Skeletal and cardiac $\alpha$-actin isoforms differently modulate myosin cross-bridge formation and myofibre force production

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Multiple congenital myopathies, including nemaline myopathy, can arise due to mutations in the $ACTA1$ gene encoding skeletal muscle $\alpha$-actin. The main characteristics of $ACTA1$ null mutations (absence of skeletal muscle $\alpha$-actin) are generalized skeletal muscle weakness and premature death. A mouse model ($ACTCCo/KO$) mimicking these conditions has successfully been rescued by transgenic over-expression of cardiac $\alpha$-actin in skeletal muscles using the $ACTC$ gene. Nevertheless, myofibres from $ACTCCo/KO$ animals generate less force than normal myofibres (20 to 25%). To understand the underlying mechanisms, here we have undertaken a detailed functional study of myofibres from $ACTCCo/KO$ rodents. Mechanical and X-ray diffraction pattern analyses of single membrane-permeabilized myofibres showed, upon maximal $Ca^{2+}$ activation and under rigor conditions, lower stiffness and disrupted actin-layer line reflections in $ACTCCo/KO$ when compared with age-matched wild-types. These results demonstrate that in $ACTCCo/KO$ myofibres, the presence of cardiac $\alpha$-actin instead of skeletal muscle $\alpha$-actin alters actin conformational changes upon activation. This later finely modulates the strain of individual actomyosin interactions and overall lowers myofibre force production. Taken together, the present findings provide novel primordial information about actin isoforms, their functional differences and have to be considered when designing gene therapies for $ACTA1$-based congenital myopathies.

INTRODUCTION

Mutations in the skeletal muscle $\alpha$-actin gene ($ACTA1$) cause a range of congenital myopathies, including the most common known as nemaline myopathy (1). Despite patients possibly exhibiting a variety of different pathological features in their skeletal muscles, most patients die within the first year of life (2). The majority of $ACTA1$ mutations are dominant; however, a series of null mutations related to recessive nemaline myopathy have been identified in several children (3). Muscles from these patients do not contain any skeletal muscle $\alpha$-actin protein but express some cardiac $\alpha$-actin instead (3). A mouse line lacking skeletal muscle $\alpha$-actin models this human condition (4). Although some animals appear normal at birth, they experience growth problems, skeletal muscle disruption and all die in the early neonatal period (days 1–9). As with the human patients, the mice naturally slightly overexpress cardiac $\alpha$-actin but this is not sufficient to preserve skeletal muscle function or lifespan (4). To rescue the animals, skeletal muscle $\alpha$-actin has to be totally replaced by cardiac $\alpha$-actin (5).

In mammals, the skeletal muscle and cardiac $\alpha$-actin isoforms (the latter encoded by $ACTC$) are co-expressed in skeletal muscles but their amounts depend on the maturation of the tissue. Thus, cardiac $\alpha$-actin predominates in fetal skeletal muscles (6) but by birth, it reaches a very low level (<5%) and is replaced by skeletal muscle $\alpha$-actin (7). Interestingly, the skeletal muscle and cardiac $\alpha$-actin protein isoforms are 99% identical (8). They only differ by four amino acids (Glu2Asp, Asp3Glu, Met299Leu and Thr358Ser). Nevertheless, these differences are likely to imply fine functional discrepancies between the two isoforms as they are present in all species, and in mice where skeletal muscle $\alpha$-actin has fully been replaced by...
cardiac α-actin, mechanically skinned myofibres produce 20–25% smaller steady-state isometric maximal forces than wild-type (WT) myofibres (5). Hence, one may hypothesize that during contraction cardiac α-actin differently regulates the formation of strongly bound myosin cross-bridges when compared with skeletal muscle α-actin and, thus, is the molecular determinant of the lower myofibre force-generating capacity in the mice. In the present study, we aimed to verify this by recording the fraction of force redevelopment (force and stiffness data). As previously shown (5), steady-state isometric force at saturating [Ca\(^{2+}\)] (pCa 4.50) was significantly smaller in ACTC\(^{-/-}\)/KO when compared with WT myofibres (Table 1). To investigate whether this is also present at non-saturating [Ca\(^{2+}\)], the relative force–pCa relationship (pCa’s between 6.30 and 4.50) was constructed and fitted using the following sigmoid Hill equation:

\[
F = \frac{[\text{Ca}^{2+}]^{nH}}{[\text{Ca}_{50}]^{nH} + [\text{Ca}^{2+}]^{nH}}
\]

where \(F\) is the force, \(-\log_{10}[\text{Ca}_{50}]\) the midpoint (pCa\(_{50}\) or Ca\(^{2+}\) sensitivity), and \(nH\) the Hill coefficient. Neither pCa\(_{50}\) nor \(nH\) were different between ACTC\(^{-/-}\)/KO and WT myofibres (Table 1), proving that steady-state isometric force is similarly affected at all [Ca\(^{2+}\)] and that the cooperative activation of myofilaments is unaltered (9).

To have insights into the mechanisms responsible for the force alteration, we performed stiffness measurements. Thus, step changes in fibre length were imposed (releases between 0.15 and 0.50% of fibre length and stretches of the same amplitudes). Stiffness was defined as the slope of the linear regression of the relationship between the peak force response and the length change (Fig. 2). Active stiffness was significantly lower at saturating [Ca\(^{2+}\)] (pCa 4.50) in ACTC\(^{-/-}\)/KO when compared with WT myofibres (Table 1). Under rigor conditions, where all myosin heads are attached due to a very slow dissociation rate (10), rigor stiffness was also significantly smaller in ACTC\(^{-/-}\)/KO when compared with WT myofibres (Table 1). These active and rigor stiffness data allowed the calculation of the fraction of strongly bound myosin cross-bridges (\(f_{xb}\)) using the following equation (11):

\[
f_{xb} = \frac{S_0/S_r}{2 - (S_0/S_r)}
\]

\(f_{xb}\) was not different between ACTC\(^{-/-}\)/KO and WT myofibres (Table 1). In addition to all the above parameters, the rate constant of force redevelopment (\(k_r\)) was also evaluated. Once steady-state isometric force was reached at saturating [Ca\(^{2+}\)] (pCa 4.50), a slackening of 20% of the original fibre length

### RESULTS

**Single membrane-permeabilized myofibre mechanics**

After permeabilization, single myofibres were isolated from the extensor digitorum longus (EDL) and mounted for analysis of mechanical properties. A total of 60 fibres were tested. For a detailed description of the myosin heavy chain (MyHC) isoform expression of these myofibres, see Figure 1. As no significant differences were seen between myofibres expressing various MyHC isoforms, all the cell types were pooled together.

**Figure 1.** MyHC isoform distribution. This figure displays the MyHC isoform distribution in EDL myofibres for WT (filled squares) and ACTC\(^{-/-}\)/KO (open squares).

<table>
<thead>
<tr>
<th>Table 1. Single myofibre mechanics</th>
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<tbody>
<tr>
<td>ACTC(^{-/-})/KO</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Maximal force (kPa)</td>
</tr>
<tr>
<td>Active stiffness (kPa.% L(_{0}^{-1}))</td>
</tr>
<tr>
<td>Rigor stiffness (kPa.% L(_{0}^{-1}))</td>
</tr>
<tr>
<td>(K_0) (AU)</td>
</tr>
<tr>
<td>(V_0) (ML.s(^{-1}))</td>
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<tr>
<td>pCa(_{50})</td>
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<tr>
<td>(nH)</td>
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</table>

For force and stiffness data, as no significant differences were seen between myofibres expressing various MyHC isoforms, all the cell types were pooled together. For \(k_r\), \(V_0\), pCa\(_{50}\) and \(nH\) values, comparisons were restricted to myofibres expressing the most common MyHC isoform in mouse EDL muscle, i.e. type IIb. Data are presented as the mean ± SEM. For each parameter, \(n\) represents the number of tested myofibres.

\(^a\)Significant difference when compared with WT (\(P < 0.05\)).
was introduced within 1–2 ms at one end of the fibre, resulting in a rapid reduction of force to near zero. This was followed by a brief period of unloaded shortening (20 ms), after which the preparation was quickly re-stretched to its original length and the force recovered to its original steady-state value. As described previously (12), $k_{tr}$ was estimated by the linear transformation of the half-time of force redevelopment ($t_{1/2}$) as follows (13):

$$k_{tr} = 0.693/t_{1/2}$$

$k_{tr}$ is known to be MyHC isoform-dependent. Hence, comparisons were restricted to myofibres expressing the most common MyHC isoform in mouse EDL muscle (type IIb). $k_{tr}$ was not different between $ACTCC^{co}$/KO and WT myofibres (Table 1), suggesting similar cross-bridge cycle turnover rates (i.e. $f_{app} + g_{app}$) between the actin isoforms (14,15). A decrease in $f_{app}$ together with an increase in $g_{app}$ cannot be totally excluded. Therefore, maximum unloaded shortening velocity ($V_0$) was then calculated. Once steady-state isometric force was reached at saturating $[Ca^{2+}]$ (pCa 4.50), nine slacks of various amplitudes were rapidly introduced (within 1–2 ms) at one end of the fibre (16). Slacks were applied at different amplitudes ranging from 7 to 13% of the myofibre length (17,18). The myofibre was re-extended between releases while relaxed in order to minimize changes in sarcomere length. During the slack test, the time required to take up the imposed release was measured from the onset of the length step to the beginning of the tension redevelopment. A straight line including four or more data points was fitted to a plot of release length versus time, using least-squares regression. The slope of the line divided by the myofibre segment length was recorded as the maximum unloaded shortening velocity ($V_0$) for that myofibre segment (16). As $k_{tr}$, $V_0$ is MyHC isoform-related. Thus, comparisons were restricted to myofibres carrying the type IIb MyHC isoform. $V_0$ was not different between $ACTCC^{co}$/KO and WT myofibres (Table 1), showing that $g_{app}$ is identical between the actin isoforms (19). Overall, $k_{tr}$ and $V_0$ results prove that the elementary kinetic steps of the cross-bridge cycles are not actin isoform-dependent.

### X-ray diffraction patterns

To gain further information on the mechanical differences between $ACTCC^{co}$/KO and WT skeletal muscles, myofibres were isolated from tibialis anterior and mounted. Low angle X-rays were then recorded (Fig. 3) and the intensity changes of two myosin meridional reflection (first and second MMs) and three actin-layer lines (second, sixth and seventh ALLs) were monitored and analysed (Fig. 3 and Table 2). All these parameters were enhanced on addition of Ca$^{2+}$. Of interest, the intensification of the sixth ALL during contraction was significantly lesser in $ACTCC^{co}$/KO when compared with WT myofibres (Table 2). As for equatorial reflections (Fig. 3), the $I_{1,1}/I_{1,0}$ intensity ratio was calculated and gave information on the mass distribution between the thick and thin filaments. The intensification of this ratio upon activation was not different between $ACTCC^{co}$/KO and WT myofibres (Fig. 4 and Table 2). $I_{1,0}$ equatorial intensities were then converted to $d_{1,0}$ lattice spacings, using Bragg’s law (20). $d_{1,0}$ lattice spacings values

![Figure 2. Fibre stiffness. This figure shows one typical peak force–length change relationships for WT (filled squares) and $ACTCC^{co}$/KO (open squares) myofibres.](image)

**Figure 2.** Fibre stiffness. This figure shows one typical peak force–length change relationships for WT (filled squares) and $ACTCC^{co}$/KO (open squares) myofibres.

![Figure 3. Low angle X-ray diffraction reflections. This figure shows diffraction patterns for WT (A and B) or $ACTCC^{co}$/KO (C and D) myofibres in resting state (A and C) or upon activation (B and D).](image)

**Figure 3.** Low angle X-ray diffraction reflections. This figure shows diffraction patterns for WT (A and B) or $ACTCC^{co}$/KO (C and D) myofibres in resting state (A and C) or upon activation (B and D).

### Table 2. Equatorial, meridional and ALLs intensifications during contraction

<table>
<thead>
<tr>
<th></th>
<th>$ACTCC^{co}$/KO</th>
<th>WT</th>
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<tbody>
<tr>
<td>$I_{1,1}/I_{1,0}$ ratio</td>
<td>0.451 ± 0.009</td>
<td>0.437 ± 0.008</td>
</tr>
<tr>
<td>First MM</td>
<td>0.168 ± 0.002</td>
<td>0.172 ± 0.002</td>
</tr>
<tr>
<td>Second MM</td>
<td>0.011 ± 0.001</td>
<td>0.006 ± 0.001</td>
</tr>
<tr>
<td>Second ALL</td>
<td>0.129 ± 0.004</td>
<td>0.164 ± 0.004</td>
</tr>
<tr>
<td>Sixth ALL</td>
<td>0.147 ± 0.003*</td>
<td>0.204 ± 0.003</td>
</tr>
<tr>
<td>Seventh ALL</td>
<td>0.021 ± 0.003</td>
<td>0.046 ± 0.002</td>
</tr>
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</table>

The intensification was calculated as the difference between the reflection upon activation and the reflection during pre-activation. All the meridional and ALL data were normalized to the sixth ALL in relaxing state. Values are presented as the mean ± SEM. A total of 38 arrays ($19 ACTCC^{co}$/KO and 19 WT) were included in the analysis.

*Significant difference when compared with WT ($P < 0.05$).
provide information on radial compressional forces and on the lattice spacing. $d_{1,0}$ lattice spacing was not significantly different between ACTCCo/KO and WT myofibres (Table 3). Similarly, the width of the 1,0 equatorial reflections (estimated via $D_{1,0}$) was not significantly different between ACTCCo/KO and WT myofibres (Table 3), suggesting a maintained thick-to-thin filament surface distance in the presence of cardiac $\alpha$-actin.

**Actomyosin modelling**

A model of strong myosin head binding to actin in the rigor-like state was used (21). In this model, incorporating Glu2Asp, Asp3Glu, Met299Leu and Thr358Ser altered the energy of some actin neighbouring amino acids (Fig. 5).

**DISCUSSION**

Understanding how skeletal muscle and cardiac $\alpha$-actin isoforms functionally differ is not only of biological interest, but also of great importance in order to establish future efficient therapeutic interventions. In the present study, we demonstrated that in ACTC$^{C\alpha}$/KO mice, the full replacement of skeletal muscle $\alpha$-actin by cardiac $\alpha$-actin finely down-regulates the contractile function at the cellular and molecular levels.

The steady-state isometric maximal force of single membrane-permeabilized myofibres from ACTC$^{C\alpha}$/KO animals was smaller when compared with myofibres from WT mice. This dysfunction may originate from either a lower number of strongly bound actomyosin interactions and/or from a smaller force per individual myosin cross-bridge. To separate between these two possibilities, stiffness measurements were performed. As active and rigor stiffness were similarly altered, and as the total sarcomeric protein content was unchanged in ACTC$^{C\alpha}$/KO rodents when compared with WT animals (5) (Supplementary Material, figure), one may suggest that the smaller force in the presence of cardiac $\alpha$-actin is largely due to a decrease in the strain of individual actomyosin interactions, i.e. less strong bindings or in other words, softer myosin cross-bridges. The $f_{kb}$ values (Table 1) as well as the collected X-ray data, i.e. the equatorial, meridional and ALLs reflections (Figs 3 and 4; Tables 2 and 3), are in line with this statement. Indeed, the $I_{1,1}/I_{1,0}$ ratio intensification during contraction was not different between ACTC$^{C\alpha}$/KO mice and WT myofibres (Table 2), suggesting an equivalent net mass transfer from the thick to thin filaments, thus a similar number of cross-bridges bound to actin monomers. In accordance with this finding, the maintained second ALL enhancement upon Ca$^{2+}$ addition (Table 2) indicate analogous tropomyosin and thin filament activations between ACTC$^{C\alpha}$/KO and WT myofibres. In addition, the mean orientation of the myosin heads strongly bound to actin filaments are unlikely to be modified (22, 23) as the first and second MM intensifications were not affected by the replacement of skeletal muscle $\alpha$-actin by cardiac $\alpha$-actin (Table 2).

Apart from these consistent data, the sixth ALL reflection enhancement during contraction was smaller in ACTC$^{C\alpha}$/KO when compared with WT myofibres (Table 2). It is well known that, upon Ca$^{2+}$ activation, the sixth ALL reflection increases, and that increase is due to actin monomers modifying their molecular shape (24), i.e. axial compression via closing-ups between subdomains 1 and 2 and between subdomains 3 and 4 (25). As the amino acids that differ between the skeletal muscle and cardiac $\alpha$-actin isoforms are located in distinct subdomains (Glu2Asp, Asp3Glu and Thr358Ser in subdomain 1 and Met299Leu in subdomain 3), they are likely to directly limit

**Table 3.** Myofilament lattice spacing

<table>
<thead>
<tr>
<th></th>
<th>ACTC$^{C\alpha}$/KO Resting</th>
<th>ACTC$^{C\alpha}$/KO Activation</th>
<th>WT Resting</th>
<th>WT Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{1,0}$ lattice spacing (nm)</td>
<td>42.25 ± 0.03</td>
<td>39.35 ± 0.02</td>
<td>41.89 ± 0.03</td>
<td>39.92 ± 0.03</td>
</tr>
<tr>
<td>$\Delta_{i}\times 10^{-4}$ (nm)</td>
<td>4.29 ± 0.03</td>
<td>6.01 ± 0.05</td>
<td>4.90 ± 0.04</td>
<td>6.06 ± 0.06</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± SEM. A total of 38 arrays (19 ACTC$^{C\alpha}$/KO and 19 WT) were included in the analysis.
the entire activation-induced closing-ups between subdomains 1 and 2 and between subdomains 3 and 4. Other residues may also be involved in this deleterious process. In fact, according to the energy computations (Fig. 5), Glu2Asp, Asp3Glu and Thr358Ser induce a modification of the stability of various neighbouring residues (Asp1, Glu99, Glu100 and Glu361 in subdomain 1). Altogether, these subtle changes may render the actin monomers stiffer, limiting the actin conformational changes during contraction directly making myosin cross-bridges less responsive, softer and weaker. Such fine alteration of the actomyosin function has been proposed in a previous study using an in vitro motility assay (26).

It should be mentioned that the present experiments were carried out EDL myofibres expressing the fast MyHC isoforms (types IIx and IIb). Therefore, it is not totally excluded that myofibres carrying the slowest MyHC isoform (type I/cardiac) would behave differently when skeletal muscle α-actin is fully replaced by cardiac α-actin. Indeed, slow and fast MyHC isoforms are highly conserved molecules but have some amino acid discrepancies in the ATP and actin binding loops as well as in the myosin light chain binding domains (27). Therefore, one may speculate that the impact on the force generating capacity is different in presence of the type I/cardiac MyHC isoform. Further experiments are required to verify this.

In conclusion, taken together, these data suggest that when skeletal muscle α-actin is replaced by cardiac α-actin in ACTC−/−/KO mice, some molecular and cellular dysfunctional processes occur. Actin monomers are less compliant upon activation, which in turn render myosin cross-bridges softer and weaker. At the myofibre level, this lowers the force-generating capacity. Hence, the skeletal muscle and cardiac α-actin isoforms differently regulate muscle contraction, which is of interest from an evolutionary biology point of view, and their function during embryonic development and in adult tissues. Moreover, this finding has to be taken into consideration when considering the incorporation of the ACTC gene as a therapy for the ACTA1-based congenital myopathies.

MATERIALS AND METHODS

Animals

Twelve-week-old WT as well as age-matched transgenic (ACTC−/−/KO) mice were included in the analyses. A complete description of the ACTC−/−/KO rodents can be found elsewhere (5). Briefly, to replace ACTA1 by ACTC in skeletal muscles, the ACTC transgenic mouse line was crossed with the ACTA1 knock-out line in which all homozygous animals die by 9 days after birth (5). The tibialis anterior and EDL muscles were dissected from ACTC−/−/KO and WT mice after sacrifice. All procedures involving animal care and handling were performed according to institutional guidelines and were reviewed and approved by the Animal Experimentation Ethics Committee of The University of Western Australia.

Muscle preparation

Specimens were placed in relaxing solution at 4°C. Bundles of ~50 myofibres were dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. They were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 h at 4°C, after which they were transferred to −20°C. In addition, the muscle bundles were treated with sucrose, a cryoprotectant, within 1–2 weeks for long-term storage (28). They were detached from the capillary tubes and snap frozen in liquid nitrogen-chilled propane and stored at −160°C.

Membrane-permeabilized myofibre mechanical recordings and analyses

On the day of experimentation, bundles were de-sucrosed, transferred to a relaxing solution and single myofibres dissected. A fibre, 1–2 mm long, was left between connectors leading to a force transducer (model 400A, Aurora Scientific) and a lever arm system (model 308B, Aurora Scientific) (29,30). The two extremities of the myofibre were tightly attached to the connectors as described previously (29). The apparatus was mounted on the stage of an inverted microscope (model IX70; Olympus). The sarcomere length was set to 2.50–2.60 μm (optimal mouse sarcomere length where the force production is the highest) and controlled during the experiment using a high-speed video analysis system (model 901A HVSL, Aurora Scientific). The diameter of the myofibre between the connectors was measured through the microscope at a magnification of x 320 with an image analysis system prior to the mechanical experiments. Myofibre depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the myofibre. The focusing control of the microscope was used as a micrometer. The cross-sectional area (CSA) was calculated from the diameter and depth, assuming an elliptical circumference, and was corrected for the 20% swelling that is known to occur during skinning (29). Diameter and depth were measured at three different locations along the length of each myofibre and the mean was considered as representative of cell dimensions. Mechanical experiments were done at 15°C and included force measurements (normalized to CSA) after various length steps at saturating [Ca2+] (pCa 4.50), as described previously (17,18,31).

After mechanical recordings, each membrane-permeabilized myofibre was placed in urea buffer [8 M urea, 75 mM dithiothreitol (DTT), 50 mM Trizma base and 104 mM SDS, 0.004% bromophenol blue] in a plastic microcentrifuge tube and stored at −160°C. MyHC isoform composition of fibres was then determined by 6% SDS – PAGE. The acrylamide concentration was 4% (w/v) in the stacking gel and 6% in the running gel, and the gel matrix included 30% glycerol. Sample loads were kept small (equivalent to ~0.05 mm of fibre segment) to improve the resolution of the MyHC bands (types I, IIA, IIX and IIB). Electrophoresis was performed at 120 V for 24 h with a Tris–glycine electrode buffer (pH 8.3) at 15°C (SE 600 vertical slab gel unit, Hoefer Scientific Instruments). The gels were silver-stained and subsequently scanned in a soft laser densitometer (Molecular Dynamics) with a high spatial resolution (50 μm pixel spacing) and 4096 optical density levels.

X-ray diffraction recordings and analyses

Two to three days prior to X-ray recordings, bundles were de-sucrosed, transferred to a relaxing solution and single
myofibres were dissected. Arrays of ~30 myofibres were set up (25,31–35). For each myofibre, both ends were clamped to half-split gold meshes for electron microscopy (width, 3 mm), which had been glued to precision-machined ceramic chips (width, 3 mm) designed to fit to a specimen chamber. The arrays were then transferred to the skinnning solution and stored at −20°C. Approximately 60 arrays were mounted.

On the day of X-ray recordings, arrays were placed in a plastic dish containing a pre-activating solution and washed thoroughly to remove the glycerol. They were then transferred to the specimen chamber, capable of manual length adjustment and force measurement (force transducer, AE801, Memscap, Bernin, France), filled with a pre-activating solution. The mean sarcosome length was measured and set to 2.50 μm. Subsequently, X-ray diffraction patterns were recorded at 15°C, first in the pre-activating solution and then in the activating solution (pCa 4.50) when maximal steady-state isometric force was reached. The activating solution was supplied to the chamber by using a remote-controlled pump.

For each array, ~10–30 diffraction patterns were recorded (depending on myofibre length) for each solution at the BL45XU beamline of SPring-8. The wavelength was 0.09 nm and the specimen-to-detector distance was 2 m. As a detector, a cooled CCD camera (C4880, Hamamatsu Photonics; 1000 × 1018 pixels) was used in combination with an image intensifier (VP5445, Hamamatsu Photonics). To minimize radiation damage, the exposure time was kept low (2 s) and the specimen chamber was moved by 100 μm after each exposure. Moreover, we placed an aluminium plate (thickness, 0.35–0.5 mm) upstream of the specimen chamber. The beam flux was estimated to be between 2.7 × 10\(^{11}\) and 4.0 × 10\(^{11}\) photons/s after attenuation, and the beam size at the sample position was 0.2 mm (vertical) and 0.3 mm (horizontal). Following X-ray recordings, background scattering was subtracted, and reflection intensities (except for equatorial reflections) were determined as described elsewhere (25,32–34,36). The intensities of equatorial reflections were determined separately. First, the background (the region outside the area of 1,0 and 1,1 reflections) was approximated by a single exponential decay function, and this was subtracted from the observed intensity profile. The resulting curve should contain the profiles of 1,0, 1,1 and 2,0 reflections of the hexagonal lattice of myofibrils, and the profile of the square lattice of Z-line components. In this curve, the 1,0 is usually the most prominent reflection, and this was first fitted by a Gaussian distribution function and was subtracted from the rest of the curve. After this, the 1,1 is the most prominent reflection, and this was again fitted by a Gaussian. The intensities of 1,0 and 1,1 were defined as the areas below the fitted Gaussian curves. The lattice spacing was calculated from the peak position of the Gaussian curve.

**Solutions**

For the mechanical and X-ray recordings, relaxing and activating solutions contained 4 mM Mg-ATP, 1 mM free Mg\(^{2+}\), 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, 324 U/ml creatine phosphokinase, 1000 U/ml catalase and KCl to adjust the ionic strength to 180 mM and pH to 7.0. DTT was also added. The pre-activating solution was identical to the relaxing solution except that the EGTA concentration was reduced to 0.5 mM. The concentrations of free Ca\(^{2+}\) were 10\(^{-9.00}\) M (relaxing and pre-activating solutions) and 10\(^{-6.30}\) to 10\(^{-4.50}\) M (activating solutions). Rigor solution had similar compositions to activating solution except that Mg-ATP, creatine phosphate and creatine phosphokinase were absent.

**Actomyosin modelling**

The model used in the present study has extensively been described elsewhere (21). It was visualized with UCSF Chimera (37) and Swiss PDB viewer. Energy computations in the absence or the presence of the actin mutation were done in vacuo with the GROMOS96 implementation of Swiss PDB viewer.

**Statistical analyses**

Data are presented as the mean ± standard error of the means (SEMs). Sigma Stat software (Jandel Scientific) was used to generate descriptive statistics. For the mechanical data, the unpaired Student’s t-test was applied, and in cases where the data did not meet the criteria of normality (Kolmogorov–Smirnov test, \(P < 0.05\)), the non-parametric Mann–Whitney rank-sum test was performed. The level of significance was set at \(P < 0.05\). Otherwise, regressions were performed and relationships were considered significantly different from zero at \(P < 0.05\).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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