Transverse Stiffness of Myofibrils of Skeletal and Cardiac Muscles Studied by Atomic Force Microscopy

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Abstract: The transverse stiffness of single myofibrils of skeletal and cardiac muscles was examined by atomic force microscopy. The microscopic images of both skeletal and cardiac myofibrils in a rigor state showed periodical striation patterns separated by Z-bands, which is characteristic of striated muscle fibers. However, sarcomere patterns were hardly distinguishable in the stiffness distributions of the relaxed myofibrils of skeletal and cardiac muscles. Myofibrils in a rigor state were significantly stiff compared with those in a relaxed state, and in each state, cardiac myofibrils were significantly stiffer compared with skeletal myofibrils. By proteolytic digestions of sarcomere components of myofibrils, it was suggested that cardiac myofibrils are laterally stiffer than skeletal myofibrils because Z-bands, connectin (titin) filament networks, and other components of sarcomere structures for the former myofibrils are stronger than those for the latter.

Key words: sarcomere, Z-band, actomyosin, connectin (titin), lateral stiffness.

Both skeletal and cardiac muscles are striated muscles, the fibers of which are composed of bundles of myofibrils. In each myofibril runs a series of sarcomeres where the contractile force is produced by the interaction between thin filaments and myosin heads extruded from thick filaments [1]. Mechanical stresses imposed on sarcomeres in functioning muscle fibers are not the same between skeletal and cardiac muscles. In skeletal muscle, the force produced is transmitted along muscle fibers to move the bones attached to the fibers, producing longitudinal stress on muscle fibers. In cardiac muscle, however, the force similarly produced is transmitted to the heart wall to squeeze out the blood filled in the heart cavity, producing longitudinal as well as transverse stresses to muscle fibers. Thus it has been known that cardiac muscle in a relaxed state is much stiffer than relaxed skeletal muscle because of the high content of connective components in the former muscle fibers [2].

The longitudinal stiffness of muscle fibers has extensively been examined in relation to the force production, which clarified that it dominantly comes from the actomyosin interaction in the overlap region of sarcomeres in skeletal as well as in cardiac muscles [3, 4]. In cardiac muscle fibers, however, the extra series elastic components further increase longitudinal stiffness [5, 6]. The transverse stiffness of skeletal muscle has been studied by osmotic compressions [7, 8] and that of myofibrils by the use of atomic force microscope (AFM) technology [9, 10]. And it has been clarified that the structure of sarcomeres was substantially less stiff in the transverse direction than in the longitudinal direction.

Although skeletal and cardiac muscles are composed of a series of similar sarcomere structures, their sarcomere components are known to have differences. Skeletal muscle fibers have narrow Z-bands with I-bands of uniform lengths, but cardiac muscle fibers have wide Z-bands with I-bands of nonuniform lengths [11]. Further nebulin present in skeletal muscle is not in cardiac muscle [12]. Connectin (titin) filaments linking between Z-bands and thick filaments in sarcomeres of cardiac muscle are shorter than those of skeletal muscle [13]; although they directly interact with thin filaments in cardiac muscle, they do not in skeletal muscle [14, 15].

In the present studies, we used AFM technology to examine in detail the transverse stiffness of single myofibrils of skeletal and cardiac muscles, whether differences exist in the mechanical strength of sarcomere structure between skeletal and cardiac muscles.

MATERIALS AND METHODS

Preparation of myofibrils. The care of the animals and the experimental protocol were approved by the Animal Care and Use Committee of Tsurumi University. The psoas muscles of young rabbit and the left ventricle muscles of neonatal rat were used. Single myofibrils were prepared...
as previously [16] by homogenizing small bundles of glycerinated muscle fibers in a relaxing solution (143 mM K+-propionate, 10 mM EGTA, 5 mM MgCl2, 5 mM ATP, and 20 mM imidazole [pH 7.0]).

Single myofibrils were attached, as detailed previously [9], to the bottom of an experimental chamber, a modified culture dish (type 1006, Falcon, NJ, USA), the bottom of which was a coverslip. During experiments, the experimental chamber was filled with a rigor solution (143 mM K+-propionate, 10 mM CaCl2, and 20 mM imidazole [pH 7.0]) containing 0.15 mg/ml calpain, and after an appropriate period of reaction, the experimental chamber was washed with a rigor solution containing 10 mM EGTA to stop the reaction. After the experimental chamber was rinsed with a rigor solution, the myofibrils were used for the AFM experiments. Trypsin treatments of myofibrils were similarly made at room temperature following Granzier and Irving [6] for myofibrils immobilized in the experimental chamber. By replacing the bathing solution with a rigor solution containing 0.25 μg/ml trypsin and after an appropriate period of reaction, the experimental chamber was washed with a rigor solution containing 0.50 μg/ml trypsin inhibitor to stop the reaction. After the experimental chamber was rinsed with a rigor solution, the myofibrils were used for the AFM experiments.

AFM measurements. The AFM measurements were made as before [9] by use of an AFM (NV2500; Olympus Optics, Tokyo) incorporated in an inverted optical microscope (IX-70; Olympus Optics, Tokyo). Commercially available cantilevers (OMCL-TR400PSA, Olympus Optics, Tokyo; spring constant, 0.02 N/m) having tips of silicon nitride (20 nm in diameter and 200 μm long) were used. Also, bead-tip cantilevers were used, which were modified by having a microbead (1 μm in diameter) at the tip of the commercial cantilever to avoid damage to the surface structure of the myofibril preparations. The contact area to be produced between the bead of the bead-tip cantilevers and the surface of the myofibrils was calculated to be about 150 nm in width. The AFM measurements of myofibrils were made at room temperature by using the former cantilevers, if not otherwise stated. The deflection of an AFM cantilever as a function of the distance between the tip of cantilever and the surface of the myofibril, called a force-distance curve, was measured by approaching the tip of the cantilever to an appropriate locus of myofibrils firmly adhered to the bottom of the experimental chamber. Based on the force-distance curves thus obtained, the transverse stiffness of myofibrils was determined based on the force applied to the tip of the cantilever to make the surface of the myofibrils indented by 25–30 nm.

SDS-PAGE analysis. An SDS-PAGE (SDS-polyacrylamide gel electrophoresis) analysis of myofibrils was made following Kimura et al. [18]. Calpain- and trypsin-treated myofibrils used for SDS-PAGE were prepared under the same proteolytic conditions employed for preparing calpain- and trypsin-treated myofibrils used for AFM experiments. Calpain treatments were made by adding calpain (final concentration of 0.15 mg/ml) to a suspension of myofibrils (2 mg/ml in a rigor solution). At an appropriate period of reaction, EGTA (final concentration of 10 mM) was added to the reaction mixture to stop the reaction. Trypsin treatments were made by adding trypsin (final concentration of 0.25 μg/ml) to a myofibril suspension (2 mg/ml in a rigor solution). After an appropriate period of reaction, trypsin inhibitor (final concentration of 0.50 μg/ml) was added to the reaction mixture to stop the reaction.

After myofibril preparations were dissolved in an SDS solution (10% SDS, 5 mM EDTA, 50 mM DTT, and 0.1 M Tris-HCl [pH 8.0]), an aliquot of the solution (containing 0.1 mg of myofibril) was applied to a 2.5%–12% gradient gel for the SDS-PAGE analysis. After the gels were stained with Coomassie Brilliant Blue, followed by destaining, they were scanned with a densitometer (LAS-1000, Fujifilm, Tokyo, Japan), and the integrated dye densities of bands corresponding to major sarcomere components were obtained [19]. Based on the densities, the relative content of sarcomere components was obtained by assuming that actin is not digested under the present proteolytic conditions [20].

Chemicals. Calpain, trypsin, trypsin inhibitor, and ATP were purchased from Sigma Chemicals (MO, USA). Other chemicals were of analytical grade and purchased from Wako Chemicals (Osaka, Japan).

RESULTS

Transverse stiffness distributions along skeletal and cardiac myofibrils

Typical AFM images of myofibrils of skeletal and cardiac muscles in a rigor state attached to the surface of a coverslip are shown in Fig. 1. They were about 1 μm in diameter and had striation patterns produced by their sarcomere structures. Band structures repeating at about 2 μm in skeletal myofibrils correspond to the Z-Z intervals, and those repeating at about 1 μm in cardiac myofibrils correspond to the Z-M-Z intervals as detailed below. Force-distance curves were measured along myofibrils in relaxed and rigor states and also along those treated with calpain in a rigor state. Typical transverse stiffness distributions of myofibrils thus obtained are shown in Fig. 2. In accord with the results of previous AFM studies of muscle
myofibrils [9, 10], rigid Z-bands could clearly be located in the transverse stiffness distributions of rigor myofibrils, but sarcomere structures were hardly distinguishable in the transverse stiffness distributions of relaxed myofibrils. For skeletal as well as cardiac myofibrils, the myofibrils in a rigor state were laterally much stiffer than those in a relaxed state, and cardiac myofibrils were significantly stiffer than skeletal myofibrils in each state.

Z-bands of rigor myofibrils were located at nearly constant intervals of about 2.0 \( \mu \text{m} \) for skeletal myofibrils, but those of cardiac myofibrils were at varied intervals of 1.8 to 2.0 \( \mu \text{m} \). The transverse stiffness distributions around Z-bands of rigor myofibrils examined in detail showed clear differences in the Z-band structures between skeletal and cardiac myofibrils (Fig. 3). Z-bands of skeletal myofibrils were about 100 nm thick, having a stiffness of 7.7 pN/nm, and those of cardiac myofibrils were about 320 nm thick, composed of 4 to 5 rigid layers with a spacing of about 20 nm, each layer having a stiffness of 25.8 pN/nm and being 60 nm wide. The transverse stiffness at the overlap regions of rigor myofibrils was fairly uniform for skeletal myofibrils, about 3.0 pN/nm, but it was fairly scattered in a range of about 4.0–11.0 pN/nm for cardiac myofibrils. The M-lines of cardiac myofibrils could be located between neighboring Z-bands and were about 200 nm wide with a stiffness of about 11 pN/nm. But the M-lines of skeletal myofibrils could not clearly be identified in the transverse stiffness distributions. In overall transverse stiffness, cardiac myofibrils in a rigor state were 3 to 4 times stiffer than rigor skeletal myofibrils.

Transverse stiffness of calpain- and trypsin-treated skeletal and cardiac myofibrils in a rigor state

After extensive calpain treatments of rigor myofibrils, the stiffness decreased to about 2–3 pN/nm along the entire lengths (Fig. 2), suggesting that Z-bands as well as M-lines had apparently disappeared from the transverse stiffness distributions of skeletal as well as cardiac myofibrils. These results indicate that the Z-bands of skeletal and cardiac myofibrils are composed of \( \alpha \)-actinin networks susceptible to calpain treatments as reported [17, 21]. To clarify in more detail how various sarcomere components contribute to the transverse stiffness of myofibrils, the myofibrils in a rigor state were treated with calpain or

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**Fig. 1.** AFM images of single myofibrils of skeletal and cardiac muscles in rigor state. (A) Skeletal and (B) cardiac myofibrils. The bar: 1 \( \mu \text{m} \).

**Fig. 2.** Typical transverse stiffness distributions along myofibrils of skeletal and cardiac muscles in relaxed and rigor states. (A) Skeletal and (B) cardiac myofibrils. (Upper traces) relaxed states, (middle traces) rigor states, and (lower traces) rigor states after calpain treatments.

**Fig. 3.** Typical transverse stiffness distributions around Z-bands of rigor myofibrils. (A) Skeletal and (B) cardiac myofibrils.
trypsin to selectively digest sarcomere components of myofibrils. The time-dependent changes in the transverse stiffness of myofibrils during the treatments were examined based on force-distance curves obtained by AFM measurements by employing bead-tip cantilevers.

First, rigor myofibrils were treated with calpain, and force-distance curves were measured at Z-bands and around the center of the overlap regions of sarcomeres of myofibrils during the treatments. Time-dependent changes of the transverse stiffness of myofibrils thus obtained are shown in Fig. 4. For skeletal and cardiac myofibrils, the transverse stiffness at Z-bands was decreased as the calpain treatments advanced, though a large drop to 11 pN/nm, from 21 pN/nm, took place at the initial stage of the treatments for cardiac myofibrils. At the overlap regions, the transverse stiffness did not change during the calpain treatments for skeletal myofibrils, though the transverse stiffness of the overlap regions dropped to 4 pN/nm, from 7 pN/nm, at the initial stage of the treatments and remained unchanged thereafter. By calpain treatments of skeletal as well as cardiac myofibrils, the transverse stiffness at Z-bands as well as at overlap regions leveled off at nearly the same value, about 2 pN/nm.

Fig. 4. Time-dependent changes of the transverse stiffness at Z-bands and overlap regions of rigor myofibrils during calpain treatments. (A) Skeletal and (B) cardiac myofibrils (n = 5). (Solid circles) Z-bands, (open circles) overlap regions.

Fig. 5. Time-dependent changes of the transverse stiffness at Z-bands and overlap regions of rigor myofibrils during trypsin treatments. (A) Skeletal and (B) cardiac myofibrils (n = 5). (Solid circles) Z-bands, (open circles) overlap regions.

Fig. 6. SDS-PAGE patterns of skeletal and cardiac myofibrils treated with calpain and trypsin. (A) Calpain- and (B) trypsin-treated myofibrils. The left and right figures are for skeletal and cardiac myofibrils, respectively. At the top of each figure are indicated the durations of digestions.
Second, rigor myofibrils were treated with trypsin, and force-distance curves were similarly measured at Z-bands and overlap regions during the treatments. The time-dependent changes of the transverse stiffness of myofibrils thus obtained are shown in Fig. 5. For skeletal as well as cardiac myofibrils, the time-dependent changes of the transverse stiffness induced by trypsin digestions were roughly similar to those induced by calpain treatments, shown above. At Z-bands, the transverse stiffness was quickly decreased and leveled off at about 3 pN/nm as the trypsin treatments advanced for skeletal myofibrils. For cardiac myofibrils, a large drop to 12 pN/nm, from 21 pN/nm, took place at the initial stage of the treatments, followed by a very gradual decrease to about 5 pN/nm. At overlap regions, the transverse stiffness for skeletal myofibrils remained almost unchanged at about 3 pN/nm during the trypsin treatments, but for cardiac myofibrils it dropped to 4 pN/nm, from 7 pN/nm, at the initial stage of the treatments and leveled off at about 3 pN/nm.

**SDS-PAGE analysis of calpain- and trypsin-treated myofibrils of skeletal and cardiac muscles**

Skeletal and cardiac myofibrils were treated with calpain and trypsin under the same conditions as those employed for AFM studies, and changes in their sarcomere components were analyzed by SDS-PAGE. Typical SDS-PAGE patterns of myofibrils thus obtained are shown in Fig. 6. It can be seen that sarcomere components of myofibrils were digested by calpain and trypsin treatments, but the digestion patterns for skeletal myofibrils were distinct from those for cardiac myofibrils. The time-dependent changes of the relative contents of major sarcomere components of skeletal and cardiac myofibrils by calpain and trypsin treatments are summarized in Figs. 7 and 8.

It can be seen that by calpain treatments, both α-actinin and connectin were digested in skeletal and cardiac myofibrils (Fig. 7). The digestion rate for α-actinin was slower than that for connectin for each myofibril preparation. Further, the digestion rates for α-actinin and connectin were significantly slower for cardiac myofibrils than for skeletal myofibrils. By means of trypsin treatments, connectin was quickly digested, but α-actinin was hardly digested in skeletal and cardiac myofibrils (Fig. 8). Filamin, present as a minor component significantly in cardiac myofibrils, was gradually digested by calpain as well as by trypsin treatments. Nebulin, present in skeletal myofibrils, was digested quickly by calpain and gradually by trypsin. In skeletal and cardiac myofibrils, the actin and myosin heavy chain (MHC) remained almost undigested by calpain and trypsin treatments [20].
Fig. 9. Schematic sarcomere structures of skeletal and cardiac muscles. (A) Skeletal and (B) cardiac sarcomeres (modified from Sanger and Sanger [27] and Neagoe et al. [28]). Note the differences between skeletal and cardiac sarcomeres at the sarcomere length, the Z-band width, and the interaction of connectin filaments with thin filaments.

**DISCUSSION**

**Characteristics of the transverse stiffness of skeletal and cardiac myofibrils**

Consistent with the results of previous AFM studies [9, 10], the AFM images of skeletal and cardiac myofibrils in a rigor state clearly showed striation patterns characteristic of striated muscle fibers. Similarly, the transverse stiffness distributions along rigor myofibrils (Fig. 2) showed periodical sarcomere structures separated by rigid Z-bands for skeletal and cardiac myofibrils, and their magnitude was several times greater for cardiac myofibrils compared with skeletal myofibrils. However, relaxed myofibrils were less rigid compared with rigor myofibrils, and their striation patterns were hardly distinguishable in their stiffness distributions for skeletal and cardiac muscles. In rigor myofibrils, the actomyosin filament lattice anchored to Z-bands would strongly support Z-bands because rigor complexes are formed between thin and thick filaments, but in relaxed myofibrils, it would not support Z-bands stably because no rigor complexes are formed. Thus the above results suggest that Z-bands in relaxed myofibrils became tilted or bent when being pressed by the tip of an AFM cantilever.

It is known that cardiac muscle fibers are longitudinally stiffer than skeletal muscle fibers [5, 6]. The present studies indicate that they are also laterally stiffer than skeletal muscle at the myofibril or the sarcomere level. In the following, we will discuss the differences in the mechanical strengths of skeletal and cardiac myofibrils in relation to known differences in their sarcomere structures [11–15] (Fig. 9).

**Proteolysis of various sarcomere components and the transverse stiffness changes of skeletal and cardiac myofibrils**

By the calpain treatments of skeletal and cardiac myofibrils in a rigor state, the transverse stiffness at Z-bands was rapidly decreased (Fig. 4) roughly in parallel with the digestion of connectin and slightly faster than the digestion of α-actinin in myofibrils (Fig. 7). And by the trypsin treatments of skeletal and cardiac myofibrils in a rigor state, the transverse stiffness at Z-bands was gradually decreased (Fig. 5) nearly in parallel with the digestion of connectin, but α-actinin in their myofibrils remained undigested (Fig. 8). Because actomyosin filaments were intact in these myofibrils (Fig. 6), Z-bands in rigor myofibrils would have been distorted or tilted by being pressed by AFM cantilevers when Z-band structures and components supporting Z-bands became degraded by proteolytic treatments. Thus the digestion of α-actinin, a major Z-band component, would directly result in the reduction of the transverse stiffness of Z-bands as observed. Similarly, because (a) connectin filaments link Z-bands and thick filaments in skeletal and cardiac myofibrils, (b) they strengthen the sarcomere structures in the longitudinal direction [5, 6], and (c) their digestions by calpain and trypsin nearly go along with the transverse stiffness changes discussed above, connectin could be a component supporting Z-band structures. As Z-band structures are complicated [22, 23], various components (such as filamin, desmin, telethonin, and nebulette) bound to Z-band would also support Z-bands mechanically. On the other hand, the transverse stiffnesses at the overlap regions of skeletal and cardiac myofibrils in a rigor state leveled off at almost the same level by extensive calpain and trypsin treatments (Figs. 4 and 5). Because the actin and myosin molecules remained almost undigested under the present proteolytic conditions (Fig. 6), the residual transverse stiffness of myofibrils after extensive digestion would come from the actomyosin lattice of sarcomere structures. Thus it is suggested that the transverse stiffness of the actomyosin filament lattice in a rigor state is almost the same for skeletal and cardiac muscles. The actomyosin filament lattice would contribute by about 100% and about 50% to the overall transverse stiffness at the overlap regions of skeletal and cardiac myofibrils in a rigor state respectively.

**Transverse stiffness differences between skeletal and cardiac myofibrils**

The present AFM studies clearly indicate that cardiac myofibrils are significantly stiffer than skeletal myofibrils. The above AFM experiments show that Z-bands of cardiac myofibrils are composed of 4 or 5 rigid layers and are 3 or 4 times wider than single-layered Z-bands of skel-
eral myofibrils (Fig. 3), which are in accord with the Z-band images of skeletal and cardiac muscles observed under an electron microscope [11]. It is conceivable that the multilayered Z-bands of cardiac myofibrils would make the sarcomere structures stiffer compared with single-layered Z-bands of skeletal myofibrils. Further, it can be noted that the proteolytic treatments more distinctly affect the stiffness of cardiac myofibrils than that of skeletal myofibrils in a rigor state. As the transverse stiffness of the actomyosin filament lattice is the same for skeletal and cardiac myofibrils in a rigor state, it is suggested that there exist extra components strengthening sarcomere structures more effectively in cardiac myofibrils. Connectin could be one such component because (a) connectin filaments of cardiac muscle are shorter than those of skeletal muscle [13], and (b) they directly interact with thin filaments in cardiac muscle, but not in skeletal muscle [14, 15, 24]. It can also be noted that the transverse stiffnesses of Z-bands change roughly in parallel with the digestion of filamin by calpain and trypsin treatments (Fig. 6). Thus filamin, rich in cardiac muscle and susceptible to calpain and trypsin treatments [25, 26], could be another component strengthening cardiac myofibrils. It is possible that other sarcomere components further contribute to strengthen the sarcomere structures more effectively in cardiac myofibrils than in skeletal myofibrils.

It was found that the force-distance curves obtained at the overlap region of myofibrils by using bead-tip cantilevers were nicely fit by the Hertz formula up to an indentation of about 200 nm. If we assume that the overlap region of myofibrils is uniform, we can calculate its Young’s modulus by applying the Hertz formula as previously [9, 10]. Young’s modulus of the overlap region of skeletal and cardiac myofibrils thus obtained was 61 and 145 kPa, respectively, in a rigor state and 5 and 61 kPa, respectively, in a relaxed state. Anyway the present studies clearly indicate that at the sarcomere level, the lateral mechanical strength of cardiac myofibril is 2 to 10 times greater than that of skeletal myofibril in a relaxed state as well as in a rigor state. Further studies are needed to clarify whether differences in the transverse stiffness of myofibrils are related with the physiological functions of skeletal and cardiac muscles.

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