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Effects of high-pressure treatment on the structure and function of myofibrils

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The effects of high pressure (40–70 MPa) on the structure and function of myofibrils were investigated by high pressure microscopy. When this pressure was applied to myofibrils immersed in relaxing solution, the sarcomere length remained almost unchanged, and the A band became shorter and wider. The higher the applied pressure, the faster the change. However, shortening and widening of the A band were not observed when pressure was applied to myofibrils immersed in a solution obtained by omitting ATP from the relaxing solution. However, even under these conditions, structural loss, such as loss of the Z-line structure, occurred. In order to evaluate the consequences of this pressure-treated myofibril, the oscillatory movement of sarcomere (sarcomeric oscillation) was evoked and observed. It was possible to induce sarcomeric oscillation even in pressure-treated myofibrils whose structure was destroyed. The pressurization reduced the total power of the sarcomeric oscillation, but did not change the average frequency. The average frequency did not change even when a pressure of about 40 MPa was applied during sarcomeric oscillation. The average frequency returned to the original when the pressure was returned to the original value after applying stronger pressure to prevent the sarcomere oscillation from being observed. This result suggests that the decrease in the number of myosin molecules forming the crossbridge does not affect the average frequency of sarcomeric oscillation. This fact will help build a mechanical hypothesis for sarcomeric oscillation. The pressurization treatment is a unique method for controlling the structure of myofibrils as described above.

Key words: structure and function of myofibrils, high-pressure microscope, sarcomeric oscillation, biorhythm phenomenon, piezophysiology

Introduction

Myofibrils comprise a contraction system in which contractile units called sarcomeres are arranged in series. Sarcomeres consist of an ordered combination of proteins such as myosin and actin. It is thought that the contractile function of myofibrils is closely related to the ordered structure of sarcomeres arranged in series. However, no

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◀ *Significance* ▶

The effect of high pressure (40–70 MPa) on the structure and function of myofibrils was investigated by high pressure microscopy. When pressure was applied to the myofibrils soaked in the relaxing solution, the sarcomere length did not change and the A-band shortened. In the case of the rigor solution, the A-band did not shorten, but the Z-line became thinner. Pressurization was proved to be a unique method for controlling the structure of myofibrils. As a result of measuring sarcomeric oscillation for functional evaluation, the total power decreased due to the pressurization process, but the average frequency did not change.

one has been able to artificially create sarcomeres in vitro, and experimental methods for confirming the relationship between the myofibrillar structure and contractile function are limited [1–7]. We wondered if the structure of myofibrils could be freely changed by the strength and time of the applied pressure without changing the composition of the solution.

Pressurization is thought to promote rigorous and uniform structural changes because it applies uniform pressure to the entire sample in solution. Pressurization at pressures below about 300 MPa has been shown to promote dissociation and unfolding of multimeric proteins [8,9]. Pressurization of muscle fibers causes weakening of the interaction between actin and myosin, weakening of the elastic connectin structure, and fragmentation [10–12]. Although it is known that pressurization changes the structure of myofibrils in this way, it has not been investigated how to slowly change the structure of myofibrils at low pressures of 80 MPa or less [12,13]. We investigated how the structure of myofibrils changes depending on the strength and time of applied pressure by using high-pressure microscopy, which allows observation of the state of biological samples while pressurizing [14–20].

Then, in order to evaluate the contractile function of myofibrils treated with high pressure, sarcomeric oscillation was induced and observed. Sarcomeric oscillation is a phenomenon in which sarcomere repeatedly contracts and relaxes [21–29]. It has been reported that myofibrils immersed in a solution that causes sarcomere to contract moderately are in a sarcomeric oscillation state even though there is no change in the calcium concentration of the solution. By observing this phenomenon, it is possible to measure both the contractile and relaxed properties of myofibrils without changing the solution [21–29]. Since pressure-treated myofibrils are considered fragile, we considered this method to be a suitable method for assessing contractile function [10–12].

Materials and Methods

Sample preparation

In this study, we used rabbit skeletal muscle (the iliopsoas muscle) as a standard model of striated muscle, which has been typical in the previous studies. The preparation of myofibrils from the rabbit iliopsoas muscle was approved by the Institutional Animal Care and Use Ethical Committee of Waseda University and was conducted according to Waseda University animal experiment rules. Experiments using myofibrils were approved by the Institutional Animal Care and Use Ethical Committee of Waseda University and the University of Tokyo. Myofibrils were obtained from the iliopsoas muscle of white rabbits decapitated after anesthesia by injecting 25 mg/kg sodium pentobarbital sodium into the ear vein. The iliopsoas muscle was split into a thin bundle and immersed in a solution of 50% (vol/vol) glycerol, 0.5 mM NaHCO$_3$, and 5 mM Glycol ether diamine tetraacetic acid (EGTA) (pH 7.6). Myofibrils were obtained by homogenizing the glycerinated muscle fibers in a solution containing 1 mM EGTA, 60 mM KCl, 5 mM MgCl, and 10 mM Tris-maleate buffer (pH 7.5).

Solution

Relaxing solution (130 mM KCl, 4 mM MgCl$_2$, 20 mM MOPS, 4 mM EGTA, and 4 mM ATP) was used to relax the myofibrils. Rigor solution (130 mM KCl, 4 mM MgCl$_2$, 20 mM MOPS, and 4 mM EGTA) was used to bring the myofibrils into the rigor state. In addition, a solution of 120 mM KCl, 4 mM MgCl$_2$, 20 mM MOPS, 4 mM EGTA, 0.2 mM ATP, 4 mM ADP, and 4 mM Pi was used to induce sarcomeric oscillation.

Optical microscopic observation of myofibrils under high pressure

The high-pressure microscope experiment was performed in an inverted microscope (Ti-E, Nikon) equipped with a high-pressure chamber, as reported in a previous paper [17–19]. The objective lens was a 40× phase-contrast objective lens (CFI S Plan Fluor ELWD ADM 40XC, Nikon), and a CCD camera (WAT-120N+, WATEC) was used for imaging. The imaging speed was set to 1 fps for the pressure experiments at 40 and 50 MPa in relaxing solution and in the pressure experiment in rigor solution and to 30 fps for the other experiments. The temperature at the time of observation was 25°C. Myofibrils were observed in a rectangular space with a height of 0.17 mm in which two cover glasses (C024321, Matsunami) and two double-sided tape layers (NW-5S, Nichiban) were stacked.

Optical microscopic observation of pressurized myofibrils under atmospheric pressure

The high-pressure treatment in the experiment without the high-pressure microscope was performed with a pressure pump (device equivalent to HP-150, Syn Corporation) provided by Masayoshi Nishiyama. Myofibrils were placed in a rectangular space created with cover glass and double-sided tape similar to the above "Optical microscopic observation of myofibrils under high pressure", and pressurization was performed in relaxing solution or rigor solution.

To observe the experiments on the sample after high-pressure treatment, an apparatus equipped with an inverted microscope (IX-71, Olympus) with a phase contrast objective lens (UPlanSapo 100XOPH, Olympus) and a high-sensitivity camera (iXon Ultra, Andor Technology) was used. The imaging speed was 20 fps. A 1550 nm laser (FPL 1055T, Thorlabs) was used to control the temperature in the observation region. The myofibrils were held in the

rectangular space created with the top cover glass and double-sided tape, set in the optical microscope, and before observation, the solution was exchanged for the solution to induce sarcomere oscillation.

Electron microscope observation

Pressure was applied to the muscle fibers under relaxing solution or rigor solution with the pressure pump used in the above "Optical microscopic observation of combining myofibrils under atmospheric pressure", and a sample was removed for use as an observation sample. This sample was fixed with 2% (vol/vol) glutaraldehyde, embedded in epoxy resin, sliced to a thickness of approximately 50 nm, and then observed with a transmission electron microscope (H-7500, Hitachi) [30].

Analysis of experimental data

The data obtained from the camera were edited with ImageJ software (National Institutes of Health). The data from the optical microscope were subjected to Gaussian filter processing $(h(x, y) = \frac{1}{2\pi\sigma^2}exp\left(-\frac{x^2+y^2}{2\sigma^2}\right), \ \sigma = 2)$ to remove image noise. The sarcomere length was measured with subpixel accuracy by estimating the peak position of the brightness distribution corresponding to the I band by fitting it to a parabolic function with respect to the brightness distribution acquired in the contraction-axis direction of the myofibrils [27–29,31]. The length and width of the A band were measured by estimating the inflection point of the luminance distribution. This estimation was performed by fitting a parabolic function to the differential data of the luminance distribution [31]. Fast Fourier transform (FFT) processing with a Hamming window was performed with LabChart 7 software (ADInstruments) to obtain the power spectrum density (PSD). In the PSD analysis, the peak frequency was defined as the frequency value, which PSD value is the highest. The peak power was defined as PSD value at the peak frequency. The average of peak frequencies was calculated as the average of the peak frequency values for each individual sarcomeres. The total power was calculated as the sum of each frequency component of the PSD. The frequency range used in the calculation was 0 to 12.5 Hz in the "Sarcomeric oscillation of pressure-treated myofibrils" experiment and 0 to 15 Hz in the "Sarcomeric oscillation in myofibrils under high pressure" experiment. The average frequency was calculated as a weighted average by dividing the sum of the product of the PSD value and the frequency value of each frequency component of the PSD by the total power. In addition, the fundamental frequency of the average PSD was calculated as the frequency corresponding to the lowest value among the maximum values with a PSD value of 0.001 [μm²/Hz] or more.

The total power was measured as the oscillational energy of sarcomeric oscillation. The peak power was measured as the strength of the fundamental frequency of sarcomeric oscillation. The average of peak frequencies was measured assuming that it corresponds to the average value of the fundamental frequencies of sarcomeric oscillation. The average frequency was measured as the average frequency of sarcomeric oscillation with various oscillation components. Since the fundamental frequency of the sarcomeric oscillations measured in this study were unstable, not only the fundamental frequency measured as the peak value of PSD but also the average frequency calculated from the entire PSD were used as the measurement index.

The electron-microscope-derived data were analyzed for the luminance distribution acquired in the contraction-axis direction of the myofibrils. The sarcomere length was measured by fitting a Gaussian function $(y = y_0 - A * exp(-(x-x_C)^2/2w^2)$ to the Z line and measuring the distance between the peak positions (values of x_C). The length of the A band was measured by fitting the sigmoid function $(y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2)$ to the boundary between the I band and the A band and measuring the distance between the inflection points (x_0 values). The p value of this sigmoid function was used as an index of the clarity of the boundary between the I band and the A band. Origin Pro 9 (OriginLab Corporation) was used for the function fitting of the Gaussian function and the sigmoid function.

Statistics

All statistical tests were performed with a library of statistical software tools (R language). The normality and homoscedasticity of the data shown in Figures 1f–h, 2i, j, 3i–k, and Supplementary Figure S5g–i were tested by the Kolmogorov-Smirnov and Bartlett tests. In the analysis of Figure 1f–h, Tukey's test or the Steel-Dwass test was performed to compare each group. Tukey's test was performed when the data in Figure 1f–h satisfied the assumptions of both normality and homoscedasticity, and the Steel-Dwass test was performed otherwise. For other statistical tests, Dunnett's test or Steel's test was performed to make a comparison with the controls.

Dunnett's test was performed when the data in Figure 3i, j, 3i–k, and Supplementary Figure S5g–i satisfied the assumptions of both normality and homoscedasticity; Steel's test was performed otherwise. When performing this test, the data in Figure 3i,j and Supplementary Figure S5g–i were compared with those of the control group, and the data in Figure 4i–k were compared with those of the 0.1 MPa group before pressurization. The significance level for all statistical analyses was 5%. The error bars in all figures indicate the standard errors.

Results

Structural changes of myofibrils during pressurization

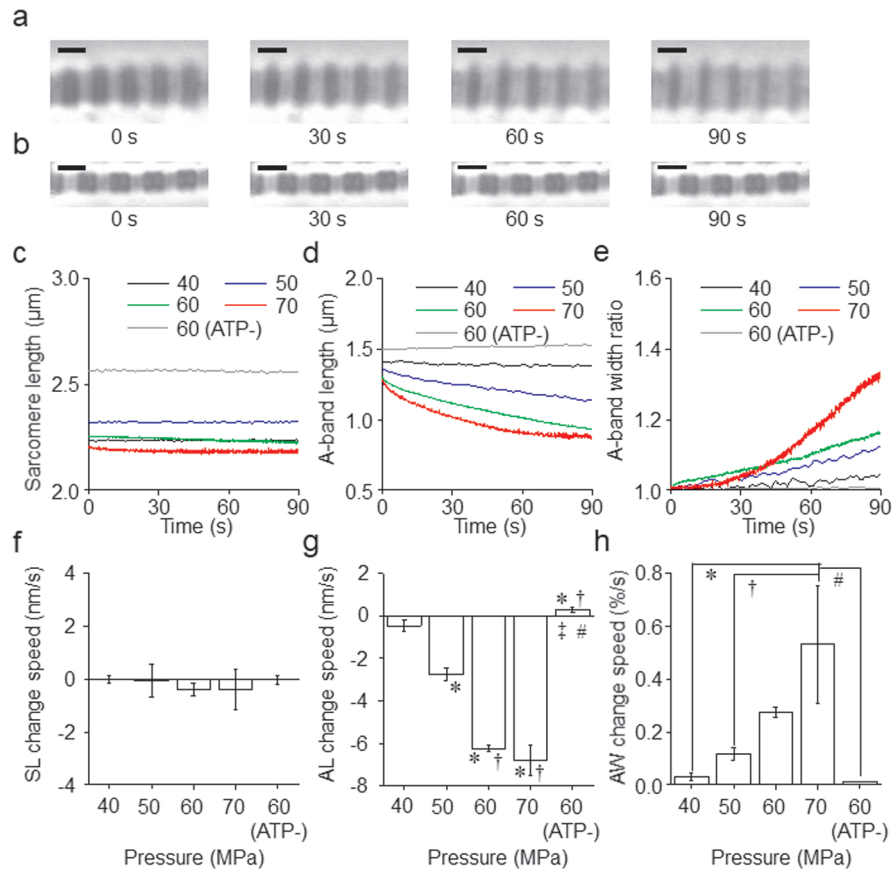When pressure was applied to the myofibrils under the

Figure 1   Structural changes in myofibrils during pressurization. (a,b) Phase contrast optical microscope images of myofibrils when a pressure of 60 MPa was applied in relaxing solution (a) and rigor solution (b). Images at 0, 30, 60 and 90 s, from left to right. The scale bar is 2 μm. (c–e) Time series changes in sarcomere length (c), A-band length (d) and A-band width ratio (e) during pressurization. The A-band width ratio was standardized based on the A-band width immediately after pressurization. The following numbers of sarcomeres were used in the analysis: 40 MPa, n=17; 50 MPa, n=13; 60 MPa, n=21; 70 MPa, n=16; 60 MPa (ATP(–)), n=16. (f–h) Rate of change in sarcomere length (c), A-band length (d) and A-band width ratio (e) during pressurization. Tukey's test was used for comparisons between groups. When there was a significant difference from the following pressure treatment groups, the following symbol marks were added: 40 MPa (*), 50 MPa (†), 60 MPa (‡), 70 MPa (#).

relaxing solution, structural changes occurred in the myofibrils depending on the strength and time of the applied pressure (Fig. 1, Supplementary Fig. S1, Supplementary Movie S1). The sarcomere length remained almost unchanged, and the A band became shorter and wider (Fig. 1c–e, f). The higher the applied pressure, the faster the shortening speed of the A band length (40 MPa: 0.5±0.3 nm/s, 50 MPa: 2.8±0.3 nm/s, 60 MPa: 6.3±0.2 nm/s, 70 MPa: 6.8±0.7 nm/s) (Fig. 1g). The rate of increase of the A bandwidth perpendicular to the sarcomere contraction axis also increased as the applied pressure increased (40 MPa: 0.03±0.01%/s, 50 MPa: 0.12±0.03%/s, 60 MPa: 0.28±0.02%/s, 70 MPa: 0.53±0.22%/s) (Fig. 1h). The A-band width was calculated as a ratio with respect to the original thickness to avoid a dependence on the number of myofibrils (which was typically one). However, when a pressure of 60 MPa was applied to myofibrils immersed in rigor solution, that is, relaxing solution without ATP,

neither shortening of the A-band length nor widening of the A band was observed (Fig. 1b, g, h, Supplementary movie S2). To confirm the structural change due to pressurization in the rigor solution, the structural change in the Z line was confirmed when a pressure of 80 MPa was continuously applied (Fig. 2). By comparing the results immediately after applying a pressure of 80 MPa and after 13.3 min, the disappearance of the dark line corresponding to the Z line was confirmed (Fig. 2).

Sarcomeric oscillation of pressure-treated myofibrils

From the above experimental results, we found that by applying a pressure of 40 MPa to myofibrils in relaxing solution, a slow structural change over the order of minutes could be induced (Fig. 1, Supplementary Fig. S1). Therefore, the myofibrils that had been subjected to a pressure of 40 MPa for 10 min and 30 min in relaxing solution were immersed in an solution to induce sarcomeric oscillation at
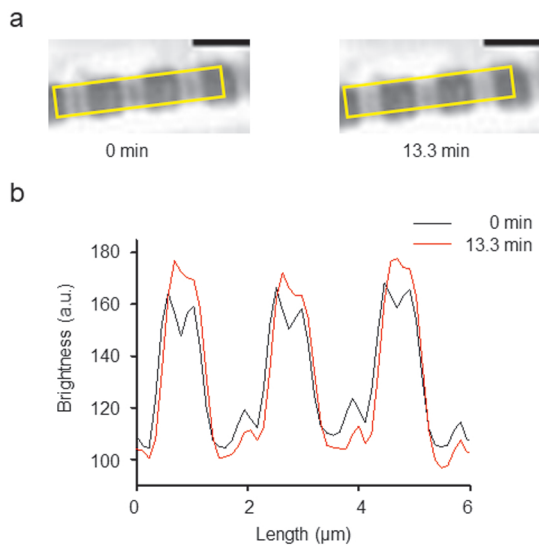
Figure 2   Structural change in the Z line by continuing to apply a pressure of 80 MPa under rigor solution. (a) Photographs of myofibrils in rigor solution at 80 MPa. Image immediately after pressurization (left) and image 13.3 min after pressurization (right). The scale bar is 2 µm. (b) Brightness distribution in the long-axis direction of the yellow frame in (a). The black line shows the brightness distribution immediately after pressurization, and the red line shows the brightness distribution 13.3 min after pressurization.

35°C, and the contraction was observed (Fig. 3). The amplitude of the sarcomeric oscillation under pressure was smaller than that of the unpressurized control group (Fig. 3a–c). As a result, the total power of the sarcomeric oscillation in the pressurization group was statistically significantly lower than before pressurization (Fig. 3j). However, the weighted average frequency and average of peak frequencies of sarcomeric oscillation did not change between the non-pressurized group and the 10-minute and 30-minute pressurized groups (Fig. 3i, k). In addition, in the average distribution of PSD of each sarcomere, it was confirmed that the pressure treatment under the relaxing solution tends to double the fundamental frequency to the vibration component (Control : 1 Hz, 10 min : 1 Hz, 30 min : 2 Hz) (Fig. 3e–g). This result suggests that myofibrils subjected to 30 minutes of pressurization in a relaxing solution have fundamental frequencies of about 1 Hz and 2 Hz, but the fundamental frequency of about 1 Hz is unstable (Fig. 3g).

The myofibrils that had been subjected to a pressure of 100 MPa for 5 min in the rigor solution were also immersed in the contraction-inducing solution at 35°C and observed (Fig. 3). The myofibrils pressurized in rigor solution exhibited no changes in the A-band length or width, but the amplitude of the sarcomeric oscillation decreased (Fig. 3d). Compared to the control results, the total PSD power was significantly reduced (Fig. 3h, j). However, the average frequency calculated from the PSD was the same

as that of the control group (Fig. 3i).

To bring the control group and various pressure-treated myofibrils into an sarcomeric oscillation state under the same solution conditions, heating to 35°C was essential. Myofibrils pressurized to 40 MPa for 30 min in relaxing solution and myofibrils pressurized to 100 MPa for 5 min in rigor solution quickly stopped sarcomeric oscillation when the temperature was returned from 35°C to 25°C (Supplementary Fig. S2). In a previous study, we found that in living cardiomyocytes, warming to a temperature close to body temperature can manifest sarcomeric oscillations [28,29]. Then, from a mathematical model that reproduces that phenomenon, it was estimated that warming the myofibrils could put the myofibrils into a sarcomeric oscillation state [29,31,32]. This time, we cannot say whether the mechanism is exactly the same as this estimation, but we succeeded in putting the myofibrils, which are no longer in the sarcomeric oscillation state at 25°C, into the sarcomeric oscillation state by warming them to 35°C.

Sarcomeric oscillation in myofibrils under high pressure

After the pressure treatment, the myofibrils maintained the ability to contract and oscillate in sarcomeres under normal pressure (Fig. 3). To learn more about the effects of pressure treatment on myofibrils, we investigated the behavior of myofibrils under sarcomeric oscillation at a pressure of approximately 50 MPa to determine whether myofibrils exhibited contractility at this pressure (Fig. 4). While observing the myofibrils in the sarcomeric oscillation state using a high pressure microscope, the change in oscillation was measured when the pressure was changed in the order of 0.1 MPa, 40 MPa, 50 MPa, and 0.1 MPa. The pressurization time of 40 MPa and 50 MPa was set to be within 30 seconds, respectively. The sarcomeric oscillation state was maintained even when a pressure of 40 MPa was applied to the myofibrils in the sarcomeric oscillation state (Fig. 4a, b, Supplementary Movies S3 and S4). However, when the applied pressure was raised to 50 MPa, the sarcomeric oscillation waveform could not be observed (Fig. 4c, Supplementary Movie S5). When the pressure was reduced from 50 MPa to 0.1 MPa (normal pressure), the oscillation waveform of sarcomeric oscillation was observed again (Fig. 4d, Supplementary Movie S6). We were also able to confirm this change through a PSD analysis (Fig. 4e–h). The average frequency calculated from the PSD did not change under pressures of 40 MPa and 0.1 MPa before and after pressurization, and the oscillation waveform of sarcomeric oscillation could be observed (Fig. 4i). At 50 MPa, where the oscillation waveform could not be observed, the average frequency calculated from the PSD was statistically significantly higher (Fig. 4i). This change is considered to be due to a significant decrease in the peak power, which is the PSD value at the peak frequency, at 50 MPa (Fig. 4g, k). In fact, the total power of
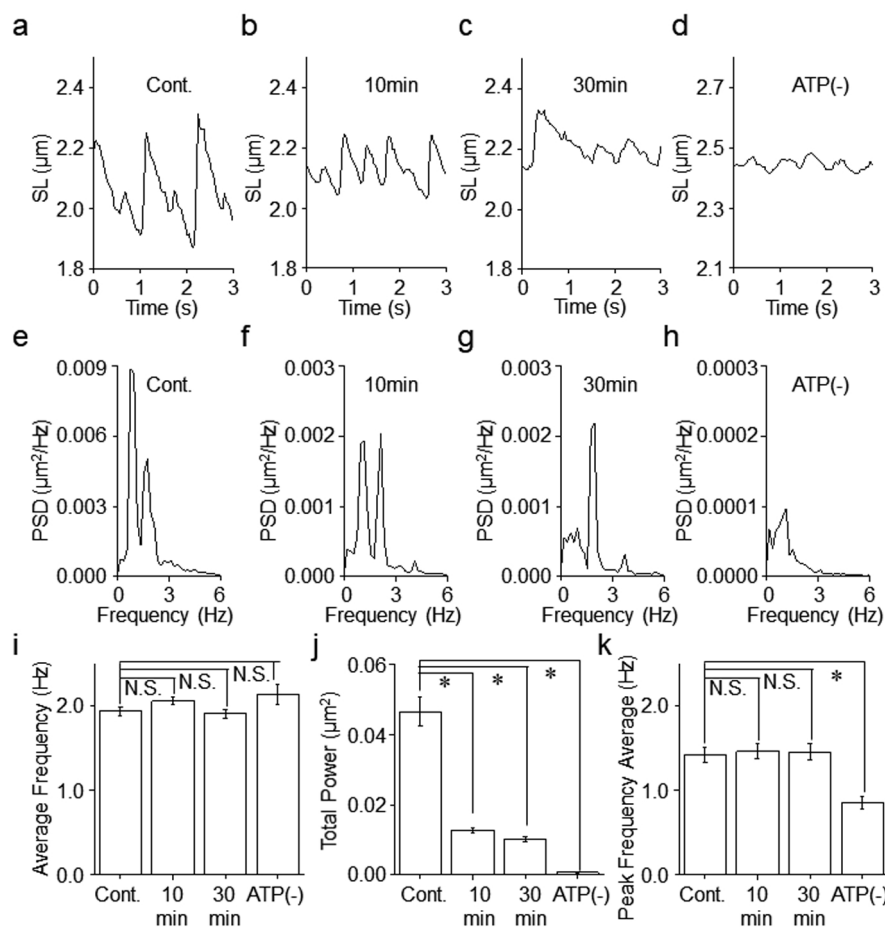
Figure 3 Changes in sarcomeric oscillation of sarcomeres caused by pressure treatment of myofibrils. (a–d) Typical waveform example of sarcomeric oscillation. The results are shown for no pressurization (a), for 40 MPa pressurization in relaxing solution for 10 min (b) or 30 min (c), and for 100 MPa pressurization in rigor solution for 5 min (d). (e–h) PSD of sarcomeric oscillation for each myofibril in a–d. Each represents the average of the PSDs of the following numbers of sarcomeres: e–g, n=30; h, n=21. (i–k) Average frequency (i) and total power (j) and average of peak frequencies (k) of sarcomeric oscillation of each myofibril in a–d. When Steel's test showed a significant difference from the control group, the result is marked with *.

the PSD has also dropped at 50 MPa (Fig. 4j). Since the myofibrils move slightly even under 50 MPa pressure, some total PSD power remains even under 50 MPa conditions (Supplementary Fig. S4). After the pressure was changed from 50 MPa to 0.1 MPa, the total power recovered to a level that was not statistically significantly different from that before pressurization (Fig. 4j). There was no statistically significant difference in total power between before pressurization and 40 MPa (Fig. 4j).

Structural analysis of pressure-treated myofibrils by transmission electron microscopy

To measure structural changes observed by a high-pressure microscope with higher spatial resolution, structural analysis of pressure-treated muscle fibers was performed by transmission electron microscopy. Using this tool, we observed the structure of sarcomeres in muscle fibers under pressure in relaxing and rigor solutions

(Supplementary Fig. S5). When pressure was applied in the relaxing solution, the boundary between the I and A bands was disturbed, and the Z line was broken (Supplementary Fig. S5a, b). On the other hand, when pressure was applied in the rigor solution, the boundary between the I and A bands was not disturbed, and no break in the Z line was observed (Supplementary Fig. S5a, c). To quantitatively analyze the change in the boundary between the I and A bands, the sigmoid function was fitted to the boundary, and the curvature p was investigated (Supplementary Fig. S5d–f). There was a statistically significant difference between the control and relaxing solution groups, but there was no statistically significant difference between the control and rigor solution groups (Supplementary Fig. S5i). That is, it could be confirmed quantitatively whether the boundary between the I and A bands was disturbed. These results are consistent with the structural changes in myofibrils observed by high-pressure microscopy.
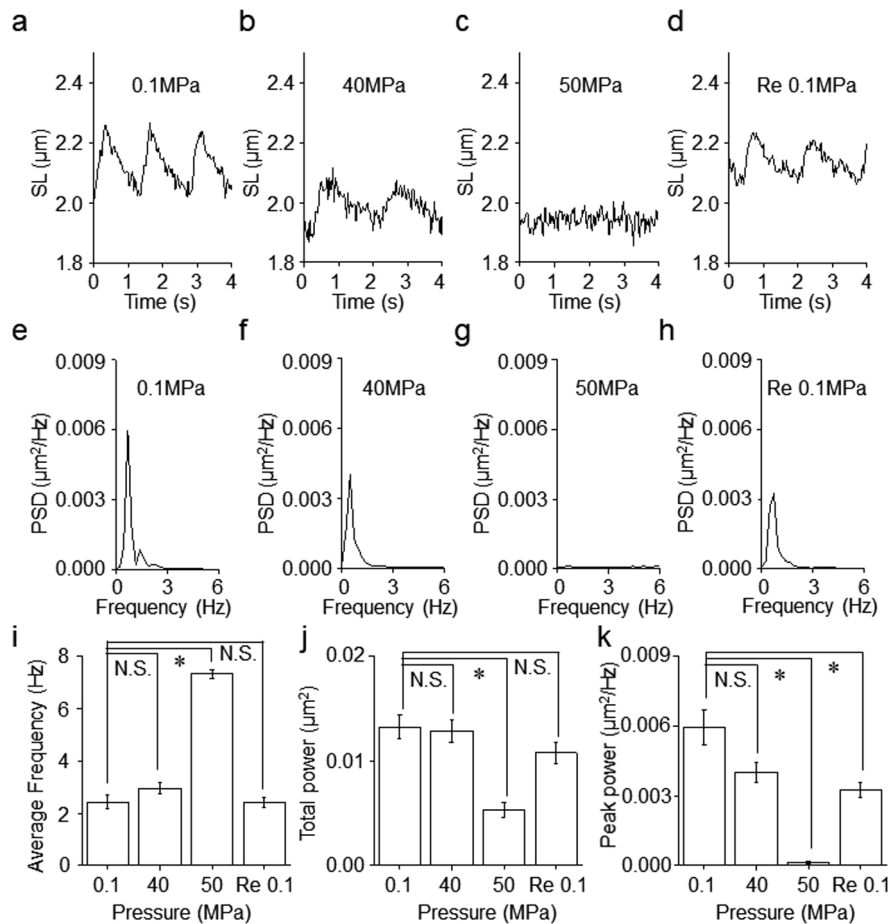
Figure 4 Characteristics of sarcomeric oscillation of during pressurization. (a–d) Typical waveform example of sarcomeric oscillation. Before pressurization (a), during pressurization at 40 MPa (b), during pressurization at 50 MPa (c), and when depressurized to 0.1 MPa (d). (e–h) PSD of sarcomeric oscillation for each myofibril in a–d. Each represents the average of the PSDs of the following numbers of sarcomeres: e–h, n=40. (i–k) Average frequency (i), total power (j) and peak power (k) of sarcomeric oscillation in each state shown in a–d. When Dunnett's test (i) and Steel's test (j, k) were significantly different from the control group, the result is marked with *. For the analysis, 20 sarcomeres were used for each group.

On the other hand, structural changes different from those in myofibrils observed by high-pressure microscopy were also observed (Supplementary Fig. S5g, h). The A-band length did not become shorter even when a pressure of 60 MPa was applied for 5 min in the relaxing solution (Supplementary Fig. S5h). The length of the sarcomeres was shorter than that of the unpressurized muscle fibers in both relaxing and rigor solutions (Supplementary Fig. S5g).

Discussion

From this study, it is clear that pressurization of 40–70 MPa in relaxing solution promotes structural changes in the direction of loss of myofibril structures over the order of seconds to minutes (Fig. 1, Supplementary Fig. S1). As a treatment for shortening the A-band length, the myosin in a myosin filament can be dissociated by immersing the myofibril in a solution with a high KCl concentration [7]. However, this treatment does not increase the A-band width [7]. Since the pressure treatment of 40 to 70 MPa also thickened the A-band width, it is considered that a structural change different from that due to this high ion concentration solution was promoted (Fig. 1). The length and width of the A band did not change in the rigor solution obtained by removing ATP from the relaxing solution, but the black line derived from the Z line was observed to change in the light microscope image (Figs. 1b and 2, Supplementary Movies S2 and 7). In particular, this change could be clearly observed at 80 MPa pressure (Fig. 2, Supplementary Movie S7). This fact shows that the pressure treatment not only promotes the dissociation of myosin but also dissociates the proteins that make up the Z line so that the structure in which myosin filaments and actin filaments are regularly bundled cannot be maintained, and the A-band width becomes thicker (Fig. 1a, e, h). The length and width of the A band did not change

when a pressure of 60 MPa was applied in rigor solution, probably because the myosin was stable in the rigor state (bound to the actin filament) and depolymerization was suppressed (Fig. 1b–h). The results showed that pressure treatment causes loss of the M-line bundling A band and the Z-line bundling I band to destroy the regular structure of myofibrils, consistent with previous studies [13]. Therefore, the change that the pressurization treatment of ~70 MPa brings to myofibrils is considered to be the change that dissociates the constituent proteins from the myofibrils. At least when the structural change is slight, the structural changes from pressurizing myofibrils are considered to be twofold. First, the shortening of myosin filaments reduces the total number of myosin molecules that can interact with actin filaments. Second, the actomyosin lattice spacing increases due to the weakening of the structure that bundles actin filaments such as the Z line and myosin filaments. These structural changes can be controlled by the magnitude and duration of the applied pressure (Fig. 1f–h). With the same solution composition, the rate of change in the A band was approximately 12.6 times different depending on whether the applied pressure was 40 MPa or 70 MPa (Fig. 1g, h). Pressurization at 40 MPa resulted in A band shortening approximately linearly for at least 30 min (Supplementary Fig. S1). Pressurization at 40 MPa over a scale of minutes slowly promoted structural changes in the myofibrils (Fig. 1c–h, Supplementary Fig. S1). We conclude that such pressurization is promising as a new structural control method for myofibrils, distinct from the method of manipulating solution composition.

Despite the above structural changes, the myofibrils that had been subjected to pressure treatment retained the ability for sarcomeric oscillation in the solution inducing this contraction at 35°C (Fig. 3). It was also found that the average frequency of the sarcomeric oscillation was the same as before the pressure treatment (Fig. 3i). This fact suggests that the cycle of sarcomeric oscillation strongly depends on the chemodynamic reaction cycle of the myosin molecule and not on whether the sarcomere structure is weakened. However, the pressure treatment reduced the total power of the sarcomeric oscillation and suppressed the rapid and large amplitude expansion (Fig. 3a–d, j). Since the sharpness of the peak frequency waveform of the PSD was lost, it is considered that the stability of the cycle was also lost (Fig. 3a–h). In a previous study investigating changes in the PSD of sarcomeric oscillation when changing the lattice spacing of actin filaments and myosin filaments of normal myofibrils by changing the dextran concentration in solution, the peak was narrowed, the frequency became sharper, and the periodic stability increased [33]. It is thought that when the lattice spacing of the filaments is narrowed, the probability of crossbridge formation between myosin and actin filaments increases, as does the number of myosins involved in oscillation

[29,31,33–36]. It can be considered that our pressurization treatment brought about the opposite change that widened the lattice spacing (Fig. 1d, e). The peak frequency became unsharp, and the periodic stability decreased due to the widening of the filament lattice spacing, the spatial limitation of the interaction between myosin and actin filaments, and the low probability of crossbridge formation [29,31,33–36]. The shortening of the A band, or myosin filament, reinforced this tendency by reducing the number of myosins that could form crossbridges (Fig. 1d). The myofibrils pressurized in rigor solution, which was obtained by removing ATP from relaxing solution, exhibited an unsharp peak frequency even though the length and thickness of the A band did not change (Fig. 1c–h, Fig. 3d, h, i, j). This was because the structure that bundles myofibrils such as the Z line was weakened, which disrupted the regular arrangement of actin filaments and myosin filaments and reduced the amount of myosin that could form a crossbridge (Fig. 2, Supplementary Movie S7). At 25°C, sarcomeric oscillation could not be observed in the myofibrils that were pressurized to 40 MPa for 30 minutes in the relaxing solution and the myofibrils that were pressurized to 100 MPa for 5 minutes in the rigor solution. Warming the solution to 35°C triggered the sarcomeric oscillation. When the solution was cooled from 35°C to 25°C, the sarcomeric oscillation stopped immediately (Supplementary Fig. S2). This was probably because the activation of myosin movement by increasing the temperature increased the number of myosins forming crossbridges [28,29,37,38]. It is considered that the probability of crossbridge formation was also increased because troponin and tropomyosin became less likely to inhibit myosin crossbridge formation due to thermal fluctuation [28,29,37,38]. In addition, the thermal fluctuation of the actin filament itself may have increased the probability of proximity to the myosin head and increased the probability of crossbridge formation.

Regarding sarcomeric oscillation during pressurization, neither the mean frequency nor the total power showed statistically significant changes at 40 MPa (Fig. 4i, j). However, when the pressure was further increased to 50 MPa, the oscillation cycle of approximately 2 Hz of the sarcomeric oscillation was lost, and the characteristic frequency peak could not be found in the PSD (Fig. 4c, g, k). However, it was confirmed that the oscillation cycle of approximately 2 Hz was returned when the pressure was lowered again to the normal pressure of 0.1 MPa (Fig. 4d, h, i). Since there was no change in the phase-contrast light microscopy images of myofibrils before and after pressurization and during pressurization, the interpretation of this result is considered to be one of the following (Supplementary Fig. S3, Supplementary Movies S3–6). The first interpretation is that pressurization deformed the sarcomere structure and disrupted the placement of myosin and actin

filaments. The number of myosins forming the crossbridge decreased even at a pressure of 40 MPa, but the number of myosins that could not maintain the oscillation decreased at a pressure of 50 MPa. The second interpretation is that pressurization deformed the structure of myosin, preventing it from exerting the full power stroke. With a pressurization of 50 MPa, it became impossible to exert a sufficiently cooperative and powerful force to oscillate the sarcomere.

By observing the transmission electron microscope image of the pressure-treated muscle fibers, it was possible to confirm the change in the boundary of the A band and the weakening of the Z line when the pressure treatment was performed in relaxing solution (Supplementary Fig. S5). However, in the pressure-treated muscle fibers, the A-band length did not become shorter even when pressure-treated in relaxing solution (Supplementary Fig. S5h). In addition, the sarcomere length was shortened in both relaxing and rigor solutions (Supplementary Fig. S5g). We consider the cause of the differences in the results to be as follows. Whether the sarcomere length is shortened depends on whether there is resistance to sarcomere contraction during pressurization. Since the myofibrils were placed on a glass slide and pressed, the friction with the glass slide is considered to be the resistance force against the sarcomere contraction. The myofibrils laid on the glass slide were resistant to sarcomere contraction because the average sarcomere length of the sarcomeric oscillation myofibrils was maintained (Fig. 1). On the other hand, the muscle fibers were pressurized in a tissue configuration and did not exhibit sufficient resistance, so the sarcomeres were considered to have been shortened (Supplementary Fig. S5g). The presence or absence of shortening of the A-band length is considered to be influenced by differences in the surrounding conditions of the sarcomere. Since myofibril is surrounded by solution, there is a low probability that myosin dissociated from the myosin filaments can be returned. On the other hand, inside the muscle fiber, since the surroundings are also muscle fibers, it is difficult to release myosin that has been temporarily dissociated to the outside, and there is a high probability that the configuration will return to the original state. Therefore, in muscle fibers, shortening of the A-band length could not be observed at a statistically significant level, but structural disorder could be confirmed.

Conclusion

By high-pressure microscopy, we succeeded in quantitatively measuring how the structure of myofibrils changes when pressurized to 40 to 80 MPa. When pressure is applied in the relaxation solution, the notable change is that the A band shortens in the direction of contraction and thickens in the vertical direction. No change in the shape of this A band was observed when pressure was applied in the relaxation solution from which ATP was removed. However, when strong pressure treatment such as 10 minutes or more was performed at 80 MPa, loss of the Z-line structure was confirmed. The progress of the change could be controlled by the strength of the applied pressure and the time. For example, the shortening speed of the A band was 12.6 times different between 40 MPa and 70 MPa. In order to evaluate the function of this pressure-treated myofibril, sarcomere induces sarcomeric oscillation that repeats contraction and relaxation, and as a result of observation, it was found that the total power of the oscillation is reduced by the pressure treatment. Interestingly, however, the weighted average and peak frequencies of the sarcomeric oscillation did not change between the non-pressurized group and the 10-minute and 30-minute pressurized groups. This result suggests that the decrease in the number of myosin molecules forming the crossbridge does not affect the average frequency of sarcomeric oscillation. Thus, we succeeded in confirming that the pressurization treatment of 40 to 80 MPa is a unique method for controlling the structure and function of myofibrils.

Author Contributions

S.A.S. designed the project, conducted the experiment, analyzed the experimental data, and wrote the paper.

Conflicts of Interest

The authors declare no competing interests.

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