinned fibers, exclude that the difference in contraction rate between atrium and ventricle is also due to other factors (e.g., differences in thin filament regulatory proteins). The physiological role of the different velocities generated by atrial and ventricular myosins is unclear. However, differences in velocity between muscles is associated with differences in tension economy. It is possible that the slow ventricular myosin is more economical than the fast atrial and therefore more adapted to sustained contractions at low energy expenditure.

Previous experiments on skinned fibers have suggested that the extent of myosin light chain-2 phosphorylation influences Ca^{2+} sensitivity and the rate of tension development [Morano et al., 1985, 1995a]. We find that the extent of myosin light chain phosphorylation was about 30% in both the atrium and ventricle, but at present we have no information regarding how the extent of light chain phosphorylation influences the velocity determined in the motility assay. The unloaded shortening velocity of skinned fibers is, however, not influenced [Morano et al., 1986], which suggests that phosphorylation is not a major determinant of motility assay velocity. We show that the difference in shortening velocity between the atrium and ventricle reflects properties of the myosin molecule. This might be associated with differences in the heavy chain and/or light chain composition. Interestingly, the light chain-1–actin interaction has recently been proposed to influence contractile function of cardiac muscle [Morano et al., 1995b; Morano et al., 1996], and the modulation of pig and human myosins may also be due to expression of isoforms of the light chains rather than in the heavy chain only.

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