Twitch and Tetanic Tension during Culture of Mature *Xenopus laevis* Single Muscle Fibres

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Abstract

Investigation of the mechanisms of muscle adaptation requires independent control of the regulating factors. The aim of the present study was to develop a serum-free medium to culture mature single muscle fibres of *Xenopus laevis*. As an example, we used the culture system to study adaptation of twitch and tetanic force characteristics, number of sarcomeres in series and fibre cross-section. Fibres dissected from m. iliofibularis (n = 10) were kept in culture at a fibre mean sarcomere length of 2.3 μm in a culture medium without serum. Twitch and tetanic tension were determined daily. Before and after culture the number of sarcomeres was determined by laser diffraction and fibre cross-sectional area (CSA) was determined by microscopy. For five fibres twitch tension increased during culture and tetanic tension was stable for periods varying from 8 to 14 days ('stable fibres'), after which fibres were removed from culture for analysis. Fibre CSA and the number of sarcomeres in series remained constant during culture. Five other fibres showed a substantial reduction in twitch and tetanic tension within the first five days of culture ('unstable fibres'). After 7–9 days of culture, three of these fibres died. For two of the unstable fibres, after the substantial force reduction, twitch and tetanic tension increased again. Finally at day 14 and 18 of culture, respectively, the tensions attained values higher than their original values. For stable fibres, twitch contraction time, twitch half-relaxation time and tetanus 10%-relaxation time increased during culture. For unstable fibres these parameters fluctuated. For all fibres the stimulus threshold fluctuated during the first two days, and then remained constant, even for the fibres that were cultured for at least two weeks. It is concluded that the present culture system for mature muscle fibres allows long-term studies within a well-defined medium. Unfortunately, initial tetanic and twitch force are poor predictors of the long-term behaviour of the fibres.

Keywords: Culture, Skeletal muscle, Single fibre, Twitch, Tetanus, Adaptation, Number of sarcomeres, *Xenopus laevis*.

Introduction

The potency of striated muscle to adapt to changing demands has been studied for more than a century. Change of number of sarcomeres in series and parallel (atrophy/hypertrophy) strongly affects muscle performance (e.g., Williams & Goldspink, 1978; Heslinga & Huijing, 1993; Burkholder & Lieber, 1998). Under normal conditions the number of sarcomeres in series and parallel adapts to functional demands. However, under pathological conditions, as for instance in case of spasticity, the addition of sarcomeres in series is hampered, which limits the range of motion. Furthermore, excessive hypertrophy of cardiomyocytes may cause heart failure. Adequate treatment of diseases mentioned requires an understanding of the adaptive mechanisms.

The conditions leading to these functional and morphological adaptations of striated muscle are known, however, the regulating mechanisms – and in particular interactions between different signal transduction pathways – are not completely understood. One reason for this is that *in vivo* the regulating factors such as strain, activation pattern and humoral factors cannot be controlled independently.

Long-term maintenance of skeletal muscle *in vitro* is an approach to manipulate adaptive stimuli in a controlled

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fashion. Overton (1902) pioneered on long-term culture of skeletal muscle and showed that after 12 days of culture, frog muscle was still excitable. Several investigators attempted to improve culture conditions to maintain whole skeletal muscle of different species in culture for longer periods (e.g., Mines, 1910; Miledi & Trowell, 1962; Harris & Miledi, 1972; McDonagh, 1984; Ohira et al., 1989). Although control over the conditions of the muscle preparations was improved compared to the *in vivo* situation, these preparations were heterogeneous with respect to fibre length, fibre type and local extra-cellular matrix composition. A system for culturing mature single muscle fibres in a completely defined medium (Lee-de Groot & van der Laarse, 1996) provides a tool to study contractile characteristics of single fibres in combination with morphological and physiological parameters under controlled manipulation of strain, activity and hormonal factors. In this system, twitch tension of single fibres of *Xenopus laevis* was stable for up to five days. However, the stimulation threshold increased slowly during culture, which was accompanied by a decrease in the content of energy rich phosphates (ATP and CrP). In order to study longitudinal adaptation of mature single muscle fibres in culture, we: (I) further developed a culture medium in which it was possible to preserve the initial stimulus threshold, (II) simultaneously monitor twitch and tetanus force characteristics and (III) determine the number of sarcomeres in series and cross-sectional area (CSA) before and after culture.

Methods

Animals and preparation of fibres

Xenopus laevis (females, 8–12 cm) were killed by decapitation and both iliofibularis muscles were excised under aseptic conditions and allowed to recover in filtered (0.2 µm), oxygenated, Ringer solution (mM: NaCl, 116.5; KCl, 2.0; CaCl$_2$, 1.9; NaH$_2$PO$_4$, 2.0; EGTA, 0.1; pH 7.2). The treatment of animals was in agreement with the guidelines, set forth by the Dutch law and was approved by the Animal Care and Use Committee of the Vrije Universiteit. After recovery of one hour in oxygenated Ringer solution, both muscles were transferred to an aseptic dissection trough similar to the one described by Lännergren and Smith (1966). Single fibres were isolated aseptically in a laminar flow cabinet under a microscope with dark-field illumination, 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 fibre to the culture chamber, fibre diameters and the number of sarcomeres in series were determined (see below).

Culture system and medium

All parts of the culture chamber (for details see Lee-de Groot & van der Laarse, 1996) were sterilised with 70% ethanol.

The fibre was mounted between a force transducer (AE801, SensoNor, Horten, Norway) and an adjustable rod at a length corresponding to a mean sarcomere length of 2.3 µm (see below). The chamber contained 0.8 ml of culture medium. The culture medium consisted of 66% DMEM/F12 (Gibco-BRL), supplemented with (final concentrations): 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. 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 filtered air (0.2 µm), containing 2.4% CO$_2$. Final pH was 7.6 and osmolarity was 235–250 mOsm kg^{-1}. The oxygen tension of the culture medium sampled from the chamber was 120 to 130 mmHg.

Measurements

The number of sarcomeres in series was determined using laser diffraction (e.g., Lieber et al., 1984). 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, the sarcomere length was determined every mm along the length of the fibre by using a HeNe laser (beam diameter 1 mm). The average of the sampled sarcomere lengths provided the most accurate estimate of the fibre's mean sarcomere length. The length of the fibre was measured under 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.

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

Twitch and tetanic force characteristics of the fibre were determined every 24 h. The fibre was stimulated (0.4 ms biphasic current pulses, 1.25 times threshold) via platinum plate electrodes flanking the fibre to produce two twitches, one tetanus (50 Hz, 260 ms duration) and another twitch. Twitch peak tension, twitch contraction time and twitch half-relaxation time were determined for the second twitch. For the tetanus, maximum tension, the time from the first stimulus pulse to the time at which 50% of maximum force was attained (tetanus 50%-contraction time) and time from the highest force after the last stimulus pulse to 10% relaxation (tetanus 10%-relaxation time) were determined. Twitch and tetanic tension were calculated by dividing fibre force by fibre CSA, determined before the fibre was mounted in the culture chamber.

Statistical analyses

Data are expressed as the mean ± S.E.M. One-way ANOVA for repeated measures was used to test for pre- and post-

culture differences in the number of sarcomeres and CSA. Two-way AVOVA with repeated measures on one factor were performed to test for differences in tetanic force during the first seven days of culture. Correlation coefficients with repeated observations were calculated using the methods described in Bland and Altman (1995).

Results

Culture period, twitch and tetanic tension

Based on the tetanic tension patterns during the first five days in culture, two groups of fibres could be discerned, which were significantly different from each other (P < 0.001). Figure 1 shows individual data of tetanic tension of all fibres during their culture period. Five fibres showed a constant tetanic tension for more than one week. In contrast, five other fibres showed a decrease in tetanic tension of more than 50% of the initial value within 2 to 5 days of culture. In order to provide a meaningful description of the mean force data, fibres were subdivided in 'stable' and 'unstable' fibres according the arbitrary criterion of a reduction of 50% in tetanic force within the first five days of culture.

Three of the stable fibres were cultured for eight days and one for 14 days, after which the fibres were removed from culture for analysis. One of the stable fibres died after eight days for no obvious reason. Figures 2A and 3A show the mean twitch and tetanic tensions of the fibres. Whereas for the stable fibres tetanic tension remained constant, twitch

tension increased during culture, which resulted in a maximum increase of the twitch to tetanus ratio of 55% during culture. It should be noted that in one case a stable tetanic tension pattern was accompanied by an unstable twitch tension pattern. This may indicate the possibility that
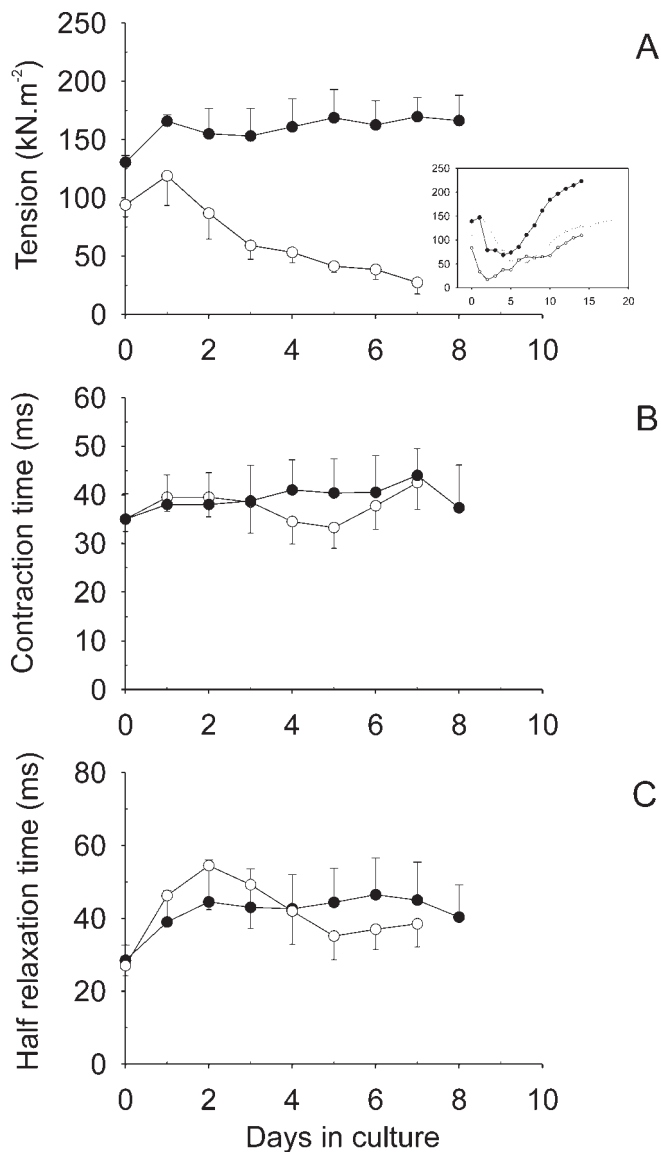
Fig. 2.   Twitch force characteristics of single muscle fibres during culture. Means ± S.E.M. are shown for (A) twitch peak tension, (B) twitch contraction time and (C) twitch half-relaxation time as a function of culture time. (●) indicates fibres (n = 5) that maintained stable tensions ('stable fibres') up to 8 days in culture, after which they were removed for analysis. (○) indicates fibres (n = 5) showing a reduction in tension during culture ('unstable fibres'). Three of these fibres died after 7–9 days in culture. Two of the unstable fibres recovered during the first week and were removed from culture after 14 and 18 days respectively. Inset in A shows twitch tension (kN.m^{-2}) of one stable fibre (●) and two unstable fibres (○) that were cultured for at least two weeks.
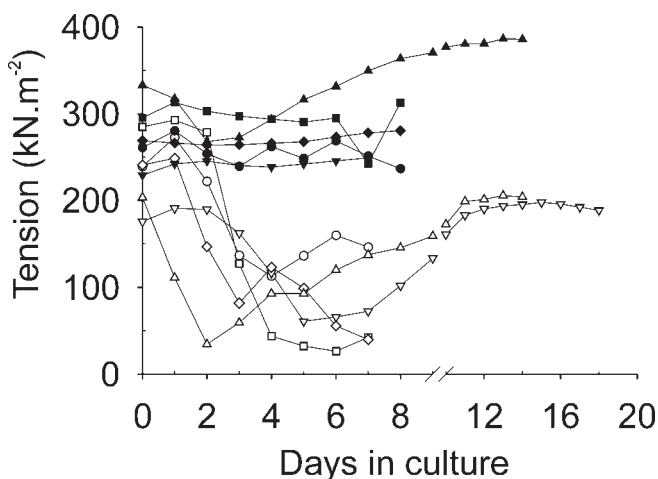

Fig. 1.   Individual data of tetanic tension during culture of single mature *Xenopus laevis* muscle fibres. Fibres were cultured for periods varying from 7 days to 18 days. On the basis of the individual tetanic tension pattern of the fibres during the culture, two groups could be discerned. Five fibres (filled symbols) showed a constant tetanic tension for 8 or 14 days, which are referred to as 'stable fibres'. In contrast, five other fibres (open symbols) showed a reduction in tetanic tension of more than 50% of the initial value during the first five days of culture. These fibres are referred to as 'unstable fibres'.
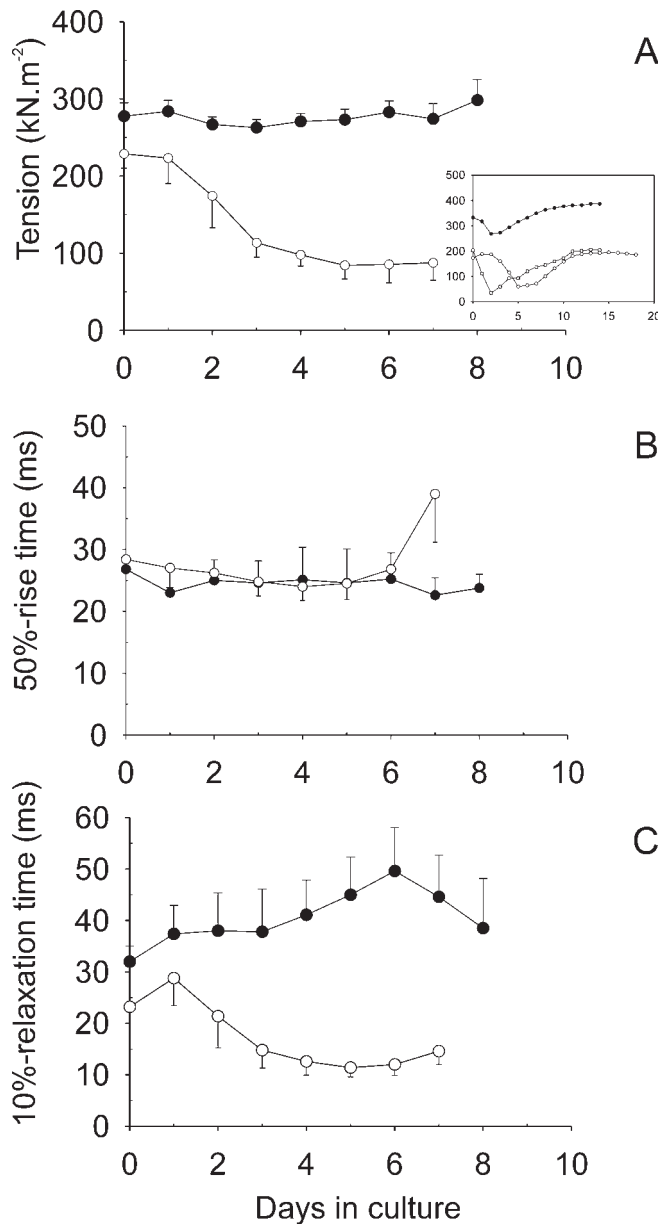
Fig. 3. Tetanic force characteristics of single muscle fibres during culture. Means ± S.E.M. are shown for (A) maximum tetanic tension, (B) tetanus 50%-rise time and (C) tetanus 10%-relaxation time as a function of culture time. Symbols are as in Figure 1. Inset in A shows tetanic tension (kN.m^{-2}) of one stable fibre (●) and two unstable fibres (○) that were cultured for at least two weeks.

days of culture. Note that twitch and tetanic tension of two unstable fibres recovered and exceeded the value attained initially (inset in Figs. 1A and 2B). This indicates that an early decrease in fibre tension is not necessarily followed by structural damage. To test whether the reduction in tension was caused by structural damage of the myofibrils, we added culture medium containing 10 mM caffeine (final concentration) to one of the fibres that showed a typical unstable tension response. Whereas tetanic tension after 7 days of culture was reduced to 16% of the original value the fibre produced 78% of its original tetanic tension during the caffeine induced contraction. This value was similar to that of freshly dissected fibres (Lännergren & Westerblad, 1989) and indicates that the sarcoplasmic reticulum (SR) contained calcium and the contractile apparatus was still functional. Furthermore, stimulation of the fibres at higher currents by increasing voltage and pulse width (4 ms) showed only a marginal increase of the tetanic tension, which indicates that direct electrical activation of the ryanodine receptor did not result in Ca^{2+} release.

Comparison of twitch and tetanic tension between both groups of fibres shows that initially the tetanic tensions between stable and unstable fibres were not significantly different (Figs. 1A and 2A). Although the tensions during culture differed, the twitch to tetanus ratio of both groups of fibres increased by the same amount.

Twitch contraction time and half-relaxation time

As mentioned above, at the start of culture mean twitch contraction time and twitch half-relaxation time of both groups of fibres was similar (Fig. 2B and 2C). According to Lännergren and Smith's criteria (1966), the fibres were classified as type 2/3. During culture twitch contraction time and twitch half-relaxation time of stable fibres increased slightly (Figs. 2B and 2C), whereas for the unstable fibres, the value of these parameters fluctuated during culture. Expression of twitch contraction time and half-relaxation time as a function of twitch peak tension shows for the stable fibres that twitch contraction time and half-relaxation time correlated positively with twitch peak tension (r = 0.51, p < 0.001 and r = 0.54, p < 0.001 respectively). For the unstable fibres only the half-relaxation time correlated significantly with twitch tension (r = 0.48, p < 0.001).

Tetanus 50%-contraction time and 10%-relaxation time

All fibres showed a decrease in tetanus 50%-contraction time after one day in culture. For both groups tetanus 50%-contraction time declined slightly further (Figs. 3B and 3C). The tetanus 50%-contraction time of the unstable fibres showed a substantial increase after six culture days.

During culture the tetanus 10%-relaxation time of the stable fibres showed an increase, whereas for the unstable fibres a decrease was found. For both groups of fibres the tetanus 10%-relaxation time correlated with tetanic tension

a train of electrical pulses did evoke a normal Ca^{2+} release, which could not be achieved by a single pulse.

For the unstable fibres twitch and tetanic tension decreased (Figs. 1A and 2A). After seven days of culture twitch and tetanic tension of these fibres attained a value of 31.4 ± 12.3% and 40.3 ± 10.9% (mean ± S.E.M.) of the maximum value, respectively. Despite this decrease, the twitch to tetanus ratio still increased up to a mean maximum value of 56%. Three of the unstable fibres died after 7 to 9

(stable fibres: r = 0.56, p < 0.001; unstable fibres: r = 0.65, p < 0.001).

Stimulus threshold

For all fibres the stimulus threshold fluctuated during the first two days and subsequently remained constant for the rest of the culture period (Fig. 4). This is a significant improvement compared to results obtained in other culture media in which the stimulus threshold increased (Lee-de Groot & van der Laarse, 1996).

Determination of number sarcomeres in series by laser diffraction

The accuracy of the estimate of the number of sarcomeres depends on the accuracy of the estimate of the fibre's mean sarcomere length and fibre length. Figure 5 shows individual data for the relationship between the number of the measurements of sarcomere length per mm fibre length by laser diffraction and the number of sarcomeres in series expressed as a percentage of the most accurate estimate of the number of sarcomeres in series (based on 1 measurement/mm). It is shown that if the fibre mean sarcomere length was calculated as the average of sarcomere lengths determined every 2 mm along the length of the fibre, the number of sarcomeres deviated only by ±1% from the most accurate estimate. At this number of determinations of sarcomere length along the fibre, the coefficient of variation of the number of sarcomeres was 0.69% (n = 10 fibres, range 0.17–1.61%). This variation is considered to be acceptable for studying longitudinal adaptation of number of sarcomeres in series in culture.
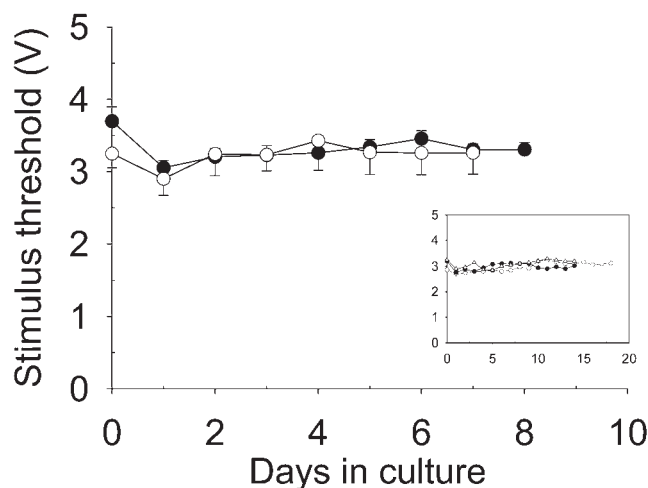
Number of sarcomeres in series and fibre CSA pre- and post-culture

The number of sarcomeres after culture could not be determined for all fibres, because four fibres died unexpectedly, which resulted in local hypercontractions (Fig. 6). For the stable fibres it is shown that after culturing the fibres at a fibre mean sarcomere length of approximately 2.3 µm, the number of sarcomeres in series was not changed (Fig. 7A). Also, for the unstable fibre, kept in culture for nearly three weeks, the number of sarcomeres was unaltered. It is concluded that long-term culture of single muscle fibres at a fibre mean sarcomere length of about 2.3 µm (~ passive slack length), has no significant effect on the number of sarcomeres in series. This means that differences in fibre twitch and tetanic tension before and after culture are not explained by alterations of the number of sarcomeres in series.

Mean fibre CSA for stable and unstable fibres is shown in Figure 7B. For the stable fibres CSA was not changed sig-
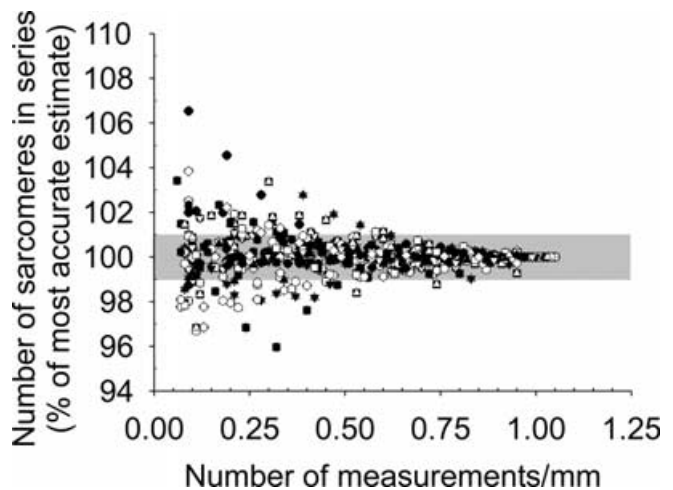

Fig. 5. The accuracy of determination of number of sarcomeres in series of single muscle fibres by laser diffraction before and after culture. Individual data (n = 53) are shown of estimates of the number of sarcomeres as a function of the number of determinations along the length of the fibre. The estimates of the number of sarcomeres in series are expressed as a percentage of the most accurate estimate, which is obtained at 1 measurement/mm.

Fig. 4. Stimulus threshold of single muscle fibres during culture. Values are Means ± S.E.M. Symbols are as in Figure 1. Inset in A shows the stimulus threshold (V) of one stable fibre (●) and two unstable fibres (○) that were cultured for at least two weeks.
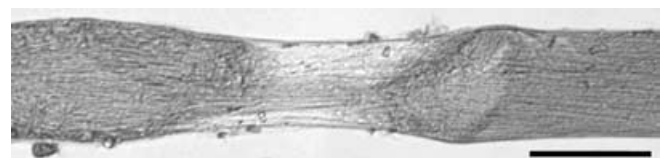

Fig. 6. Photograph of a single muscle fibre that died during culture. Fibres that died during culture developed typical hypercontractions, making the post-culture determination of the number of sarcomeres in series impossible. Scale bar = 100 µm.
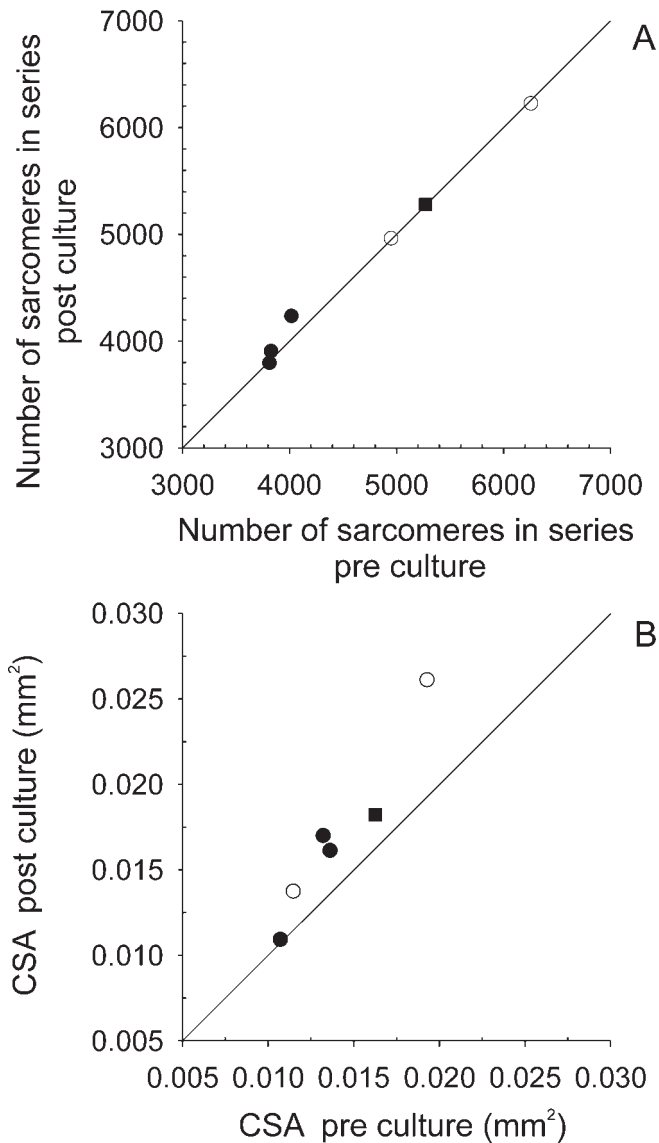
Fig. 7. Comparison of the number of sarcomeres in series (A) and CSA (B) before and after culture. Fibres were kept at a mean sarcomere length of 2.3 μm. The post-culture number of sarcomeres in series and CSA is plotted against the pre-culture values. (●) stable fibres cultured for 8 days, (■) stable fibre cultured for 14 days, (○) unstable fibres cultured for 14 and 18 days. The lines indicates y = x.

nificantly. The two unstable fibres showed after 14 and 18 days culture an increase in fibre CSA of 20% and 35.5%, respectively. This suggests that for the unstable fibres the reduction in tension was not due to atrophy of the fibre.

Discussion

Culture system

Using the culture medium described, it is possible to maintain mature single muscle fibres up to at least 8 days at a con-

stant tension and stimulus threshold. This was not possible for all fibres, as half of the fibres that were cultured showed a transient increase in tension during the first day. Besides the two patterns described it is worth to mention that at the start of this study we also cultured fibres that died within a period of three days. This very short survival period was presumably due to micro-damage at the sarcolemma (e.g., motor-endplate is very fragile) caused during the dissection procedure. However, the incidence rate of such fibres was reduced to zero when fibres were prevented from excessive stretching during the dissection.

The present stable tension patterns during culture are different from those of fibres cultured in the previously used diluted (60%) Leibowitz's medium, supplemented with 5 mM phosphate and 20 mM creatine (final concentration, Lee-De Groot & van der Laarse, 1996). In the latter medium, fibre twitch tension could only be kept constant for five days while the stimulus threshold increased gradually from the start of culture. As these changes were accompanied by reduction in ATP and CrP contents, it is obvious that these fibres exhausted metabolically. The striking stability of fibre tension and stimulus threshold of the stable fibres suggests that in the present, enriched and bicarbonate-buffered medium, fibres were able to maintain their metabolic status. Why half of the cultured fibres were not able to maintain tension and integrity is unclear. At the start of culture, tension of the unstable fibres was similar to that of the stable fibres and within the range of natural variation as reported in other studies (Lee-de Groot & van der Laarse, 1996; Buschman et al., 1997). This suggests that the initial metabolic status of the unstable fibres did not depress tension. Furthermore, the caffeine induced contraction showed normal tensions, which indicates that the contractile apparatus of the unstable fibres was still functional and that the SR could still release Ca^{2+}. Therefore, we suggest that the reduction in twitch and tetanic tension during subsequent days is mainly due to defects in excitation-contraction coupling. The finding that stimulation of the fibres at higher electrical current and pulse width could not activate the ryanodine receptor suggests a malfunction of this receptor in the unstable fibres, of which fibres may recover, as shown for two unstable fibres. An impaired Ca^{2+} release can also explain the observed higher relaxation rates of the unstable fibres while producing lower tensions (Wahr et al., 1998). Besides a reduction in Ca^{2+} release during culture, a decrease in tension due to changes in calcium-sensitivity cannot be excluded. Further investigation is necessary for a complete understanding of the reduction in tension.

Unfortunately, the twitch and tetanic tension characteristics during the first days in culture do not provide objective indicators of the survival time.

Effects of Culture

Laser diffraction is commonly used to estimate the number of sarcomeres in series (e.g., Koh & Herzog, 1998; Burke-

holder & Lieber, 1998) and the reported accuracy was shown to be about 2 to 3%. When the fibre's mean sarcomere length was determined by measuring sarcomere length at least every 2 mm along the fibres, the coefficient of variation of the determination of number of sarcomeres was 0.69%. We conclude that for the comparison of the number of sarcomeres in series before and after culture, laser diffraction provides sufficiently accurate estimates.

Under the present conditions in culture at a mean sarcomere length of 2.3 μm, the number of sarcomeres remained unchanged for at least two weeks. This is not surprising as the mean sarcomere length was near optimum. However, it was not expected that after one to three weeks of culture, fibres being kept at a very low degree of activity of three twitches and one tetanus daily did not show any sign of reduction in CSA or tension. An unequivocal effect of denervation of mammalian and anuran muscle *in vivo* is a reduction in CSA and tetanic force (e.g., Muscatello et al., 1965; Sarnat et al., 1977; Finol et al., 1981; Gundersen, 1985; Anzil & Wernig, 1989). Denervation atrophy in rat and frog skeletal muscle has been shown to occur within one week and two weeks, respectively (Gundersen, 1985; Sarnat et al., 1977).

In its natural surrounding, *Xenopus laevis* prefers temperatures of about 20°C (McCoid & Fritts, 1980), which indicates that the culture temperature was physiological. At this temperature, the *in vivo* rate of protein turnover within muscle tissue of bullfrogs is about 10% per week (Sayegh & Lajtha, 1989). Therefore, the present findings suggest that during culture either protein synthesis had increased or protein degradation had decreased. It is well known that the magnitude of atrophy during *in vivo* joint immobilisation is largely determined by the length at which the muscle is immobilised. Immobilisation at low length resulted in atrophy, whereas at high length muscle CSA increased (Spector et al., 1982; Williams & Goldspink, 1978). If prevention of fibre atrophy in culture is due to fibre length, this implies that the length dependent stimulus for protein synthesis is present at a mean sarcomere length of 2.3 μm. However, the insulin in the culture medium also may stimulate protein synthesis (Airhart et al., 1982; Gulve & Dice, 1989).

In vivo denervation of skeletal muscle also causes a prolonged twitch contraction time and half-relaxation time, which may be caused by an increased sensitivity of the excitation-contraction coupling (Dulhunty, 1985; Finol et al., 1981). The increase of contraction time and half-relaxation time of fibres kept in culture suggests that similar changes occur *in vitro*.

In conclusion, we developed a culture system in which mature single muscle fibres can be maintained functionally stable for long-term within a well-defined medium. This system allows longitudinal studies of the regulating mechanisms of muscle adaptation.

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