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# Length-dependent activation and auto-oscillation in skeletal myofibrils at partial activation by $Ca^{2+}$

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**Abstract**

The length-dependent activation of skeletal myofibrils was examined at the single-sarcomere level with phase-contrast microscopy at sarcomere length (SL) >2.2 μm. At the maximal activation by $Ca^{2+}$ (pCa 4.5) the active force linearly decreased with increasing SL, while at partial activation by $Ca^{2+}$ (pCa 6.1–6.5) the larger active force was generated at longer SL. Throughout these experiments, the distribution of SL was kept homogeneous upon activation. In addition, we found that the spontaneous oscillation of force and SL frequently occurs in the SL range 2.2–2.6 μm at pCa 6.1–6.2. Either changes in $[Ca^{2+}]$ or osmotic compression of the myofilament lattice induced by the addition of dextran T-500, affected both the length dependence of activation and the occurrence of auto-oscillation. These results suggest that the force-generating properties of sarcomeres in striated muscle are determined not only by $[Ca^{2+}]$, but also by the lattice spacing as a function of SL.


*Keywords:* Muscle contraction; Force–length relationship; Spontaneous oscillation; Lattice spacing; Dextran

In 1972, Endo reported that under partial $Ca^{2+}$-activation skinned skeletal muscle generates larger active force at longer SL, when a fiber is stretched above its slack length [1,2]. This nonlinear relationship between the active force and the length of overlap between the thick and thin filaments, termed the length-dependent activation, suggests that the contractile system itself possesses the SL-dependent self-regulatory mechanisms, in addition to the regulation by $Ca^{2+}$.

On the other hand, microscopic observations revealed that under partial $Ca^{2+}$-activation, cardiac fiber [3,4] and myofibrils [5] exhibit steady auto-oscillation of force and SL (Ca-SPOC). Though Ca-SPOC is yet to be found in the fast skeletal muscle [4,6], the similar type of oscillation can be observed when skeletal myofibrils are partially activated by adding MgADP to the relaxing solution (ADP-SPOC) [7,8].

The actual properties of single sarcomeres in muscle fibers can be masked by averaging out the properties of over thousand of sarcomeres, connected in parallel and in series, due to the structural instability and/or the concentration gradient of $Ca^{2+}$ and substrates within the fiber space [1]. Hence, the SL- and $[Ca^{2+}]$-dependent properties of sarcomeres should be examined at the single-sarcomere level.

In this study, we examined the force–length relationship in rabbit skeletal myofibrils (~1 μm in diameter) that contained only 10–20 sarcomeres connected in series, at various $[Ca^{2+}]$. The advantages of using myofibrils are that the sarcomeres are uniformly activated with a rapid solution exchange system, and that simultaneously with the active force measurement the individual SLs are monitored with the phase-contrast microscopy [7].

Applying this technique, we confirmed that the larger force production at longer SL observed under partial

*Abbreviations:* SL, sarcomere length; CSA, cross-sectional area.

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$Ca^{2+}$-activation [1,2] is the property of the single sarcomeres themselves. In addition, steady auto-oscillation (Ca-SPOC) was observed in the region of weak forces near the slack SL. Either changes in $[Ca^{2+}]$ or osmotic compression of the myofibrils by dextran strongly affected both the length-dependent activation and the occurrence of Ca-SPOC. These results suggest that both $[Ca^{2+}]$ and the lattice spacing modulate the SL-dependent properties of activation in sarcomeres.

## Materials and methods

*Preparation of myofibrils.* The single myofibrils or their thin bundles were prepared by homogenizing psoas muscle of white rabbits according to the method described previously [7]. All procedures were performed according to the Regulations for Animal Experimentation at Waseda University. The fibers were stored in glycerol solution and used for myofibril preparation within 10 days. Just before the force measurements, myofibrils were chemically skinned with 0.1% (v/v) Triton X-100 for 10 min in the experimental chamber.

*Solutions.* Rigor solution contained 2 mM free $Mg^{2+}$, 20 mM MOPS, and 4 mM EGTA. Relaxing solution contained 2 mM free $Mg^{2+}$, 20 mM MOPS, 4 mM EGTA, 1 mM DTT, and 1 mM MgATP. $Ca^{2+}$-activating solutions contained 2 mM free $Mg^{2+}$, 20 mM MOPS, 4 mM EGTA, 1 mM DTT, 1 mM MgATP, and various concentrations of $CaCl_2$ to adjust pCa (4.5–6.5). The ionic strength was adjusted with KCl to 150 mM (rigor solution) or 180 mM (relaxing and activating solutions). The pH values of all solutions were adjusted to 7.0 by KOH at room temperature. Some experiments were performed with the addition of 1% (w/v) dextran T-500 (D1037, Sigma–Aldrich) in the activating solution. ATP was purchased from Roche. For the description of ryanodine experiments, see Supplementary methods.

*Measurement of force–length relationship.* A full description of the experimental procedure is given in Ref. [7]. Briefly, a single myofibril or a thin bundle of 2–3 myofibrils containing 10–30 sarcomeres was suspended between a pair of glass microneedles, one of which was >50 times stiffer than the other, on an inverted phase-contrast microscope (TE2000, Nikon). The exchange of activating and relaxing solutions was performed within 30 ms by a dual laminar flow system [9]. The solutions flowed perpendicular to the long axis of the myofibril with the velocity of 15 μm/ms. The myofibrillar force was estimated from the deflection of the flexible needle with the stiffness of 0.1–1.0 μN/μm (≈10–100 μg/μm; resonant frequency of 3.0–10.0 kHz in solution). The active isometric force was estimated by subtracting the resting force from the total (resting + active) force at the activation plateau. When the myofibrils oscillated, the active force was determined by averaging the force during the steady oscillation. The cross-sectional area (CSA) of the myofibrils was estimated from the phase-contrast image by assuming that the myofibril is a uniform cylinder. The average SL and the SD of the distribution of SL were calculated by measuring individual SLs from the distance between the centers of mass of the adjacent A-bands in the intensity profile. The RMS noise of individual SLs was within ±40 nm. All experiments were carried out at 23 ± 1 °C. For more details, see Supplementary methods.

*Data analysis.* The force–pCa relationship (inset in Fig. 3) was fitted by non-linear regression to the Hill equation, $F = F_0/(1 + 10^{-n(pCa_{50}-pCa)})$, where $F_0$ is the maximal active force at pCa 4.5, $n$ is the Hill coefficient, $pCa_{50}$ is the $[Ca^{2+}]$ at the half maximal active force.

## Results

### *Spontaneous oscillation at partial activation by $Ca^{2+}$*

The single myofibrils or thin bundles composed of 2–3 myofibrils were activated with various $[Ca^{2+}]$ (between pCa 4.5 and 6.5) in the presence of 1 mM MgATP. At pCa 6.1–6.2, spontaneous oscillation of force and SL was consistently observed near the slack length (Fig. 1A and Supplementary movie S1). The properties of the oscillation observed here indicate that it can be classified as SPOC (spontaneous oscillatory contraction [10]) that we have reported previously.

During SPOC each sarcomere repeated the cycle of slow shortening and rapid lengthening phases in a sawtooth waveform (Fig. 1B). The period of oscillation was 1.1 ± 0.3 s, while the peak-to-peak amplitude was 165 ± 71 nm per sarcomere (means ± SD; $n = 10$ from 10 myofibrils upon the first activation of each myofibril at pCa 6.1). In addition, the lengthening phase transmitted to the adjacent sarcomeres along the myofibril over 10 sarcomeres with the transmission time of <30 ms per sarcomere (so called SPOC wave). Meanwhile, the active force oscillated between 8.4 ± 4.6 kN/m$^2$ at minimums and 55.0 ± 26.9 kN/m$^2$ at maximums, while the average active force was 29.5 ± 14.3 kN/m$^2$. In some cases a small fluctuation of SL was observed in the phase-contrast image at pCa 6.3, but the amplitude of the fluctuation was statistically insignificant compared with the RMS noise level in steady contraction. These data were classified as contraction in the following analysis.

The steady oscillatory waveform was maintained for over 10 s without attenuation, and reproduced well over several repeated activation/relaxation cycles (Fig. 1C). In some cases, however, the oscillation was intermittent with irregular waveform (Fig. 1D). In such cases, after a steady force plateau lasting for a few seconds, the active force suddenly dropped, accompanied by the lengthening of a sarcomere and subsequent transmission of the lengthening phase to the adjacent sarcomeres along the myofibril. Besides, when the myofibrils was continuously activated for longer than 20 s (Fig. 1E), the waveform of force oscillation gradually changed with time, with the amplitude of oscillation becoming larger and the period becoming longer. Finally, all the sarcomeres reached a steady force plateau. Those myofibrils, in which the oscillation once disappeared, did not generate SPOC after subsequent activations, showing steady contraction instead. Since the resting force did not significantly differ before and after the activation, we infer that the regulatory properties of the thin filaments were kept intact.

The SPOC is possibly not caused by the oscillation of local concentration of ligands, such as $Ca^{2+}$ and ATP, inside the myofibrils, because the solutions were continuously replenished by the laminar flow, which allowed these small molecules to diffuse inside the myofibrillar space of 2–4 μm$^2$ within milliseconds. The residual sarcoplasmic reticulum is not involved either, because neither the functional inhibition of $Ca^{2+}$-channel by 100 μM ryanodine nor the stronger buffering of free $Ca^{2+}$ by 10 mM EGTA affected the characteristics of oscillation (data not shown). Hence, we conclude that the generation of the spontaneous oscillation is attributable to the intrinsic properties of the contractile system itself.

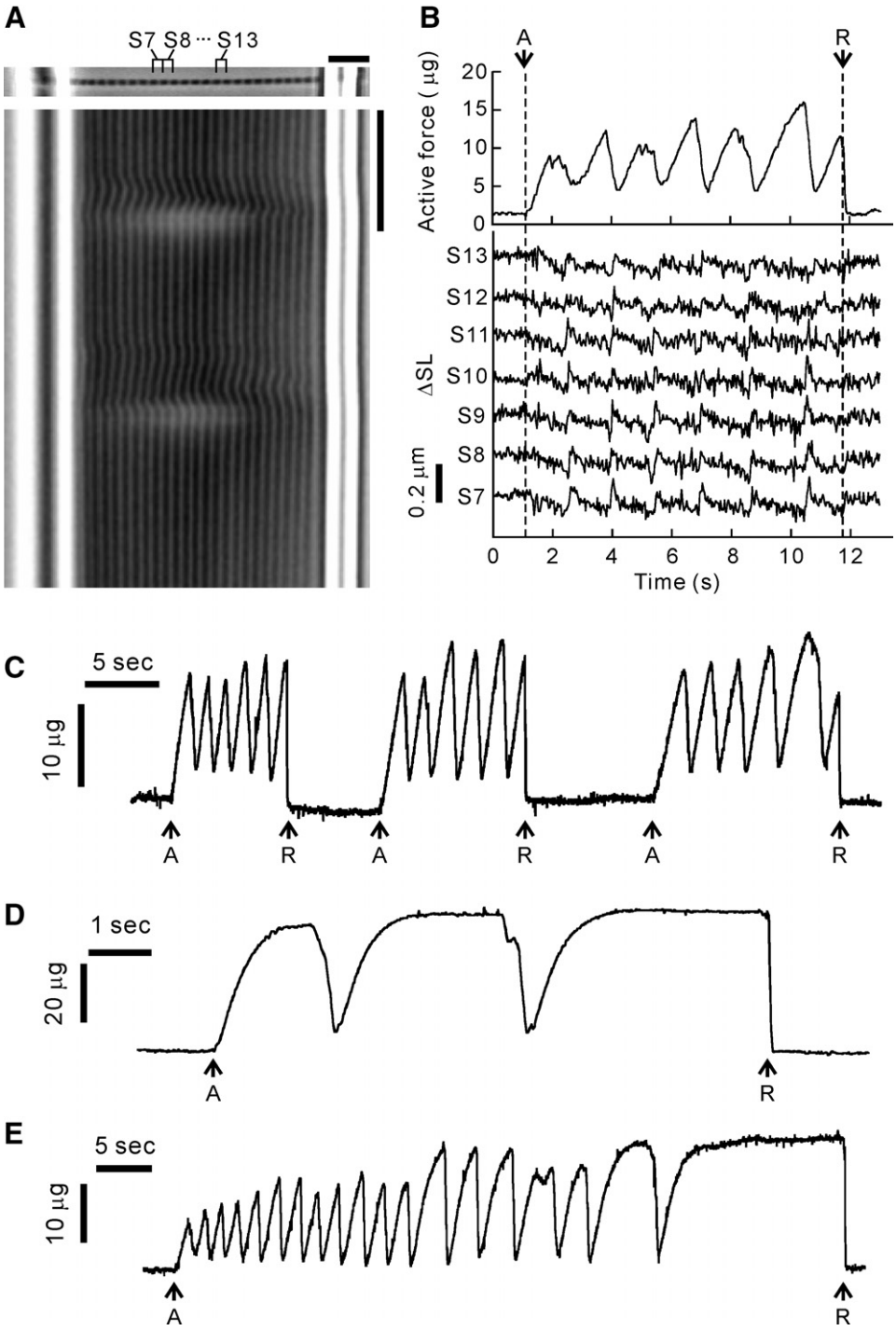


Fig. 1. Spontaneous oscillation at partial activation by $Ca^{2+}$. (A) The phase-contrast image (top) and the kymograph (bottom) of the single myofibril generating spontaneous oscillation at pCa 6.1. The needle on the right is flexible. The average SL during relaxation was 2.64 μm; CSA was 2.46 $\mu m^2$. The horizontal scale bar is 10 μm, and the vertical scale bar is 1 s. (B) The typical time courses of force (upper) and length change of each sarcomere (lower). The sarcomere number to the left from each SL trace corresponds to that in the upper micrograph in (A). The myofibrils were activated and relaxed at the arrows A and R, respectively. (C–E) Various types of force oscillation observed at pCa 6.1. The data were obtained from different myofibrils with CSA of 3.27, 3.15, and 3.39 $\mu m^2$, respectively. For details, see text.

*Length-dependent activation at various [$Ca^{2+}$]*

We next studied the effect of [$Ca^{2+}$] on the active isometric force at various SLs. At the saturating level of activation (pCa 4.5) the developed force was smaller at longer SL (Fig. 2A). On the other hand, at the low level of activation (pCa 6.2) the developed force became larger at longer SL (Fig. 2B), as previously found by Endo in skinned single

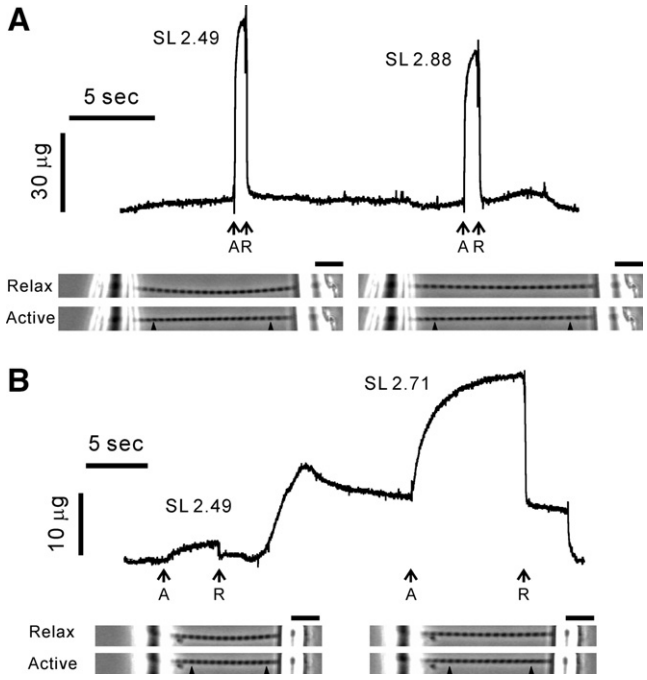


Fig. 2. SL-dependent force development of myofibrils at two different [$Ca^{2+}$]. The time courses of force development at a short and a medium SLs at pCa 4.5 (A) and pCa 6.2 (B) are shown. The phase-contrast images of myofibrils during relaxation and at the activation plateau are shown below each force trace. The myofibril was stretched in relaxing solution between the first and the second activations. The average SL and the SD at the plateau of activation were 2.49 ± 0.16 μm and 2.88 ± 0.11 μm in (A) ($n = 17$ sarcomeres), and 2.49 ± 0.04 μm and 2.71 ±0.04 μm in (B) ($n = 11$ sarcomeres). SL was analyzed in the region between the arrowheads in the micrographs. The arrows A and R in the force traces mean the same as in Fig. 1. The CSA and the flexible needle stiffness were 2.50 $\mu m^2$ and 72.2 μg/μm in (A), 3.33 $\mu m^2$ and 21.5 μg/μm in (B).

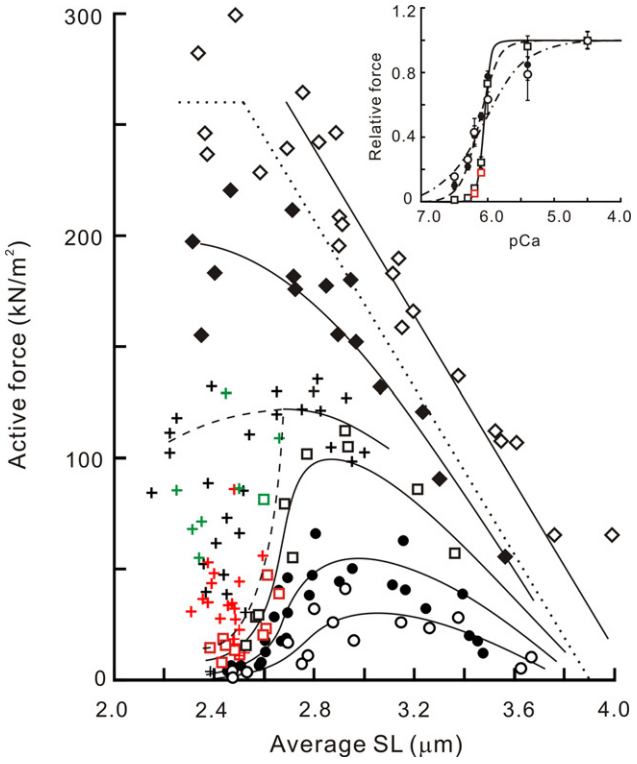


Fig. 3. Force–SL relationship at various [$Ca^{2+}$]. The active isometric force at pCa 4.5 ($n = 3$ myofibrils; open diamonds), pCa 6.0 ($n = 4$ myofibrils; filled diamonds), pCa 6.1 ($n = 10$ myofibrils; crosses), pCa 6.2 ($n = 4$ myofibrils; open squares), pCa 6.3 ($n = 4$ myofibrils; filled circles), and pCa 6.5 ($n = 3$ myofibrils; open circles) are plotted against the average SL. Two to eight data were obtained from each myofibril. The lines were drawn by eye. For dashed lines, see Discussion. The red and green symbols indicate the average force during the steady and the intermittent oscillations, respectively (see Fig. 1 and text). The data at pCa 5.4 are not shown for clarity. (Inset) The force–pCa relationships within three regions of SL (SL 2.3–2.6 μm (open squares), 2.7–3.0 μm (filled circles), and 3.1–3.4 μm (open circles)). The plots are means ± SEM at each pCa. The lines are drawn according to the Hill equation. The red symbols indicate the average force during the oscillation.

fibers [1,2]. We confirmed that the striation pattern of sarcomeres was kept homogeneous during activation (micrographs in Fig. 2).

The relationship between the active isometric force and the average SL obtained between pCa 4.5 and 6.5 is summarized in Fig. 3. At the maximal activation (pCa 4.5) the active force decreased proportionally to the length of overlap between the thick and thin filaments, similarly to intact [11] and skinned [12] fibers. The maximal active force obtained at SL 2.3–2.6 μm was 258 ± 14 kN/$m^2$ (means ± SEM; $n = 5$ from 3 myofibrils). On the other hand, at partial activation (pCa 6.1–6.5) the active force was not proportional to the length of overlap, but reached maximum at shorter overlap (i.e., at longer SL). Thus, the SL at which the maximal active force was generated shifted rightward as the [$Ca^{2+}$] decreased. SPOC occurred between strong and weak contractions at pCa 6.1–6.2 and SL 2.3–2.6 μm (Fig. 3 and inset).

The SD of the distribution of SL throughout a myofibril was typically kept within ±100 nm on the descending limb of the force–length relationship at pCa 4.5 (Supplementary Fig. S1), where the experimentally obtained relationship shifted rightward by ~150 nm from that estimated from the length of overlap between the thick and thin filaments [13] (dotted line in Fig. 3). It is partly attributable to the overestimation of the active force due to shortening of the sarcomeres located out of the observable region, in contact with glass needles. Since the SD was typically within ±50 nm at low levels of activation, we conclude that the relationship is reliable, at least with the ±150 nm accuracy.

The $Ca^{2+}$-sensitivity and cooperativity of force development (inset in Fig. 3) were SL-dependent, as also shown by Endo [1]. When the force–pCa relationship is analyzed for the data showing steady contraction, the Hill coefficient $n$ was 8.7, 3.1, and 1.4, and $pCa_{50}$ was 6.04, 6.14, and 6.06 at SL 2.3–2.6, 2.7–3.0, and 3.1–3.4 μm, respectively. For comparison, the values of $n$ and $pCa_{50}$ at SL 2.3–2.6 μm, obtained from the data showing steady oscillation at pCa 6.1–6.2, were 10.8 and 6.03, respectively.

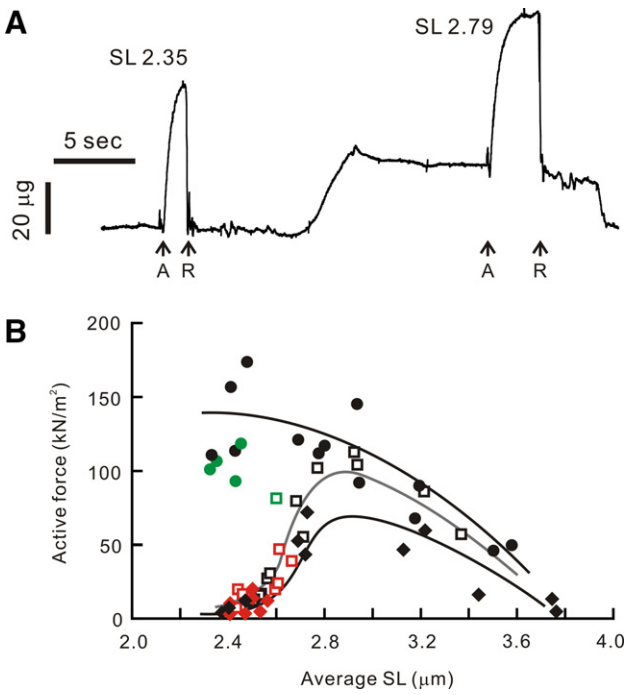


Fig. 4. Effect of dextran. (A) A typical time course of force development at two different SLs, 2.35 and 2.79 μm, at pCa 6.2 in the presence of 1% dextran. (B) The force–length relationship in the presence of 1% dextran at pCa 6.2 (filled circles; $n = 3$ myofibrils) and pCa 6.3 (filled diamonds; $n = 3$ myofibrils). For comparison, the relationship obtained at pCa 6.2 in the absence of dextran is replotted from Fig. 3 (open squares). The definition of red and green symbols is the same as in Fig. 3. The lines were drawn by eye. The CSA of the myofibril in (A) was 5.73 μm$^2$. The stiffness of the flexible needle was 21.5 μg/μm.

### *Effect of dextran*

To see the contribution of the interfilament spacing between the thick and thin filaments to the mechanism of the length-dependent activation, the effect of dextran T-500 was examined at partial $Ca^{2+}$-activation (Fig. 4). Dextran osmotically compresses the lattice spacing of muscle fibers [14] and myofibrils [7]. At pCa 6.2, the addition of 1% (w/v) dextran substantially (more than 4-fold) increased the active force at SL 2.3–2.6 μm, whereas hardly affected it at SL >3.0 μm, so that the force–length relationship approached the linear dependence. In addition, the occurrence frequency of SPOC reduced prominently, and the observed oscillation was of the intermittent (Fig. 1D), rather than the steady, type (Fig. 1C). On the other hand, we found that when the level of activation was decreased by increasing pCa from 6.2 to 6.3, both the length-dependent activation and SPOC were observed even in the presence of 1% dextran.

## Discussion

Our microscopic measurements of the force–length relationship on myofibrils (Fig. 3) confirmed that the larger force production at longer SL observed under partial $Ca^{2+}$-activation [1,2] is attributable to the property that the individual sarcomeres themselves intrinsically possess. In addition, we found that the spontaneous oscillation of force and SL (Ca-SPOC) occurs in the vicinity of the maximal overlap between the thick and thin filaments (Fig. 1), where the active force was kept at a low level (Fig. 3). Both the linear force–length relationship and the level of active force at the maximal activation (Fig. 3) were comparable with fiber studies, implying that the characteristics of the mechanical performance of the contractile system observed in this study are well conserved between muscle fibers and myofibrils.

Endo reported at least two different types of the force-SL dependence near the slack SL at the same activating conditions (partial activation at pCa 6 in Fig. 1 of Ref. 2). We observed the same characteristics under similar activating conditions (see the data at pCa 6.1 in Fig. 3). Moreover, our single-sarcomere analysis revealed the reason for this biphasic property: the upper dashed curve (black crosses in Fig. 3) was attributable to the normal contraction without oscillation, while the lower dashed curve (red crosses in Fig. 3) corresponded to the typical Ca-SPOC. In the region between these two curves the intermittent oscillation was observed (green crosses in Fig. 3).

The force–pCa relationship (inset in Fig. 3), and in particular the Hill coefficient, was affected when the myofibrils exhibited SPOC instead of steady contraction. Therefore, one should confirm under the optical microscope whether SPOC occurs or not, when studying the [$Ca^{2+}$]-dependent properties of sarcomeres.

At partial activation by $Ca^{2+}$ (pCa 6.1–6.5), the force-generating capacity of a unit length of overlap was increased up to 5-fold with increasing SL (Fig. 3). Besides, the osmotic compression of the myofilament lattice enhanced the active force (Fig. 4), as previously reported in fiber studies [15]. Therefore, although several factors are likely to be responsible for the length-dependent activation (for review, see Ref. [16–18]), one crucial factor must be the length-dependent changes in the lattice spacing, which modulate the probability of the myosin heads binding to the thin filament. In fact, a simple model we proposed previously, which is based on the lattice spacing-dependent changes in the probability of cross-bridge formation, successfully explained the force–length relationship at partial activation and its dependence on [$Ca^{2+}$] [19].

We recently reported that the ADP-bound cross-bridges cooperatively activate the thin filament independently of $Ca^{2+}$, and that such activation is sensitive to the sub-nm changes in the lattice spacing [7]. It is likely that the $Ca^{2+}$-affinity of troponin is length-independent in skeletal muscle [20,21]. Hence, the length-dependent activation is implemented in the cooperative increase of the affinity of cycling cross-bridges to the thin filament in response to the changes in the lattice spacing, which results in the observed dependence of the Hill coefficient on SL (inset in Fig. 3).

The length-dependent activation was weakened not only by compressing the lattice spacing with 1% dextran, but by

increasing [$Ca^{2+}$] as well (Figs. 3 and 4). In addition, the effect of the lattice compression was compensated by the decrease in [$Ca^{2+}$] (Fig. 4). These results imply that change in [$Ca^{2+}$] produces the effect similar to that seen after the addition of dextran, by which the optimal lattice spacing is modulated.

It is generally recognized that under physiological conditions muscle adopts either of the two states, contraction or relaxation, depending on [$Ca^{2+}$]. However, the spontaneous oscillation (Ca-SPOC) occurred under the conditions between relaxation and contraction, at sub-μM level of [$Ca^{2+}$] (Fig. 3). The oscillatory contractions under constant [$Ca^{2+}$] are also reported in slow skeletal [6], cardiac [3–5], and insect flight muscle [22], indicating that all the contractile systems of striated muscle possess the ability to auto-oscillate.

The length-dependent activation (Fig. 3) and its modulation by the lattice compression (Fig. 4) suggest that SPOC is driven by the shortening deactivation and lengthening activation of sarcomeres [5], that is, by the length-dependent changes in the $Ca^{2+}$-sensitivity of force, which is attributable to the changes in the lattice spacing. Based on this property, the sarcomeres are deactivated and activated, respectively, as the shortening and lengthening proceed, allowing maintaining stable oscillation.

It is known that the filament lattice of skinned fibers and myofibrils is swollen compared with intact muscle, due to a disruption of its membranous structure [23]. Nevertheless, we stress that both the length-dependent activation and SPOC occurred in the region of higher pCa values even with the compressed myofilament lattice (Fig. 4). Besides, the alteration of SL induces larger changes in the lattice spacing in intact muscle than in skinned preparations; hence, we infer that these cooperative phenomena observed at partial $Ca^{2+}$-activation are also characteristic of intact muscle.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.123.

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