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Effects of strain on contractile force and number of sarcomeres in series of *Xenopus laevis* single muscle fibres during long-term culture

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Abstract

The aim of the present study is to test whether mechanical strain uniquely regulates muscle fibre atrophy/hypertrophy and adaptation of the number of sarcomeres in series within mature muscle fibres *in vitro*. Mature single muscle fibres from *Xenopus laevis* illiofibularis muscle were cultured (4–97 days) while kept at negative strain (~20% below passive slack length, 'short fibres') or at positive strain (~5% over passive slack length, 'long fibres'). Before and after culture the number of sarcomeres in series was determined using laser diffraction. During culture, twitch and tetanic force characteristics were measured every day.

Survival time of long fibres was substantially less than that of short fibres. Of the long fibres 40% died or became inexcitable within 1 week, whereas this did not occur for short fibres. During culture, twitch and tetanic force of all short fibres increased substantially. Regression analysis showed that the post-culture number of sarcomeres in series was not significantly changed compared to the number before culture. It is concluded that culture at negative strain does not result in atrophy or a reduction of the number of sarcomeres in series, even after 97 days. For the long fibres we did not detect any hypertrophy as tetanic force remained stable or decreased slowly, while twitch force varied. Regression analysis of the change of the number of sarcomeres in series as a function of the culture time showed a positive slope ($P = 0.054$). Two out of four long fibres that were cultured for at least 2 weeks showed an increase in the number of sarcomeres of 4–5%. Compared with *in vivo* adaptation to mechanical stimuli this is much less than would be expected. The data suggest that strain may not be the only factor that regulates hypertrophy and the number of sarcomeres in series.

Introduction

Major determinants of the mechanical properties of striated muscle are the muscle fibre cross-sectional area (CSA) and the number of sarcomeres arranged in series (e.g. Williams and Goldspink, 1971; Crawford, 1973; Tardieu *et al.*, 1974; Heslinga and Huijing, 1993). The potential for adaptation of healthy normal muscle to altered functional demands is enormous (e.g. Goldspink, 1971; Tardieu *et al.*, 1973; Spector *et al.*, 1982; Heslinga and Huijing, 1993; Burkholder and Lieber, 1998). Under pathological conditions such as cerebral palsy, spinal cord disease, lung emphysema or chronic heart failure, adaptation, i.e. change in muscle fibre diameter (atrophy/hypertrophy) or length adaptation of muscle fibres by change of the number of sarcomeres in series is limited. The consequences of such limitations may be impeded motor function (Yokochi *et al.*, 1995) or exercise intolerance (e.g. Clark and Coats, 1994; Casaburi, 2000). Restoration of muscle properties under pathological conditions, requires understanding of the underlying mechanisms of adaptation.

Several physiological factors, such as strain (e.g. Williams and Goldspink, 1978; Spector *et al.*, 1982), excursion (e.g. Koh and Herzog, 1998), activity (e.g. Goldspink *et al.*, 1974; Hayat *et al.*, 1978) and hormones (e.g. McKoy *et al.*, 1999), have been identified that play a role in the regulation of hypertrophy and the number of sarcomeres in series. However, the type of stimuli and the mechanism by which hypertrophy and the number of sarcomeres in series is regulated, is poorly understood. A reason for this is, that *in vivo* independent

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manipulation of the regulating factors, such as strain, activity and hormones is lacking. Furthermore, within a muscle the physiological conditions of the fibres are not homogeneous. For instance, heterogeneity of sarcomere lengths within muscle has been demonstrated within *in vivo* muscle as well as *in situ* muscle (e.g. van Eijden and Raadsheer, 1992; Zuurbier and Huijing, 1993; Willems and Huijing, 1994; Pappas *et al*., 2002; Ahn *et al*., 2003). In addition, mechanical interaction between neighbouring muscles and extramuscular connective tissue, causes unequal forces to be exerted at proximal and distal tendons of muscles in the rat anterior tibial compartment (e.g. Huijing and Baan, 2001; Maas *et al*., 2001; Huijing and Baan, 2003) as well as substantial variation of sarcomere length along muscle fibres (e.g. Yucesoy *et al*., 2003, in press). As a consequence the local sarcomere strain in the muscle fibres may be substantially different from the strain applied to the whole muscle fibre.

On the basis of the *in vivo* results regarding adaptation of sarcomere number, strain is indicated as being the most likely factor for regulation. Independent control of overall muscle fibre strain is possible within a culture system for mature single skeletal muscle fibres using a serum-free medium (Lee-de Groot and Van der Laarse, 1996; Jaspers *et al*., 2001). Two weeks of culture of muscle fibres at a length corresponding to a mean sarcomere length of 2.3 μm, was shown not to result in an altered number of sarcomeres in series (Jaspers *et al*., 2001). The aim of the present study is to test the hypothesis that strain is the regulating factor for the number of sarcomeres in series. For this purpose single muscle fibres of the *Xenopus laevis* were maintained in culture at negative and positive strains (i.e. below and over a fibre's mean sarcomere length of 2.3 μm). If strain is the regulating factor for muscle fibre CSA and the number of sarcomeres in series, it is expected that after a certain period the initially short and long fibres will have adapted the number of sarcomeres such that mean sarcomere length in these fibres is similar.

Methods

Animals and culture system

Treatment of animals was in accordance with the guidelines and regulations concerning animal welfare and experimentation set forth by Dutch law, and approved by the Committee on Ethics of Animal Experimentation at the Vrije Universiteit. In brief, after cooling in ice water for about 15 min, *Xenopus laevis* (females, 8–12 cm body length) were killed by decapitation and both iliofibularis muscles were excised. The muscles were allowed to recover for about 1 h in sterilised (0.22 μm filter), oxygenated Ringer solution (mM: NaCl, 116.5; KCl, 2.0; $CaCl_2$, 1.9; NaH_2PO_4, 2.0; EGTA, 0.1; pH 7.2). After recovery, both muscles were transferred to aseptic dissection troughs similar to the one described by Lännergren and Smith (1966). Single fibres V = (type 2 or 3) were isolated aseptically in a laminar flow cabinet under a microscope with dark-field illumination. This was done by cutting away the muscle fibres around the target fibre, using fine-tipped forceps and scissors. Small platinum hooks were tied to the trimmed down tendons using 20 μm diameter sterile polyamide thread. The platinum hooks and tools for dissection were sterilised using 70% ethanol. Before transferring the isolated fibre to the culture chamber, fibre diameters and the number of sarcomeres in series were determined (see below).

All parts of the culture chamber (for details see Lee-de Groot and Van der Laarse, 1996) were sterilised with 70% ethanol. The fibre was mounted at the appropriate length between a force transducer (AE801, SensoNor, Horten, Norway) and an adjustable rod. The chamber contained 0.8 ml of culture medium. This medium consisted of 66% DMEM/F12 (GibcoBRL) containing 100 U ml^{-1} penicillin/100 μg ml^{-1} streptomycin, 5 mM sodium phosphate, 1 mM creatine, 0.5 mM L-carnitine and 1 μg ml^{-1} insulin. After the fibre was mounted at the appropriate length, the culture chamber was transferred to an incubator kept at 20°C and tubing for gas and culture medium supply were connected. Fresh culture medium was continuously pumped through the chamber at a rate of about 0.5 ml h^{-1} and was equilibrated with air, containing 2.4% CO_2. Final pH was 7.6 and osmolarity was 235–250 m Osm kg^{-1}. The oxygen tension of the culture medium sampled from the chamber was 120–130 mmHg.

Measurements

The number of sarcomeres in series was determined using laser diffraction. Before and after culture, fibres were set to a length at which sarcomere length in the middle of the fibre was approximately 2.3 μm. At this fibre length, mean length of sarcomeres within the beam (diameter 1 mm) length was determined every 2 mm along the length of the fibre by using a HeNe laser. Subsequently, the length of the fibre was measured with the microscope using an ocular scale. The number of sarcomeres in series was calculated by dividing fibre length by the fibre's mean sarcomere length. In order to obtain an estimate of the accuracy of the determination of the number of sarcomeres in series, repeated measures of the number of sarcomeres in series were performed on freshly dissected muscle fibres ($n = 47$).

The smallest and largest diameters of the fibre were measured at three positions along the length of the fibre. The CSA of the fibre was calculated for each location assuming an ellipsoidal cross-section. Fibre CSA was taken as the mean of these three values.

After measurement of CSA and the number of sarcomeres in series, fibres were mounted in culture and set just over slack length, which corresponded to a mean sarcomere length of 2.3 μm (this length is referred to as "$l_{2.3\ \mu m}$"). At this length, we measured twitch and tetanic force characteristics. Subsequently, either a negative strain (~20% below $l_{2.3\ \mu m;}$ referred to as "short fibres"), or a positive strain (~5% over $l_{2.3\ \mu m}$; referred to

as "long fibres") was applied to the fibres. At these strains twitch and tetanic force characteristics of the fibre were determined every 24 h. The fibre was stimulated by 0.4 ms biphasic current pulses, 1.25 times above threshold voltage *via* platinum plate electrodes flanking the fibre, to produce two twitches, one tetanus (50 Hz, 260 ms duration) and a post-tetanic twitch (300 ms after the last pulse of the tetanus). Peak force, contraction time and half-relaxation time were measured for the second twitch and maximum tetanic force was determined. Twitch and tetanic tension were calculated by dividing fibre force by fibre CSA at $l_{2.3\ \mu m}$ as determined before the fibre was mounted in the culture chamber.

To determine the twitch and tetanic length–force characteristics of freshly dissected fibres, twitch and tetanic force of five fresly dissected fibres were measured at different fibre lengths (80, 95, 100, 105 and 110% of $l_{2.3\ \mu m}$, in this order). Fibres were allowed to rest for two min between tetanic contractions.

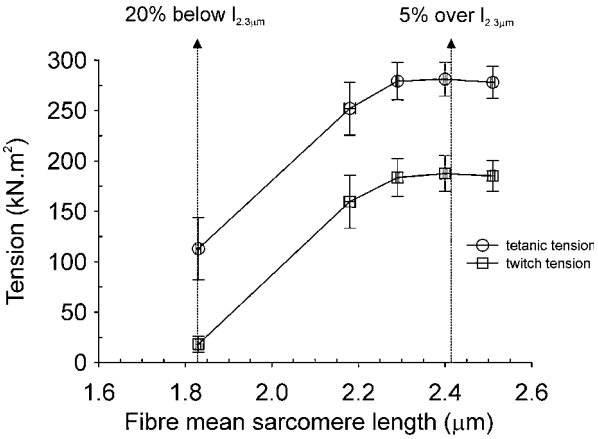

Fig. 1. Twitch and tetanic length–force characteristic of *Xenopus laevis* single muscle fibres. Mean active twitch and tetanic tension (±SEM) of freshly dissected fibres ($n = 5$) are expressed as a function of passive fibre mean sarcomere length (mean ± SEM). The experimental lengths at which the short and long fibres were cultured are indicated by vertical arrows.

Morphological examination

Post-culture, fibres were fixed (4% (v/v) formaldehyde and 15% (v/v) alcohol) at room temperature (RT) for 1 day and collected on a glass slide. The fibre morphology was examined with a Leica DMRB microscope using brightfield illumination (Wetzlar, Germany). Images were obtained with different objectives (×20, ×40 and ×100) and a monochrome charge-coupled device camera (Sony XC-77CE; Towada, Japan) connected to a LG-3 frame grabber (Scion; Frederick, MD) in an Apple Power Macintosh computer. After examination, three long fibres were embedded in 15% (w/v) gelatin in Ringer, and frozen in liquid nitrogen. Subsequently, serial cross-sections (10 μm thick) were cut and collected on slides, air-dried and fixed at room temperature. After this, sections were stained with hematoxylin and eosin (HE), dehydrated, and mounted in Entellan (Merck). These sections were used to localise myonuclei.

Statistical analyses

Data are expressed as the mean ± SEM. To test for significant differences ($P < 0.05$) in force data during culture within the short and long fibres one-way analyses of variance (ANOVA) for repeated measures were performed. Regression analysis was performed to test for significant changes in the number of sarcomeres in series during culture. The significance of the correlation between twitch force and half-relaxation time as well as between culture period and stimulus threshold was calculated according to Bland and Altman (1995).

Results

Culture conditions

Figure 1 shows twitch and tetanic length–tension characteristics of freshly dissected fibres expressed as a function of the fibre's mean sarcomere length ranging from about 80% below $l_{2.3\ \mu m}$ to 110% over $l_{2.3\ \mu m}$. According to Gordon *et al.* (1966), maximum overlap of actin and myosin filaments occurs between sarcomere lengths of 2.05–2.2 μm. Fibres were cultured at either 20% below or 5% over $l_{2.3\ \mu m}$. As filaments lengths of frog and toad are similar (Page and Huxley, 1963; Smith and Ovalle, 1973), this means that at their experimental lengths the fibres were on average approximately 15% below or 15% over the length of maximum overlap between actin and myosin.

Fibres were maintained in culture at the experimental length for different times. Within the group of long fibres, 60% of the fibres could be maintained in culture for at a least 5 days while being excitable. The remaining 40% of the long fibres became inexcitable or developed hypercontractions between day three and six of culture. In contrast, none of the short fibres developed hypercontractions within the 5 days of culture and all remained excitable. The mean initial tetanic tension at $l_{2.3\ \mu m}$ of both short and long fibres was 246.7 ± 9.7 kN m^{-2}, which indicates that fibres were metabolically normal after being transferred to the culture chamber. The time periods during which fibres were maintained at their experimental lengths differed. The long fibres were maintained in culture for periods varying from 5 to 15 days. One short fibre was kept in culture for 97 days.

Morphological changes

Initially, in the passive state the short fibres were sagging in the culture medium. During culture, macroscopic examination of the fibres showed that the sagging of the fibres was abolished as all short, passive fibres were hanging straight or almost straight. In order to explain the straightening of the muscle fibres, we measured the sarcomere lengths for one muscle fibre in the straightened situation. This showed that the mean sarcomere

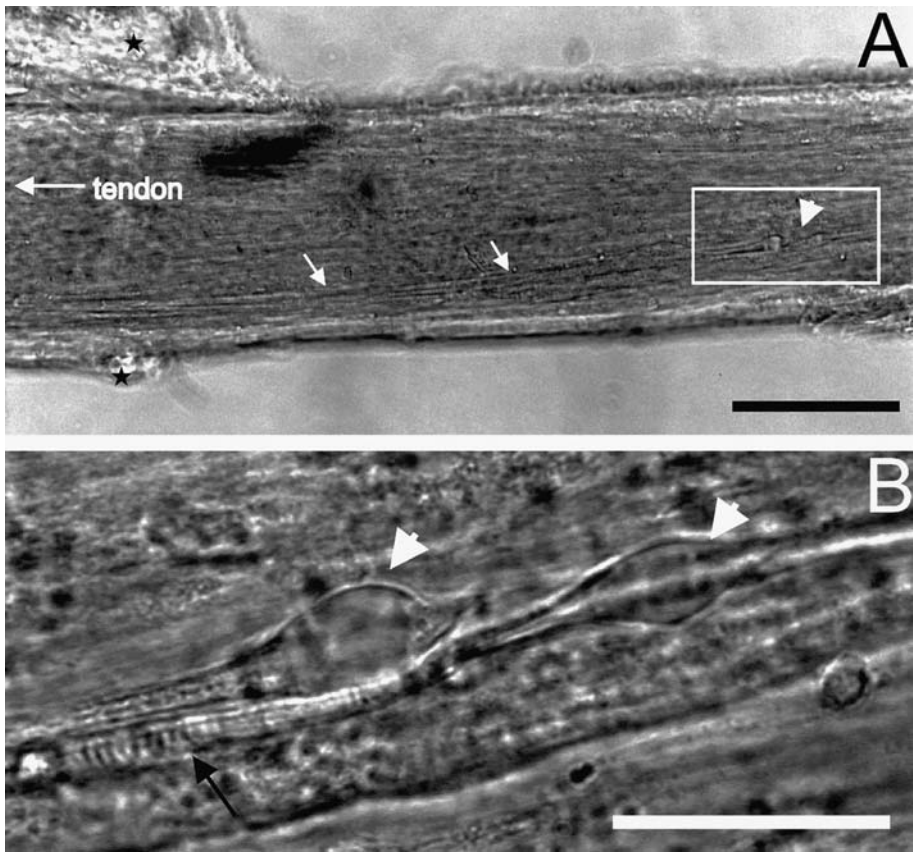

Fig. 2. Myogenic outgrowths during long-term culture. (A) Post-culture macroscopic examination of the short fibre that was cultured for up to 97 days revealed the presence of two extremely thin muscle fibres along its length (diameter 2.0–2.5 μm). These fibres originated from retraction cloths near the left tendon (located about 200 μm to the left) and ended in spherical heads. In these images, due to focussing on the thin fibres, no clear striations are visible in the target fibre. Outside the target fibre, segments of previously neighbouring but cut fibres are visible (☆). (B) Spherical heads at the end of the fibres (enlargement of window in A) are shown at a higher magnification. To the left of the heads, clear light–dark striations were visible. Scales bars A: 100 μm; B: 25 μm.

length was reduced by the magnitude of the applied negative strain.

Light microscopic examination of the morphology of the short and long fibres after culture was also compared with that of freshly dissected fibres. Post-culture, the morphological features of the short fibres cultured for up to 21 days were not visibly different from that of freshly dissected fibres. However, for the short fibre, being cultured for up to 97 days, the post-culture macroscopic examination revealed the presence of two extremely thin muscle fibres (diameter 2.0–2.5 μm), which seemed to originate from retraction clots of cut neighbouring muscle fibres at the tendon (Figure 2A). These thin fibres ended as spherical structures along the originally cultured target fibre (Figure. 2B). Behind the spherical structure striations were present at a larger actin/myosin spacing than within the originally cultured fibre.

All long fibres developed cytoplasmic bulges below the sarcolemma during culture (Figure 3). Examination at high magnification revealed that at the location of the bulge, myofibrils were either completely or partially disrupted from the sarcolemma (Figure 3B and C). Furthermore within every bulge, round structures could be discerned, some of which were enclosed in a membrane (Figure 3D). HE staining of serial sections of long fibres showed a high density of nuclei within these bulges, suggesting proliferation of satellite cell nuclei. It should be noted that such bulges were never observed in short fibres or in freshly dissected fibres. We conclude that these specific morphological features are induced by the passive strain imposed on the fibre and/or the long culture time.

Physiological status of fibres during culture

The stimulus threshold of short and long fibres generally showed only a slight, significant increase during culture (<1% per day, Figure 4). Thus, the stimulus threshold, which is related to the metabolic status, is fairly constant.

At the start of culture, the twitch force of the short fibres was 0.15 ± 0.5 mN (i.e. 7.8% of the initial value at $l_{2.3\ \mu m}$). During culture, for all short fibres twitch peak force increased substantially, which resulted in a mean maximum value of 31.3 ± 7.3% of the initial value at slack length (Figure 5A, $P = 0.02$). For the long fibres, at the start of culture twitch peak force was 1.91 ± 0.33 mN (i.e. 105.6 ± 2.1% of the initial value at $l_{2.3\ \mu m}$). During culture twitch force showed an increase of 32.4 ± 16.0% ($P = 0.002$).

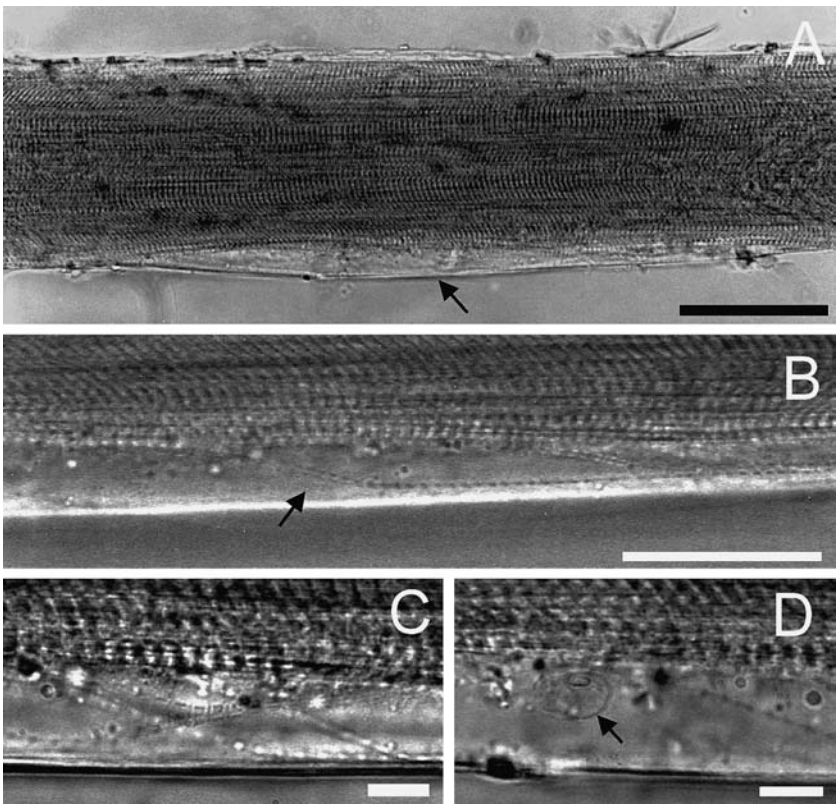

Fig. 3. Bulges below the sarcolemma of single fibres cultured at positive strain. (A) An example of a subsarcolemmal bulge observed along the length of the long fibres after culture (arrow indicates sarcolemma, i.e. plasmamembrane plus basal lamina and endomysium). (B, C) Observation at higher magnification shows that at the location of the bulges, myofibrils were completely or partially disconnected from the sarcolemma (arrow). (D) Within the bulges, nuclei were discerned indicating activation of satellite cells. Scale bars A: 100 μm; B: 50 μm; C: 10 μm; D: 10 μm.

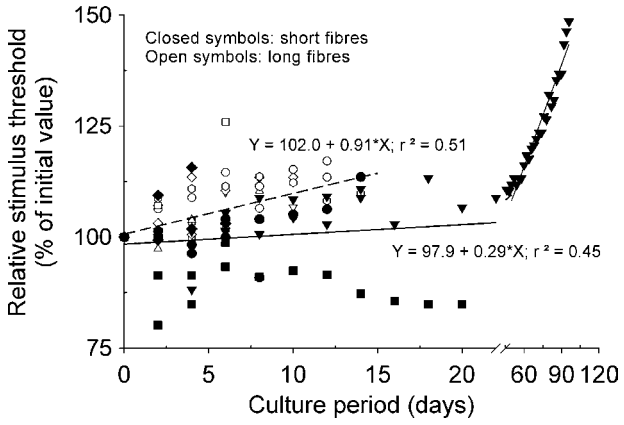

Fig. 4. Stimulus threshold of single muscle fibres during culture at negative and positive strains. Individual data, expressed as a percentage of the initial value, are plotted as a function of the duration of the culture period. Muscle fibres were cultured at positive strain (~5% over $l_{2.3\ \mu m}$; open symbols) or at negative strain (~20% below $l_{2.3\ \mu m}$; closed symbols). $l_{2.3\ \mu m}$ refers to a fibre length at mean sarcomere length of 2.3 μm, which is just over fibre passive slack length. Regression lines indicate for both short (solid line) and long fibres (dotted line) a significant, slight increase in stimulus threshold during the first month of culture. After about 2 months, the stimulus threshold of the short fibre that was cultured for up to 97 days increased faster. Note the change in scale in the horizontal axis after the break.

Acutely after applying the negative strain, for some fibres twitch peak force was nearly zero and due to this twitch contraction time (T_c) and half-relaxation time ($T_{0.5r}$) could not be measured. After 1 day at negative strain all fibres produced measurable twitch forces with twitch contraction times of 37.9 ± 3.2 ms (Figure 5B). During the subsequent days at short length T_c of all fibres increased significantly ($P = 0.005$) and reached a maximum value of 51.6 ± 6.4 ms. The T_c of the long fibres was 33.1 ± 2.9 ms at the start of culture. During the following days, T_c of these fibres increased significantly ($P < 0.001$) up to a mean maximal value of 51.9 ± 3.5 ms.

The twitch half-relaxation time ($T_{0.5r}$) of the short fibres increased also during the culture period (Figure 5C). After one day in culture $T_{0.5r}$ of the short fibres was 20.5 ± 3.6 ms and increased significantly ($P = 0.001$) to a maximum value of 33.4 ± 3.6 ms. For the long fibres, $T_{0.5r}$ reached a maximum value of 61.4 ± 5.5 ms, which was significantly ($P < 0.001$) higher than the initial value of 55.5 ± 5.5 ms, but the pattern was less consistent than that for the short fibres. Regression analysis for repeated measures showed that for short as well as long fibres the change in $T_{0.5r}$ correlated with a change in twitch force ($P < 0.0001$).

After being set at the experimental length in culture, mean tetanic force of the short fibres attained a value of 1.4 ± 0.3 mN (i.e. 39.6 ± 6.0% of the initial value at

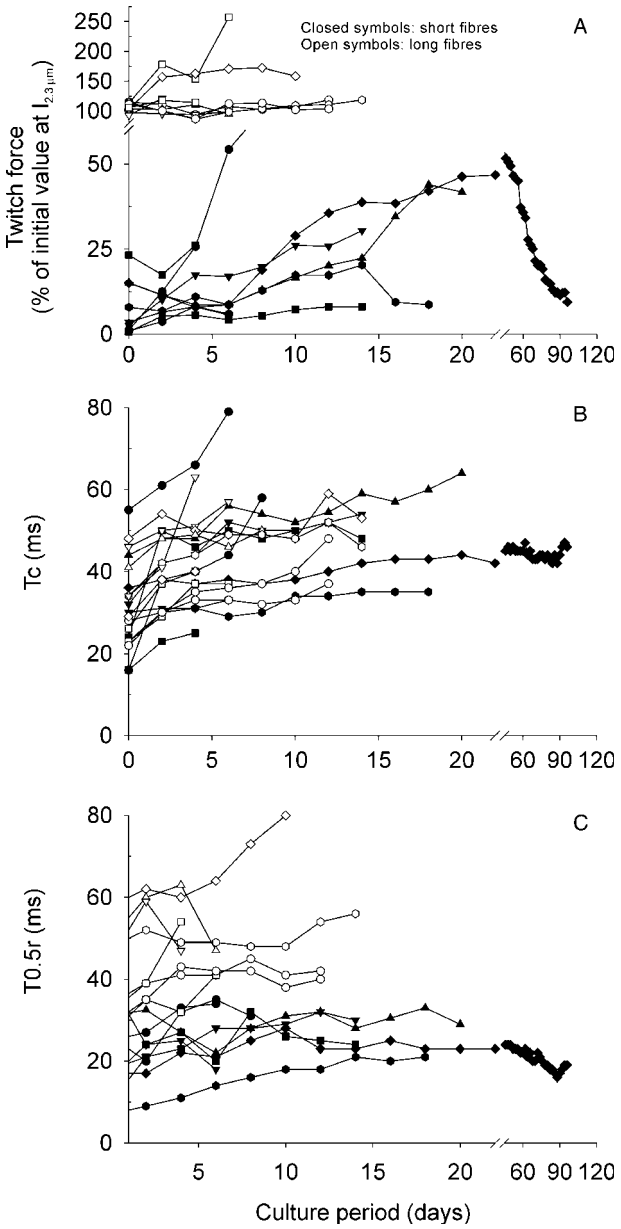

Fig. 5. Individual data of twitch force characteristics of single muscle fibres during culture at low and high strains. Muscle fibres were cultured at positive strain (~5% over $l_{2.3\ \mu m}$; open symbols) or at negative strain (~20% below $l_{2.3\ \mu m}$; closed symbols). $l_{2.3\ \mu m}$ refers to a fibre length at mean sarcomere length of 2.3 μm, which is just over fibre passive slack length. (A) Twitch peak force of short and long fibres during culture is expressed as a percentage of the initial value at $l_{2.3\ \mu m}$. (B) Twitch contraction time (ms). (C) Twitch half-relaxation time (ms). Note the change in scale in the horizontal axis after the break.

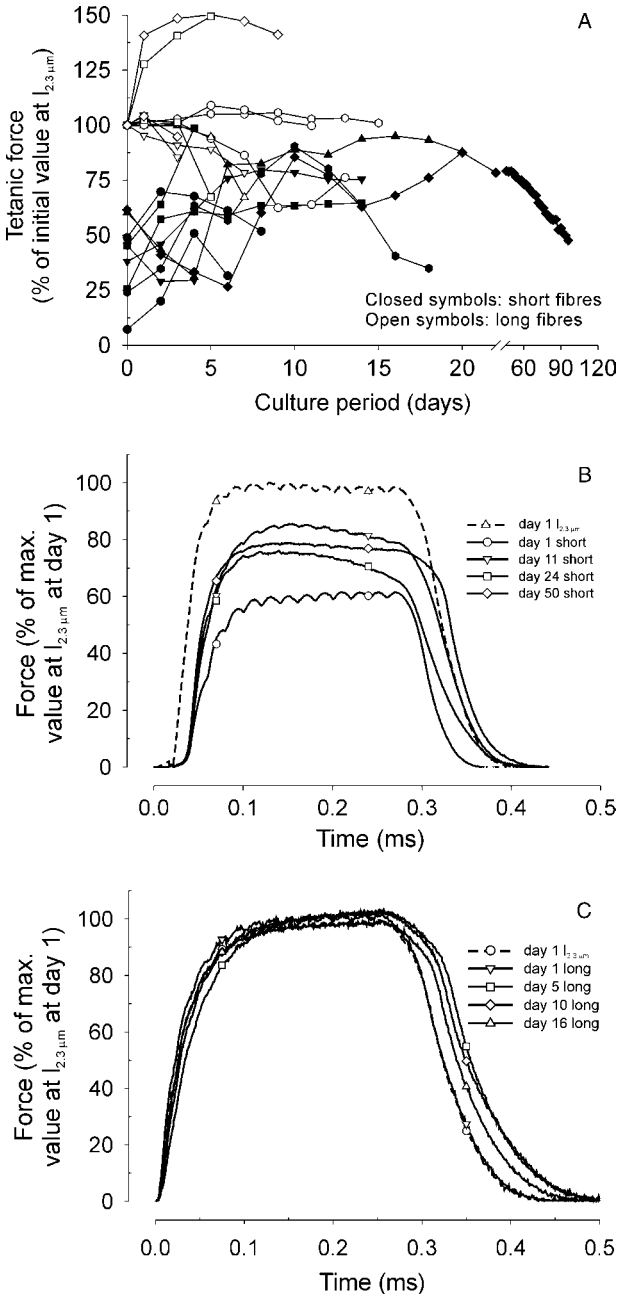

Fig. 6. Individual tetanic forces of single muscle fibres during culture at negative and positive strains. Muscle fibres were cultured at positive strain (~5% over $l_{2.3\ \mu m}$; open symbols) or at negative strain (~20% below $l_{2.3\ \mu m}$; closed symbols). $l_{2.3\ \mu m}$ refers to a fibre length at mean sarcomere length of 2.3 μm, which is just over fibre passive slack length. (A) Tetanic force during culture is expressed as a percentage of the initial value at $l_{2.3\ \mu m}$. Note the change in scale in the horizontal axis after the break. (B) Individual tetani of a short fibre at different time points during the culture. (C) Individual tetani of a long fibre at different time points during the culture.

$l_{2.3\ \mu m}$). During culture, for all short fibres tetanic force increased substantially, up to a mean maximum value of 2.6 ± 0.4 mN (i.e. 86.9 ± 6.8% of the value at $l_{2.3\ \mu m}$, $P < 0.003$, Figure 6A). The increase in tetanic force was accompanied by an altered time course of the tetanus. During culture, tetanus half rise time increased substantially (Figure 6B). The twitch–tetanus ratio for the short fibres showed a significant increase from a mean value of 0.10 at the start of culture to a mean maximum value of 0.27 during culture. However, we could not find a change of the electro-mechanical delay during culture ($P = 0.003$). In contrast to the substantial increase in tetanic force of the short fibres, tetanic force of the long fibres at the end of culture was not significantly changed (Figure 6A). Despite the different force patterns of the long fibres, the twitch to tetanus ratio increased too, but less than for the short fibres.

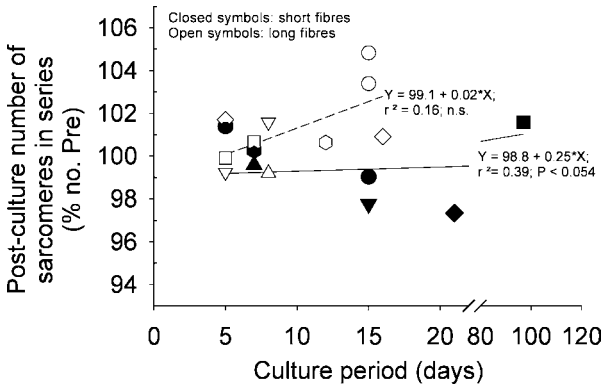

Fig. 7. The number of sarcomeres in series within single muscle fibres cultured at negative and positive strains. Muscle fibres were cultured at positive strain (~5% over $l_{2.3\ \mu m}$; open symbols) or at negative strain (~20% below $l_{2.3\ \mu m}$; closed symbols). $l_{2.3\ \mu m}$ refers to a fibre length at mean sarcomere length of 2.3 μm, which is just over fibre passive slack length. The number of sarcomeres in series of the individual muscle fibres after culture is expressed as a percentage of the number before culture. The relationship between the culture period and the change in the number of sarcomeres in indicated by regression lines (solid line, short fibres; dotted line, long fibres).

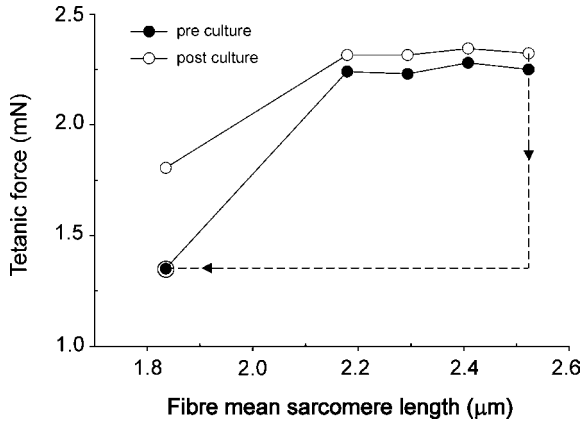

Fig. 8. Length-force data of a single muscle fibre before and after culture at negative strain. Before and after culture (21 days) for one of the short fibres tetanic force was measured at fibres lengths ranging from 80 to 105% of $l_{2.3\ \mu m}$ with 2 min rest after each tetanus. At the end of culture the fibre was hanging straight because the sarcomeres were kept at the shortened length and tetanic force was substantially higher than the initial value. At higher fibre lengths tetanic force was only slightly increased. Following stimulation of the fibre at higher lengths, tetanic force was assessed again at 80% of $l_{2.3\ \mu m}$ (process is indicated by arrows). At this length the tetanic force was now similar as the initial value, indicating that the force enhancement was lost.

Although for short and long fibres twitch and tetanic force showed different patterns, for both conditions the increases in T_c and $T_{0.5r}$ suggest alterations of excitation–contraction (E–C) coupling and/or calcium sensitivity.

Number of sarcomeres in series

If fibres that were maintained at negative strain during culture reduced their number of sarcomeres in series this would have resulted in an increase of the fibre's sarcomere lengths. As a consequence tetanic force would have increased. During culture, the short fibres became straight and short, while tetanic force increased substantially. However, irrespective of the culture period, for all short fibres regression analysis showed that the number of sarcomeres in series was not reduced in proportion to the applied negative strain of 20% (Figure 7). This indicates that the observed changes in fibre length and tetanic force were not caused by a reduction in the number of sarcomeres in series. As the substantial increase in force of the short fibres is not due to a reduction in the number of sarcomeres in series, it is conceivable that tetanic force was increased over the whole range of fibre lengths. To test this, additional force measurements were performed for one fibre that had been cultured for 21 days to determine the effect of culture on fibre length–force characteristics. Before and after culture the fibre twitch and tetanic length–force characteristics were determined over the length range from 80 to 110% of $l_{2.3\ \mu m}$. Post-culture, after stimulation of the fibre at 110% $l_{2.3\ \mu m}$ the fibre was stimulated again at the cultured length of 80% $l_{2.3\ \mu m}$. Figure 8 shows tetanic force before and after 21 days culture. It is shown that at the end of the culture, tetanic force at the fibre's mean sarcomere length of approximately 1.8 μm was 34% higher than the value before culture. However, at a sarcomere length range of 2.18–2.5 μm tetanic force was only slightly (2–3%) higher. Subsequent stimulation of the fibre at a sarcomere length of 1.8 μm, showed that force enhancement was lost at that sarcomere length as tetanic force was similar to that before culture. Macroscopic examination of the passive fibre subsequent to stimulation at higher lengths revealed that fibre length was increased, as the fibre was sagging again in the medium like it had been at the beginning of the culture period.

For the long fibres, the number of sarcomeres in series was not changed during the first week of culture (Figure 7). For the culture period up to 2 weeks, regression analysis did not show a significant positive correlation between culture period and change in the number of sarcomeres in series ($P = 0.054$). However, 2 of the four long fibres that were cultured for 2 weeks showed an increase of 4–5% in the number of sarcomeres in series. Based on the error of the determination of the number of sarcomeres in series ($-0.25 \pm 1.14\%$, mean ± SD, $n = 47$ fibres), it is concluded that positive strain resulted in a significant increase of the number of sarcomeres in series ($P < 0.0002$). In a search for an explanation for the difference in effect between muscle fibres we found that the half-relaxation times of these muscle fibres were much lower than those of the other fibres. It is suggested that addition of sarcomeres in series is induced by positive strain, but in fast fibres only.

Discussion

Culture system and survival time

Single mature muscle fibres were cultured in a well-defined medium in order to study longitudinal effects of

fibre strain on force and number of sarcomeres in series. The finding that the survival time of long fibres was substantially less compared to that of short fibres indicates that muscle fibres cultured above slack length are more vulnerable to damage. One reason for such enhanced risk of being damaged could be metabolic exhaustion due to the 'Feng effect' (e.g. increase in metabolic rate of some amphibian muscles upon stretch, Feng, 1932; Euler, 1935). However, for *Xenopus* muscle fibres, measurement of oxygen consumption as a function of fibre length did not reveal a Feng effect (Jaspers *et al*., 2000). Acute metabolic exhaustion induced by lengthening is therefore not indicated as a factor.

It cannot be excluded that cytosolic [Ca^{2+}] increased due to the strain applied to the long fibres. Free cytosolic [Ca^{2+}] in frog fibres has been shown to increase when fibres are stretched over a sarcomere length of 2.4 μm (Snowdowne, 1986). Increases in intracellular [Ca^{2+}] may activate calpains, which are involved in degradation of a variety of muscle proteins including the cytoskeletal proteins (e.g. Shevchenko *et al*., 1998; Belcastro *et al*., 1998; Berchtold *et al*., 2000). Due to this, muscle fibre integrity may be affected. This mechanism could take more than several hours to become effective and cannot be excluded on the basis of the oxygen consumption measurements.

Another potential explanation for the low survival time of the long fibres is that intracellular mechanical overload of myofibrils causes local damage to membranes, transverse tubules or sarcoplasmic reticulum (SR), which results in local loss of Ca^{2+} homeostasis. At the sarcomere length–force curve's descending limb, non-uniform sarcomere length is more likely to occur during stimulation than at the ascending limb, which may result in extreme, local extension of sarcomeres. This concept was expounded by Morgan (1990) in order to explain fibre damage after eccentric contractions. During culture of long fibres, the mean sarcomere length was about 15% higher than the length of optimal actin and myosin filament overlap. In case of extreme local extension of sarcomeres resulting in damage to the sarcolemma, Ca^{2+} will enter the fibre, which may cause hypercontractions and activation of calpains.

The bulges observed for the long fibres (Figure 3) indicate locations along the fibre where myofibrils are disconnected from the sarcolemma. Such disruptions could make the fibre plasma membrane highly vulnerable to micro-lesions and enhance the risk of hypercontractions. To answer the question whether local myofibrillar disruption from the sarcolemma is caused by Ca^{2+} mediated activation of calpains, mechanical overload, or both, requires further investigation.

Fibre morphology

The morphological appearance of thin muscle fibres aligning the cultured fibre resembles myogenic outgrowth (referred to as 'muscle sprouts') similar to that found in cultures of muscle fibres from adult rat and frog (Bekoff and Betz, 1977; Glavinovic *et al*., 1983). Whereas the sprouts during culture of frog and rat muscle fibres were in cytoplasmic continuity with the parent muscle fibre, the origin of these fibres in our culture system is not known yet. Based on the microscopic images (e.g. Figure 2) we suggest that the myogenic outgrowths represent *de novo* formation of muscle fibres.

Force enhancement during culture at negative strain, despite an unaltered sarcomere number

A surprising finding is that the short fibres showed a substantial increase in twitch and tetanic force, despite the fact that the number of sarcomeres in series was not reduced. The observation that during several days of culture the initially sagging muscle fibres had straightened and shortened implies that after fibre contraction, the sarcomere length was reduced by an internal force of unknown origin. Adjustment in proteins such as the folding and unfolding of titin (see for review Wang *et al*., 2001) or rearrangements of collagen fibres in the extracellular matrix may determine passive sarcomere length.

The observations that long-term force enhancement was accompanied by straightening of the fibres suggests a relationship between these phenomena. Straightening of the muscle fibres can explain an increase in twitch peak force and twitch to tetanus ratio, as straight fibres do not have to shorten before producing force. However, the increase in tetanic force is not explained by a reduced passive fibre length, because fibre force reached a plateau during the tetanus. The mechanisms underlying these phenomena are not clear, but the magnitude of the effects warrants further investigation.

No major atrophy and reduction in number of sarcomeres during culture at negative strain

As yet adaptation of the number of sarcomeres in series in mature muscle fibres, due to manipulation of physiological conditions has only been studied in whole muscle *in vivo*. For valid comparisons of the present effects of strain on single muscle fibres *in vitro* and those on muscle fibres *in vivo*, the data should be compared to *in vivo* muscle with a low degree of pennation such as *m. soleus* in rat and cat (cf. Swatland, 1980; Huijing and Woittiez, 1984).

Several experiments on different species were performed in which the *m. soleus* was immobilised at low length by maximum ankle plantar flexion (Tabary *et al*., 1972; Goldspink *et al*., 1974; Williams and Goldspink, 1978; Tabary *et al*., 1981; Spector *et al*., 1982; Heslinga and Huijing, 1993). These experiments revealed consistent reductions in the number of sarcomeres in series of *m. soleus*, which were in the order of magnitude of 25–40% within 1 day to 4 weeks of immobilisation. Simultaneously, the CSA of *m. soleus* was decreased by 35–40% (Spector *et al*., 1982; Heslinga *et al*., 1995). Our

finding that tetanic force and the number of sarcomeres in series within single muscle fibres were not reduced, even after 97 days, is in striking contrast with the substantial reductions observed *in vivo*. As there is no obvious explanation for the discrepancy between the present results and those obtained *in vivo*, several factors that are related to the process of protein turnover are discussed:

Effects of activity. The rate of atrophy and reduction of the number of sarcomeres in series i*n vivo* are co-determined by the degree of contractile activity. Four weeks immobilisation of denervated cat *m. soleus* at short length yielded a reduction of the number of sarcomeres in series (Goldspink *et al*., 1974), similar to that achieved as normal innervated *m. soleus* that was immobilised at low length for only 1 week (Hayat *et al*., 1978). Furthermore, for guinea pig muscle that was constantly activated at low length, a 20% reduction in the number of sarcomeres in series was achieved within 12 h (Tabary *et al*., 1981). From these results, it is concluded that breakdown of sarcomeres in fibres, being kept at low length, is enhanced by fibre activity, but contractile activity is not a requirement for the reduction of the number of sarcomeres in series.

Temperature. Because for amphibia muscle protein turnover rate at physiological temperatures is about three times lower than for mammalian muscle (Sayegh and Lajtha, 1989), it is conceivable that within *Xenopus* muscle the process of breakdown of sarcomeres occurs more slowly than within mammalian muscle. Although temperature and the low degree of activity may have inhibited the breakdown of proteins, some atrophy was expected at least after more than 3 months of culture at negative strain. *In vivo*, dry weight of denervated frog muscle was shown to be reduced by 20% within 2 months (Muscatello *et al*., 1965). Therefore, it can be excluded that the lack of atrophy is due to the culture temperature of 20°C.

Effects of hormones and metabolites. Muscle protein turnover rate may be affected by numerous hormones (auto-, para- or endocrine) and metabolically related agents (see for review Sugden and Fuller, 1991). The only known factor, added to the medium, which may be involved in the protein turnover rate is the insulin in the culture medium. A high insulin concentration stimulates protein synthesis and inhibits protein degradation in cultures of mammalian, undifferentiated, myotubes (Gulve and Dice, 1989; Vandenburgh *et al*., 1991; Perrone *et al*., 1995) and in cultured whole muscle (Ohira *et al*., 1989). As the concentration of insulin in our culture medium is similar to that showing stimulatory effects on protein synthesis in myotubes, it is possible that for the short fibres the absence of any reduction in the number of sarcomeres in series and tetanic force was due to an insulin effect. It is not fully clear as yet by what mechanisms insulin affects the protein turnover rate within mature muscle fibres (e.g. Sugden and Fuller, 1991). Expression of insulin-like growth factor I (IGF-1) has shown to be enhanced in muscle subjected to stretch and/or intense stimulation (Yang *et al*., 1996, 1997; Goldspink, 1999) and infusion *in vivo* of IGF-1 stimulates muscle hypertrophy (Adams and McCue, 1998; McCall *et al*., 1998). As insulin and IGF-1 bind to each other's membrane receptors (Shimizu *et al*., 1986), insulin may mimick the effects of autocrine IGF-1. In addition, insulin may also stimulate initiation of mRNA translation (Monier and Le Marchand-Brustel, 1982). Therefore, we hypothesise that insulin plays an important role in the process of atrophy and adaptation of sarcomeres in series *in vitro*.

Effects of culture at positive strain

Immobilisation of soleus muscle in a lengthened position *in vivo* results in an increase in tetanic force and number of sarcomeres in series (Williams and Goldspink, 1978; Spector *et al*., 1982). *In vivo*, increased workload and prolonged muscle stretch are accompanied by satellite cell proliferation and fusion with the target muscle fibres (e.g. Winchester *et al*., 1991; Carson and Alway, 1996; Phelan and Gonyea, 1997). Furthermore, irradiation studies have suggested that satellite-cell incorporation is required for the induction of muscle fibre hypertrophy (Rosenblatt *et al*., 1994; Phelan and Gonyea, 1997; Barton-Davis *et al*., 1999). The question arises whether the applied positive strain during culture activated the satellite-cells within the muscle fibres. The lack of hypertrophy caused by a lack or insufficient satellite cell proliferation and fusion can be explained in two ways: (1) Several growth factors have been identified, which are involved in the stimulation of activation and fusion of satellite cells in an autorcrine or paracrine way (see for review, Hawke and Garry, 2001). In case of positive strain stimulating secretions of any of these factors to the culture medium, this could lead to extremely low concentrations due to the relatively high volume of culture medium or due to the fact that they are washed away. (2) The finding that dystrophic mice did not express mechano growth factor (MGF) upon stretch (Goldspink *et al*., 1996) and that MGF is a potent stimulus for satellite-cell proliferation (Yang and Goldspink, 2002) suggests an important role for mechanical signalling *via* dystrophin in the process of hypertrophy. Culture of single muscle fibres implies interference with the extracellular matrix, in particular the endomysium surrounding the muscle fibre. Despite the dissection of the muscle fibres, they are still surrounded by the basal lamina and a network of collagen fibres (Figure 9) with likely intact trans-sarcolemmal connections. This implies that mechanical signalling *via* trans-sarcolemmal structures such as integrins and dystroglycans was still possible. However, as the mechanical interaction of the target fibre with its neighbouring muscle fibres is prevented, mechanical signals applied to the muscle fibre that originate from shear forces applied to the endomysium no longer exist. Effects of such mechanical interaction are unknown as

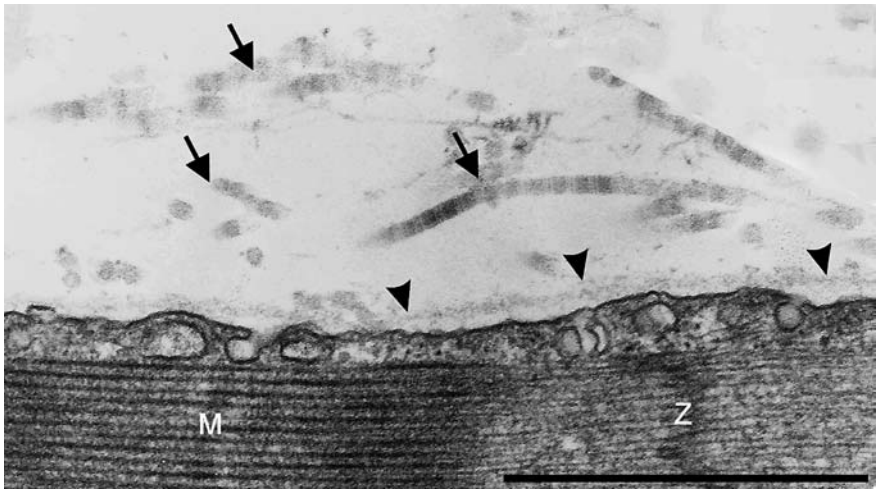

Fig. 9. Illustration of the endomysium surrounding a muscle fibre after isolation. Electron-micrograph of a longitudinal section of an isolated *Xenopus laevis* muscle fibre (obtained according to the methods described by Borgers *et al.,* 1984). Isolation of a muscle fibre by dissection implicates interference with the endomysium stroma, surrounding the muscle fibre. The image shows that despite the dissection, the muscle fibre is still surrounded by the basal lamina (arrowheads) and collagen fibres of the endomysium (arrows). M bands (M) and Z bands (Z) are indicated. Scale bar is 1 μm.

yet, but could be studied by culture of muscle fibres, which are adhered to a laminin substrate for example.

The finding that the faster fibres showed an increase in the number of sarcomeres after 2 weeks of culture at positive strain, whereas the slower muscle fibres did not, suggests a fibre type-dependent time scale of adaptation of the number of sarcomeres upon culture at positive strain. This finding is in accordance with the observation that mammalian fast muscle fibres are much more responsive to hypertrophic stimuli than slow muscle fibres (e.g. Mitchell *et al*., 2002; Oishi *et al*., 2002). In addition, the increase in the number of sarcomeres without hypertrophy upon positive strain suggests that these processes are regulated by different signal transduction pathways.

Removal of interactions with neighbouring muscles and extramuscular connective tissue

Regarding the discrepancy between results of the *in vivo* experiments described in literature and our single muscle fibre cultures, it should be realised that length changes applied to a muscle *in vivo* may not be homogeneously distributed over the muscle fibres. Recently, heterogeneity of sarcomere strain has been measured within *in vivo* passive and active muscles (e.g. Pappas *et al*., 2002; Ahn *et al*., 2003). *In vivo,* muscles are mechanically linked to neighbouring muscles and extramusclar connective tissue causing mechanical interactions between connected structures. These interactions may co-determine muscle force and cause unequal force exertions at proximal and distal tendons (Huijing and Baan, 2001; Maas *et al*., 2001) and heterogeneity of sarcomere strain along the length of the fibre (e.g. Yucesoy *et al*., 2003). As a consequence, the local sarcomere strain within muscle fibres may be substantially different from the strain applied to the whole muscle fibre. It is a possibility that atrophy/hypertrophy and adaptation of sarcomeres in series are triggered by local strains due to mechanical interaction with neighbouring muscles and/or connective tissue.

In conclusion, this study shows the possibility of longitudinal, *in vitro* investigation of the effects of strain on twitch and tetanic force and adaptation of the number of sarcomeres in series. The discrepancy in effects between *in vitro* and *in vivo* effects of strain indicates that strain is not the only factor that regulates hypertrophy and the number of sarcomeres in series. A combination of *in vivo* experiments and culture of mature, single muscle fibres is required to obtain a profound insight in the mechanisms underlying atrophy/hypertrophy and adaptation of the number of sarcomeres in series.

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References

Adams GR and McCue SA (1998) Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. *J Appl Physiol* **84:** 1716–1722.

Ahn AN, Monti RJ and Biewener AA (2003) In vivo and in vitro heterogeneity of segment length changes in the semimembranosus muscle of the toad. *J Physiol (Lond)* **549:** 877–888.

Barton-Davis ER, Shoturma DI and Sweeney HL (1999) Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. *Acta Physiol Scand* **167:** 301–305.

Bekoff A and Betz W (1977) Properties of isolated adult rat muscle fibres maintained in tissue culture. *J Physiol (Lond)* **271:** 537–547.

Belcastro AN, Shewchuk LD and Raj DA (1998) Exercise-induced muscle injury: a calpain hypothesis. *Mol Cell Biochem* **179:** 135–145.

Berchtold MW, Brinkmeier H and Muntener M (2000) Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol Rev* **80:** 1215–1265.

Bland JM and Altman DG (1995) Calculating correlation coefficients with repeated observations: Part 1 – Correlation within subjects. *BMJ* **310:** 446.

Borgers M, Thone F, Verheyen A and Ter Keurs HE (1984) Localization of calcium in skeletal and cardiac muscle. *Histochem J* **16:** 295–309.

Burkholder TJ and Lieber RL (1998) Sarcomere number adaptation after retinaculum transection in adult mice. *J Exp Biol* **201:** 309–316.

Carson JA and Alway SE (1996) Stretch overload-induced satellite cell activation in slow tonic muscle from adult and aged Japanese quail. *Am J Physiol* **270:** C578–C584.

Casaburi R (2000) Skeletal muscle function in COPD. *Chest* **117:** 267S–271S.

Clark A and Coats A (1994) Mechanisms of exercise intolerance in cardiac failure: abnormalities of skeletal muscle and pulmonary function. *Curr Opin Cardiol* **9:** 305–314.

Crawford GN (1973) The growth of striated muscle immobilized in extension. *J Anat* **114:** 165-183.

Euler USV (1935) Some factors influencing the heat production of muscle after stretching. *J Physiol (Lond)* **84:** 1–14.

Feng TP (1932) The effect of length on the resting metabolism of muscle. *J Physiol (Lond)* **74:** 441–454.

Glavinovic MI, Miledi R and Nakajima Y (1983) Morphological and physiological changes in dissociated adult frog muscle fibres after prolonged culturing. *Proc R Soc Lond B Biol Sci* **219:** 91–101.

Goldspink G (1971) Changes in striated muscle fibres during contraction and growth with particular reference to myofibril splitting. *J Cell Sci* **9:** 123–137.

Goldspink G (1999) Changes in muscle mass and phenotype and the expression of autocrine and systemic growth factors by muscle in response to stretch and overload. *J Anat* **194:** 323–334.

Goldspink G, Tabary C, Tabary JC, Tardieu C and Tardieu G (1974) Effect of denervation on the adaptation of sarcomere number and muscle extensibility to the functional length of the muscle. *J Physiol (Lond)* **236:** 733–742.

Goldspink G, Yang SY, Skarli M and Vrbova G (1996) Local growth regulation is associated with an isoform of IGF-1 that is expressed in normal muscle but not in dystrophic muscle when subjected to stretch. *J Physiol (Lond)* **496p:** 10.

Gordon AM, Huxley AF and Julian FJ (1966) The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J Physiol (Lond)* **184:** 170–192.

Gulve EA and Dice JF (1989) Regulation of protein synthesis and degradation in L8 myotubes. Effects of serum, insulin and insulin-like growth factors. *Biochem J* **260:** 377–387.

Hawke TJ and Garry DJ (2001) Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* **91:** 534–551.

Hayat A, Tardieu C, Tabary JC and Tabary C (1978) Effects of denervation on the reduction of sarcomere number in cat soleus muscle immobilized in shortened position during seven days. *J Physiol* (*Paris*) **74:** 563–567.

Heslinga JW and Huijing PA (1993) Muscle length-force characteristics in relation to muscle architecture: a bilateral study of gastrocnemius medialis muscles of unilaterally immobilized rats. *Eur J Appl Physiol* **66:** 289–298.

Heslinga JW, te Kronnie G and Huijing PA (1995) Growth and immobilization effects on sarcomeres: a comparison between gastrocnemius and soleus muscles of the adult rat. *Eur J Appl Physiol* **70:** 49–57.

Huijing PA and Baan GC (2001) Extramuscular myofascial force transmission within the rat anterior tibial compartment: proximo-distal differences in muscle force. *Acta Physiol Scand* **173:** 297–311.

Huijing PA and Baan GC (2003) Myofascial force transmission: muscle relative position and length determine agonist and synergist muscle force. *J Appl Physiol* **94:** 1092–1107.

Huijing PA and Woittiez RD (1984) The effect of muscle architecture on skeletal muscle performance: a simple planimetric model. *Netherlands J Zool* **34:** 21–32.

Jaspers RT, Feenstra HM, Huijing PA and van der Laarse WJ (2000) Effects of stretch on mature single muscle fibres of Xenopus laevis in long term culture are not explained by the Feng effect. *J Mus Res Cell Motil* **21:** 799.

Jaspers RT, Feenstra HM, Lee-De Groot LM, Huijing PA and van der Laarse WJ (2001) Twitch and tetanic tension during culture of mature *Xenopus laevis* single muscle fibres. *Arch Physiol Biochem* **109:** 410–417.

Koh TJ and Herzog W (1998) Excursion is important in regulating sarcomere number in the growing rabbit tibialis anterior. *J Physiol* (*Lond*) **508:** 267–280.

Lännergren J and Smith RS (1966) Types of muscle fibres in toad skeletal muscle. *Acta Physiol Scand* **68:** 263–274.

Lee-de Groot MB and Van der Laarse WJ (1996) Twitch characteristics and energy metabolites of mature muscle fibres of Xenopus laevis in culture. *J Muscle Res Cell Motil* **17:** 439–448.

Maas H, Baan GC and Huijing PA (2001) Intermuscular interaction via myofascial force transmission: effects of tibialis anterior and extensor hallucis longus length on force transmission from rat extensor digitorum longus muscle. *J Biomech* **34:** 927–940.

McCall GE, Allen DL, Linderman JK, Grindeland RE, Roy RR, Mukku VR and Edgerton VR (1998) Maintenance of myonuclear domain size in rat soleus after overload and growth hormone/IGF-I treatment. *J Appl Physiol* **84:** 1407–1412.

McKoy G, Ashley W, Mander J, Yang SY, Williams N, Russell B and Goldspink G (1999) Expression of insulin growth factor-1 splice variants and structural genes in rabbit skeletal muscle induced by stretch and stimulation. *J Physiol* (*Lond*) **516:** 583–592.

Mitchell PO, Mills ST and Pavlath GK (2002) Calcineurin differentially regulates maintenance and growth of phenotypically distinct muscles. *Am J Physiol Cell Physiol* **282:** C984–C992.

Monier S and Le Marchand-Brustel Y (1982) Insulin affects only initiation and not elongation in protein synthesis in soleus muscles of lean and obese mice. *FEBS Lett* **147:** 211–214.

Morgan DL (1990) New insights into the behavior of muscle during active lengthening. *Biophys J* **57:** 209–221.

Mozdziak PE, Pulvermacher PM, Schultz E and Schell K (2000) Hoechst fluorescence intensity can be used to separate viable bromodeoxyuridine-labeled cells from viable non-bromodeoxyuridine-labeled cells. *Cytometry* **41:** 89–95.

Muscatello U, Margereth A and Aloisi M (1965) On the differential response of sarcoplasm and myoplams to denervation in frog muscle. *J Cell Biol* **27:** 1–24.

Ohira Y, Ohira M, Chen M and Holloszy JO (1989) Organ culture of frog muscle: maintenance of mass, enzyme levels, and contractile force. *J Appl Physiol* **67:** 466–471.

Oishi Y, Imoto K, Ogata T, Taniguchi K, Matsumoto H and Roy RR (2002) Clenbuterol induces expression of multiple myosin heavy chain isoforms in rat soleus fibres. *Acta Physiol Scand* **176:** 311–318.

Page SG and Huxley HE (1963) Filament lengths in striated muscle. *J Cell Biol* **19:** 369–390.

Pappas GP, Asakawa DS, Delp SL, Zajac FE and Drace JE (2002) Nonuniform shortening in the biceps brachii during elbow flexion. *J Appl Physiol* **92:** 2381–2389.

Perrone CE, Fenwick-Smith D and Vandenburgh HH (1995) Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-like growth factor binding proteins from differentiated skeletal muscle cells. *J Biol Chem* **270:** 2099–2106.

Phelan JN and Gonyea WJ (1997) Effect of radiation on satellite cell activity and protein expression in overloaded mammalian skeletal muscle. *Anat Rec* **247:** 179–188.

Rosenblatt JD, Yong D and Parry DJ (1994) Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. *Muscle Nerve* **17:** 608–613.

Sayegh JF and Lajtha A (1989) In vivo rates of protein synthesis in brain, muscle, and liver of five vertebrate species. *Neurochem Res* **14:** 1165–1168.

Shevchenko S, Feng W, Varsanyi M and Shoshan-Barmatz V (1998) Identification, characterization and partial purification of a thiol-protease which cleaves specifically the skeletal muscle ryanodine receptor/Ca^{2+} release channel. *J Membr Biol* **161:** 33–43.

Shimizu M, Webster C, Morgan DO, Blau HM and Roth RA (1986) Insulin and insulinlike growth factor receptors and responses in cultured human muscle cells. *Am J Physiol* **251:** E611–E615.

Smith RS and Ovalle WK Jr. (1973) Varieties of fast and slow extrafusal muscle fibres in amphibian hind limb muscles. *J Anat* **116:** 1–24.

Snowdowne KW (1986) The effect of stretch on sarcoplasmic free calcium of frog skeletal muscle at rest. *Biochim Biophys Acta* **862:** 441–444.

Spector SA, Simard CP, Fournier M, Sternlicht E and Edgerton VR (1982) Architectural alterations of rat hind-limb skeletal muscles immobilized at different lengths. *Exp Neurol* **76:** 94–110.

Sugden PH and Fuller SJ (1991) Regulation of protein turnover in skeletal and cardiac muscle. *Biochem J* **273:** 21–37.

Swatland HJ (1980) Analysis of growth in a complex muscle (*M. supracoracoideus, Anas platyrhynchos*). *Growth* **44:** 139–146.

Tabary JC, Tabary C, Tardieu C, Tardieu G and Goldspink G (1972) Physiological and structural changes in the cat's soleus muscle due to immobilization at different lengths by plaster casts. *J Physiol* (*Lond*) **224:** 231–244.

Tabary JC, Tardieu C, Tardieu G and Tabary C (1981) Experimental rapid sarcomere loss with concomitant hypoextensibility. *Muscle Nerve* **4:** 198–203.

Tardieu C, Tabary JC, Gagnard L, Lombard M, Tabary C and Tardieu G (1974) Change in the number of sarcomeres and in isometric tetanic tension after immobilization of the cat anterior tibialis muscle at different lengths. *J Physiol* (*Paris*) **68:** 205–218.

Tardieu G, Tabary JC, Tardieu C, Tabary C, Gagnard L and Lombard M (1973) Adjustment of the number of sarcomeres of the muscle fiber to its required length. Consequence of this regulation and of its disorders observed in neurology. *Rev Neurol* (*Paris*) **129:** 21–42.

van Eijden TM and Raadsheer MC (1992) Heterogeneity of fiber and sarcomere length in the human masseter muscle. *Anat Rec* **232:** 78–84.

Vandenburgh HH, Karlisch P, Shansky J and Feldstein R (1991) Insulin and IGF-I induce pronounced hypertrophy of skeletal myofibers in tissue culture. *Am J Physiol* **260:** C475–C484.

Wang K, Forbes JG and Jin AJ (2001) Single molecule measurements of titin elasticity. *Prog Biophys Mol Biol* **77:** 1–44.

Willems ME and Huijing PA (1994) Heterogeneity of mean sarcomere length in different fibres: effects on length range of active force production in rat muscle. *Eur J Appl Physiol Occup Physiol* **68:** 489–496.

Williams PE and Goldspink G (1971) Longitudinal growth of striated muscle fibres. *J Cell Sci* **9:** 751–767.

Williams PE and Goldspink G (1978) Changes in sarcomere length and physiological properties in immobilized muscle. *J Anat* **127:** 459–468.

Winchester PK, Davis ME, Alway SE and Gonyea WJ (1991) Satellite cell activation in the stretch-enlarged anterior latissimus dorsi muscle of the adult quail. *Am J Physiol* **260:** C206–C212.

Yang S, Alnaqeeb M, Simpson H and Goldspink G (1996) Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. *J Muscle Res Cell Motil* **17:** 487–495.

Yang S, Alnaqeeb M, Simpson H and Goldspink G (1997) Changes in muscle fibre type, muscle mass and IGF-I gene expression in rabbit skeletal muscle subjected to stretch. *J Anat* **190:** 613–622.

Yang SY and Goldspink G (2002) Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. *FEBS Lett* **522:** 156–160.

Yokochi K, Yokochi M and Kodama K (1995) Motor function of infants with spastic hemiplegia. *Brain Dev* **17:** 42–48.

Yucesoy CA, Koopman HJFM, Baan GC, Grootenboer HJ and Huijing PA (2003) Effects of inter- and extramuscular myofascial force transmission on adjacent synergistic muscles: assessment by experiments and finite element modeling. *Biomech* **36:** 1797–1811.

Yucesoy CA, Maas H, Koopman HJFM, Grootenboer HJ and Huijing PA (2003) Extramuscular myofascial force transmission: experiments and finite element modelling. *Arch Physiol Biochem* **111:** in press

Zuurbier CJ and Huijing PA (1993) Changes in geometry of actively shortening unipennate rat gastrocnemius muscle. *J Morphol* **218:** 167–180.