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Twitch characteristics and energy metabolites of mature muscle fibres of *Xenopus laevis* in culture

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Summary

Mature, high-oxidative, skeletal muscle fibres of *Xenopus laevis* were kept in culture in L-(Leibovitz's)-15 medium supplemented with creatine and antibiotics and some other additions. Single fibres were mounted at a fixed sarcomere length in a flow-through culture chamber which accommodates stimulus electrodes and a force transducer. Twitch characteristics were determined daily. Depending on culture conditions, fibres remained excitable electrically for up to two weeks at 20° C when foetal calf serum and/or phosphate were added to the culture medium. During the second week, fibres lost phosphocreatine and ATP, but relatively small changes (if any) in total creatine, glycogen and protein contents, fibre volume and dry weight occurred. Succinate dehydrogenase activity decreased after 9 days – when ATP was reduced already. Fibres which were inexcitable electrically contracted normally when exposed to caffeine, indicating that excitation–contraction coupling failed and that the contractile apparatus was still functional.

Introduction

The contractile properties of heart and skeletal muscle fibres can adapt to changing functional demands. Although a number of regulating factors have been identified, like strain, amount of activity, and hormone levels, fairly little is known about the molecular mechanisms which regulate gene expression in growing and mature muscle fibres, and almost nothing is known about interactions between various regulating factors (Booth & Thomason, 1991; Pette & Vrbová, 1992).

Investigation of adaptive processes of muscle in intact animals is hampered by the fact that it is impossible to manipulate the regulating factors independently, while experiments on isolated muscle cells have the disadvantage that the load of the cells cannot be controlled accurately because the tendons – required for mechanical measurements – are lost in enzymatic isolation procedures. The aim of the present study is to improve this situation by developing a culture system for mature muscle fibres which allows control over extracellular conditions, contractile activity and the strain imposed on muscle cells.

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It has been demonstrated previously that mature rat muscle (Miledi & Trowell, 1962) and anuran muscle (Harris & Miledi, 1972; McDonagh, 1983; Ohira *et al.*, 1989) can be kept in culture for periods of 1 week up to 2 months. Although control over extracellular conditions in these preparations is improved compared to the *in vivo* situation, these preparations remain heterogeneous with respect to muscle fibre type and extracellular fluid composition (due to diffusion gradients). Therefore, we developed a culture system for a single muscle fibre preparation. In theory, a culture system for this well-defined preparation allows mechanistic studies of gene expression in individual muscle fibres as well as the study of interactions between different regulators acting on these cells. Because we are interested in the determinants of the volume density of mitochondria and mitochondrial distribution, we use slow-twitch, high oxidative muscle fibres (type 3) from *Xenopus laevis*, which can be isolated by dissection. This choice has the advantage that many basic morphological, contractile and metabolic characteristics have been described already (Lännergren & Smith, 1966; Smith & Ovalle, 1973; Lännergren & Hoh, 1984; van der Laarse *et al.*, 1989a, b; Westerblad *et al.*, 1991; Nagesser *et al.*, 1993). A

preliminary report of this work has been published (Lee-de Groot & van der Laarse, 1994).

Materials and methods

Preparation

Xenopus laevis (females, 8–12 cm) were killed by decapitation and pithed. Both iliofibularis muscles were excised under aseptic conditions and allowed to recover in filtered (0.22 μm), oxygenated, Ringer solution (in mM: NaCl, 116.5; KCl, 2.0; $CaCl_2$, 1.9; NaH_2PO_4, 2.0; EGTA, 0.1; pH 7.2). The dissection of muscle fibres was carried out under aseptic conditions in a laminar flow cabinet equipped with a dissection microscope fitted with dark-field illumination. Single, slow-twitch, high oxidative muscle fibres from the tonic bundle with an opaque appearance – type 3 (cf. Lännergren & Smith, 1966) – were dissected in a dissection trough (cf. Lännergren & Smith, 1966), using fine-tipped forceps and scissors. Small platinum hooks were tied to the trimmed down tendons using 20 μm diameter sterile polyamid thread. The platinum hooks and the tools for dissection were sterilized using 96% ethanol. The fibre was set at a sarcomere length of 2.3 μm as judged by laser diffraction. The length, the smallest and largest diameters of the fibre were measured under the microscope (magnification 100 ×) using an ocular scale. The cross-sectional area and volume of the fibre were calculated assuming an ellipsoidal cross-section.

Culture chamber

The culture chamber was machined out of a solid block of polysulphon. The metal parts were made of stainless steel (except for the stimulus electrodes which were platinum). The parts of the culture chamber were sterilized with 96% ethanol. The chamber was allowed to dry in a laminar flow cabinet and filled with sterile Ringer's solution. The fibre was transferred to the culture chamber and mounted at a sarcomere length of 2.3 μm between a force transducer (AE801, SensoNor, Horten, Norway) and an adjustable rod (Fig. 1). The distance between the platinum plate electrodes flanking the fibre was 5 mm, the length of the trough was 30 mm, and the chamber contained 0.8 ml of culture medium. The lid was put in place, the culture chamber was transferred to an incubator kept at 20° C, and tubing for gas and culture medium supply and leads were connected. Culture medium consisted of 60% (v/v) L-(Leibovitz's)-15 medium supplemented with 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (GIBCO BRL, Breda, the Netherlands) (cf. Glavinović *et al.*, 1983) and creatine (final concentration 20 mM), with or without the following additions: sodium phosphate (final concentrations 5 or 10 mM), and/or 2.5% (v/v) foetal calf serum (FCS). When FCS was added, 4 μg ml^{-1} 1-(β-D-arabinofuranosyl)-cytosine (AraC) was added as well to prevent connective tissue growth. When phosphate was not added to the culture medium, the phosphate concentration was 0.9 mM. Final pH was 7.6 and osmolality was 220–240 mOsm kg^{-1}. Fresh culture medium was continuously pumped through the culture chamber at a rate of about 0.5 ml h^{-1}, and air, saturated with water vapour, flowed continuously over the medium in the chamber. The PO_2 of the culture medium sampled from the chamber after equilibration was about 130 mmHg.

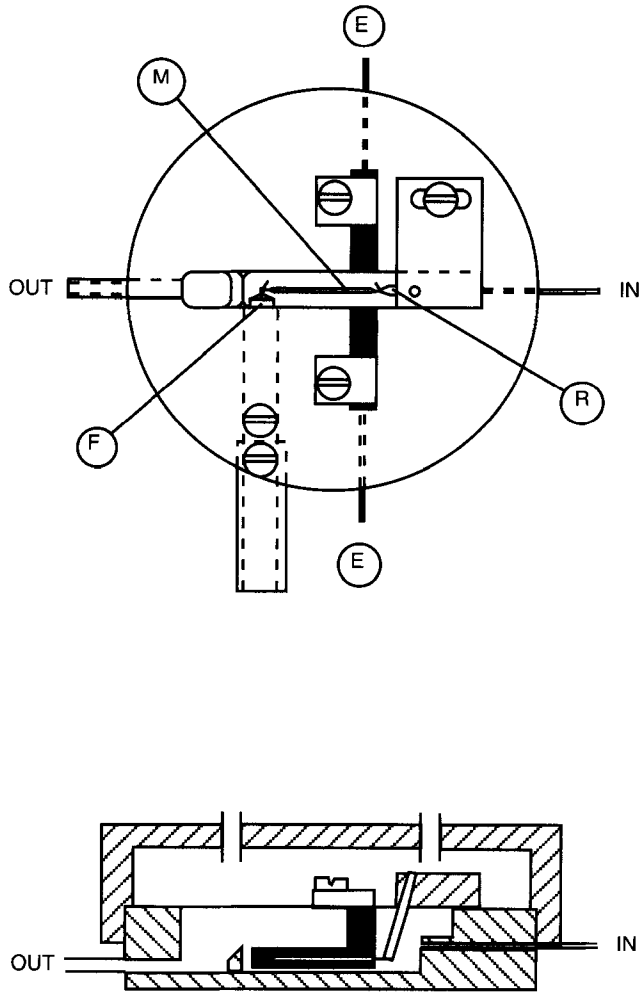

Fig. 1. Schematic drawing of the culture chamber for mature, single muscle fibres. The muscle fibre (M) is mounted in a trough between a force transducer (F) and a rod (R), the position of which allows setting the fibre length. Culture medium is pumped continuously through the trough; a dam in the trough keeps the meniscus of the culture medium at a fairly constant level. The fibre can be stimulated electrically via platinum plate electrodes (E) flanking the fibre. The lid is equipped with connections for gas in- and outflow.

Measurements

Fibres were stimulated by symmetric, biphasic, square pulses (total duration 0.4 ms, if not indicated otherwise). Each day, the stimulus threshold was determined, and the fibres were stimulated to produce a train of 10 twitches at 1 Hz. After A to D conversion, force was sampled at 1 kHz using a computer. For the second twitch in the series, twitch force, twitch contraction and half relaxation time were determined. If not indicated otherwise, the fibre was removed from the culture chamber when force was decreased to less than half of original. The fibre was

transferred to the dissection trough again, the sarcomere length was set at 2.3 μm and the dimensions of the fibre were measured in Ringer's solution as described above. Then the fibre was freeze-clamped using solid CO_2, freeze dried, and stored at −80° C. Depending on the dry weight of the fibre (10–25 μg), protein content and/or the phosphagen contents, which require about 10 and 15 μg dry weight, respectively, were determined. A small part (2 mm, corresponding to about 3 μg) was used for histochemistry. Fibres exposed to caffeine were discarded.

For the protein assay, parts of fibres were dissolved in 1 N NaOH for 20 min at 60° C, and the protein content of the fibres was determined, using bovine serum albumin as standard (Lowry *et al.*, 1951).

For the metabolic assays, parts of fibres were homogenized on ice in 75 μl 0.1 M perchloric acid solution for 1 min, and 60 μl of the homogenate was neutralized with 15 μl 3 M $KHCO_3$. The $KClO_4$ precipitate was spun down and the creatine (Cr), phosphocreatine (CrP), ATP, ADP, AMP and IMP contents in the supernatant were determined using high performance liquid chromatography (Juengling & Kammermeier, 1980).

For the histochemistry, parts of fibres were embedded in 15% (w/v) gelatine in 0.1 M Tris-maleate buffer containing 15 mM EGTA (pH 7.5) and frozen in liquid nitrogen. Sections, 10 and 16 μm thick, were cut in a cryostat at −23° C and collected on slides treated with Vectabond (Vector Laboratories, Birlingame, CA). The 10 μm sections were stained for glycogen using the PAS reaction (van der Laarse *et al.*, 1992) and the 16 μm sections were stained for succinate dehydrogenase activity, which is proportional to the maximum obtainable rate of oxygen consumption (van der Laarse *et al.*, 1989a).

Statistics

Analysis of variance was used to detect statistical significance. When the data were not normally distributed, Wilcoxon's signed-ranks test was used.

Results

In initial experiments muscle fibres were cultured in 60% (v/v) L-15 medium because Glavinović and colleagues (1983) demonstrated previously that dissociated muscle fibres from *Rana temporaria* remained intact in this culture medium at 20° C for at least 11 days. However, we found that the stimulus threshold increased slowly by about 0.5% of original per h during the first 36 h, after which it started to increase very rapidly by about 50% of the original per h. Despite the fact that the fibres became inexcitable electrically, they appeared to be intact under the microscope. Addition of 20 mM Cr (final concentration) to the culture medium delayed the occurrence of the rapid rise of the stimulus threshold considerably (see below). For this reason we decided to routinely supplement culture media with 20 mM Cr (cf. Szymanski, 1989).

Twitch tension of fibres in the culture chamber either stabilized after one day in culture (see below) or decreased continuously to zero during the first to the fifth day. Within both groups of fibres two subgroups could be discerned: one subgroup in which twitch tension was independent of the amplitude and the width of the biphasic stimulus pulse, and another subgroup in which tension increased with both the amplitude (up to about twice the original threshold) and the width (up to about 3 ms) of the stimulus pulse. These four types of behaviour have been observed irrespective of the additions to L-15 medium, suggesting that the quality of the preparation rather than the composition of the culture medium causes this. We suppose that fibres in which twitch tension does not stabilize during the first days in culture have been slightly but irreversibly damaged during dissection. When we added culture medium containing 10 mM caffeine (final concentration) to two fibres which did not stabilize during the first days in culture and which were inexcitable electrically after 3 days, a tension of about 220 $kN\,m^{-2}$ was produced. This is similar to the tension of fully rested fibres exposed to caffeine (Lännergren & Westerblad, 1989), which indicates that the contractile apparatus was still functional and that excitation-contraction coupling in these two fibres failed. The metabolite contents of other fibres, similarly inexcitable, indicate major changes compared to control values (Fig. 5).

The effect of culture medium composition on twitch parameters has been investigated in some detail using fibres in which force production stabilized during the first days in culture. These results are shown in Fig. 2, which excludes fibres in which the force response became dependent on the amplitude and width of the stimulus pulse (see above). The dashed curves in Fig. 2a–d give results obtained in 60% L-15 medium supplemented with Cr and antibiotics. Twitch tension is fairly constant during the first 3 days in culture and then decreases at a rate of about 20% of the maximum tension per day, whereas twitch contraction time and half relaxation time increase slightly during the first day and subsequently remain fairly constant. The stimulus threshold increases by about 10% of original per day – similar to the rate observed in L-15 medium lacking Cr. Two fibres, cultured in L-15 medium with Cr, which were inexcitable electrically after 10 and 11 days, but appeared intact under the microscope, were freeze-clamped at day 13 and 18, respectively. The ATP contents of these fibres were 8.7 and 2.9 and the CrP contents were 18 and 15 $\mu mol\,g^{-1}$ dry weight, respectively, indicating that a considerable fraction (about 80% of the original phosphagen content) was lost under these conditions. Rather than identifying the cause of these changes, we attempted to improve culture conditions

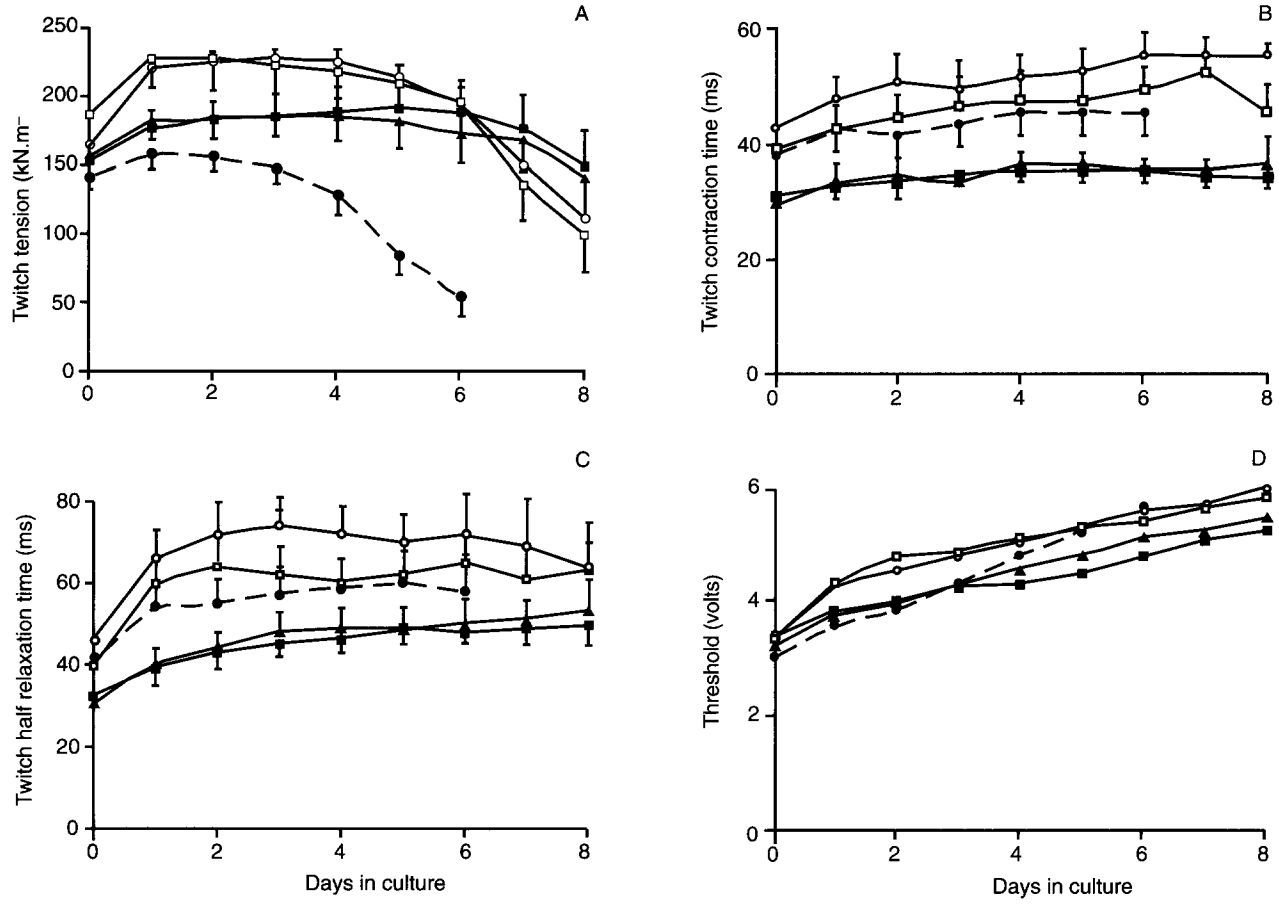

Fig. 2. Twitch tension (a), twitch contraction time (b), twitch half-relaxation time (c) and stimulus threshold (d) of single muscle fibres kept in culture at 20° C and pH 7.6 in 60% (v/v) L-(Leibovitz's)-15 medium plus 20 mM creatine (final concentration), 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (dashed curve; values are mean ± SEM for $n = 9$ fibres), and with various additions to this medium (final concentrations): ■: 5 mM phosphate ($n = 6$); ▲: 10 mM phosphate ($n = 6$); ○: 2.5% (v/v) FCS + 4 μg ml^{-1} AraC ($n = 7$); □: 5 mM phosphate + 2.5% FCS + 4 μg ml^{-1} AraC ($n = 7$). Error bars are not shown in (d) for clarity; the SEs are about 5% of the mean.

by adding FCS and/or sodium phosphate to the culture medium.

Figure 2 shows that the addition of FCS and/or phosphate to the culture medium initially enhances twitch tension compared to culture media lacking these additions and delays the tension decrease. Addition of FCS to the culture medium increases twitch contraction time, whereas addition of phosphate to the culture medium has the opposite effect (see below). The same is true for the half relaxation time. Stimulus threshold increases irrespective of the additions to the medium (Fig. 2d), although the rate of increase is slowed down by the addition of FCS and/or phosphate.

Figure 2 also shows that it made no difference whether the extracellular phosphate concentration is increased to 5 or 10 mM. We observe interactions when phosphate and FCS are added together: the effect of phosphate on twitch tension is not observed in the presence of FCS, but twitch contraction time and half relaxation time are in between the values observed in medium containing FCS and in media containing 5 or 10 mM phosphate.

When culture medium containing 10 mM caffeine (final concentration) was added to two fibres of which twitch force had decreased to almost zero, a similar tension (about 200 kN m^{-2}) as found in control fibres was produced, indicating again that the contractile apparatus was still functional and that excitation-contraction coupling failed (Fig. 3).

Figure 4a shows the effect of culture conditions on the volume of the muscle fibres. The effects, if any, are fairly small: Wilcoxon's signed-ranks test detected a 9% increase in the volume of fibres during the second week in culture medium with 5 mM phosphate ($n = 6$, $p < 0.05$), a 16% decrease in volume in culture medium with FCS ($n = 5$, $p < 0.05$), and a 10% decrease in volume in culture medium with both FCS and 5 mM phosphate ($n = 7$, $p < 0.01$).

Figure 4b shows that, during the second week in culture, fibres in culture medium with FCS, irrespec-

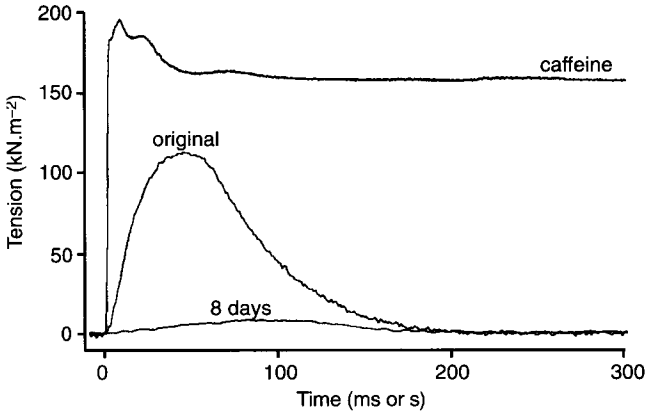

Fig. 3. Typical twitches of a muscle fibre kept in culture medium with 10 mM phosphate on the first and the eighth day in culture, and a caffeine contracture on the eighth day. Note that the time-axis is in milliseconds for the twitches and in seconds for the caffeine contracture.

tive of the phosphate concentration, have a 16% higher dry weight per unit volume during the second week in culture compared to fibres cultured without FCS ($F_{1,26} = 19.8$, $p < 0.001$).

Figure 4c shows that protein content normalized by the dry weight of fibres, during the second week in culture with FCS, irrespective of the phosphate concentration, was 9% less compared to the value in fibres processed immediately after dissection ($F_{1,20} = 19.7$, $p < 0.001$). Protein content per dry weight of fibres cultured in medium with 5 mM phosphate does not change, but culture medium with 10 mM phosphate results in an 11% decrease ($F_{1,11} = 16.0$, $p < 0.005$).

Figure 4d shows that protein content normalized by the volume of fibres, during the second week in culture, with 5 or 10 mM phosphate decreased by 17% compared to control fibres ($F_{1,15} = 25.5$, $p < 0.001$), whereas it remains constant when FCS

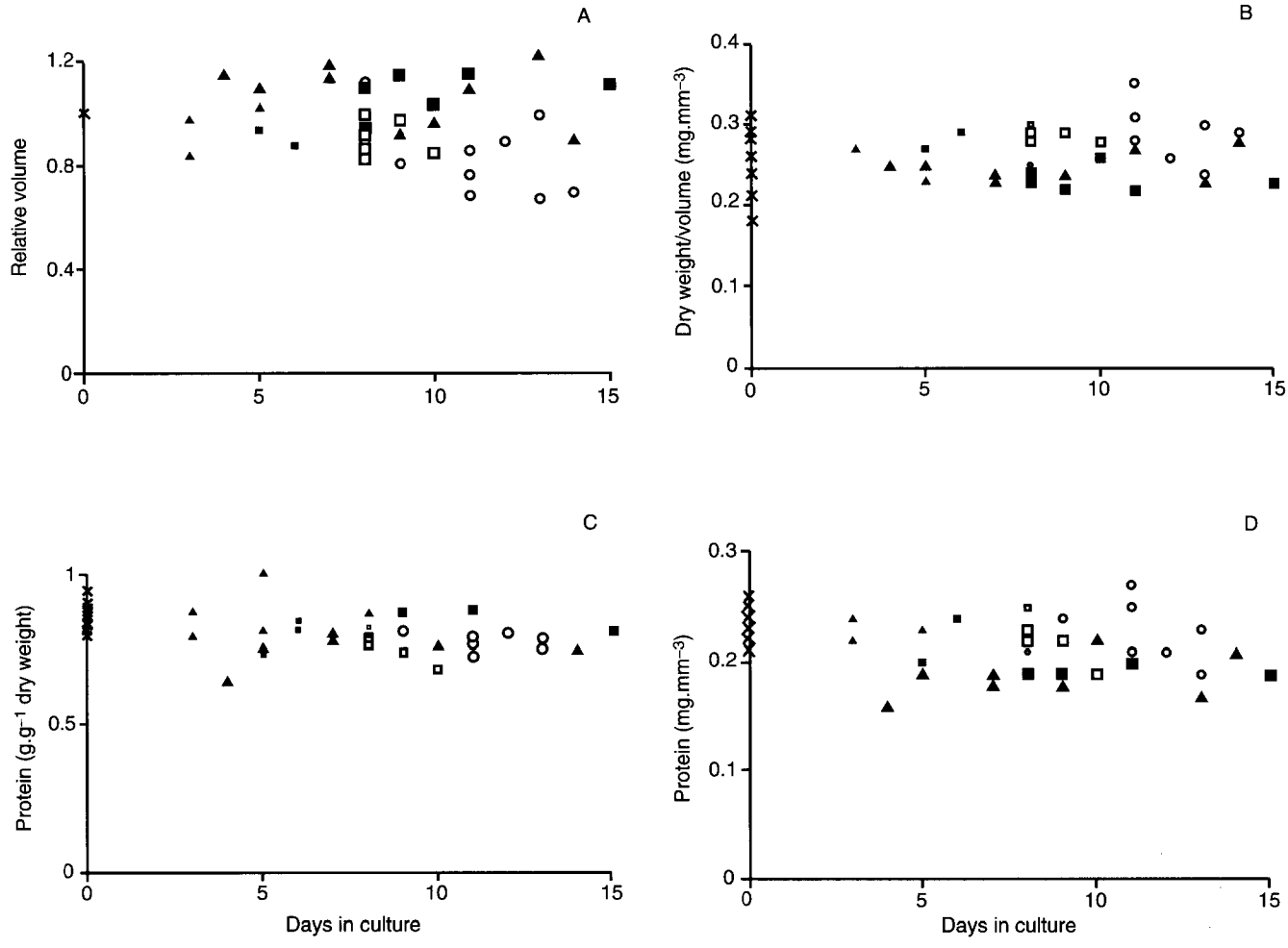

Fig. 4. Fibre volume relative to that on the day of dissection (a), dry weight per volume (b), protein content per dry weight (c), and protein content per volume (d), as a function of the time in culture. Note that the fibre volume was determined in Ringer's solution. The crosses indicate control values measured in fibres freeze-dried immediately after dissection; the meaning of the other symbols is the same as in Fig. 2. The small-sized symbols indicate fibres which either did not stabilize or developed activation failure (see Text).

was added. Five fibres (results not illustrated) were cultured for 4 to 10 days in medium with 5 mM phosphate and 1 mU ml^{-1} insulin. We found that the addition of insulin did not alter the protein content (0.84 ± 0.11 g protein g^{-1} dry weight) or the fibre volume (1.07 ± 0.11, relative to the original).

Because the protein content per unit dry weight is only slightly affected by different culture conditions, and because the determination of fibre volume is rather inaccurate (Blinks, 1965), it was decided to normalize metabolite contents on fibre dry weight. Figure 5a–d shows that the Cr content of fibres kept in culture increases relative to original, whereas CrP content and the ratio CrP/total Cr decrease. Total creatine increases 20% ($F_{1,47} = 9.5$, $p < 0.005$, Fig. 5c). These results indicate already that major changes in energy metabolism occur during culture. Figure 5e shows that the ATP content also decreases.

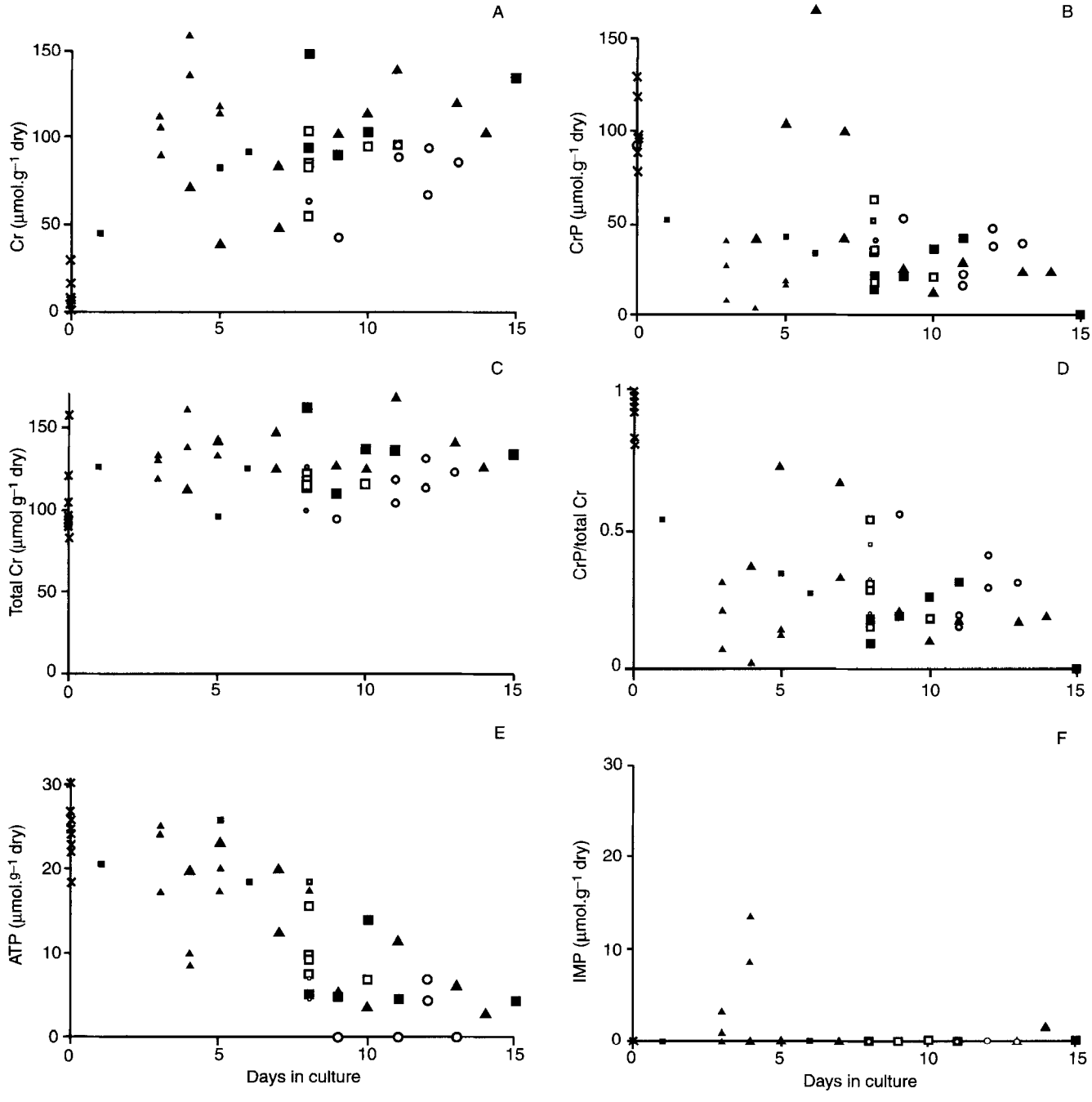

Fig. 5. Creatine content (a), phosphocreatine content (b), total creatine (Cr + CrP) content (c), phosphocreatine normalized by total creatine (d), ATP content (e), and IMP content (f) of single muscle fibres as a function of the time in culture. The crosses indicate control parameters measured in fibres freeze-dried immediately after dissection; the meaning of the other symbols is the same as in Fig. 2. The small-sized symbols indicate fibres which either did not stabilize during the first days in culture or developed activation failure (see Text).

The ADP content (2.1 ± 1.0 μmol g^{-1} dry weight) did not change during culture and AMP remains undetectable (i.e. <1 μmol g^{-1} dry weight). Because the IMP content of the majority of fibres is very low (Fig. 5f), these results indicate that nucleotides are lost – or bound – during culture. Figure 5e indicates that ATP loss was considerable after 7 days in culture. The results presented in Fig. 5 also indicate that the decrease in twitch tension is not usually due to acute metabolic fatigue as described previously (Nagesser *et al.*, 1992, 1993). Interestingly, five fibres, four of which did not stabilize during the first days in culture, contained both IMP and CrP, a phenomenon we never observed before in acute fatigue experiments (Nagesser *et al.*, 1992, 1993). This indicates metabolic compartmentalization in these fibres.

Figure 6 shows cross-sections of fibres incubated for succinate dehydrogenase and stained for glycogen using the PAS reaction. The results indicate that glycogen is not depleted in cultured fibres, even after 13 days, when twitch force is severely depressed. However, the succinate dehydrogenase activity, which is normal after 9 days in culture, is reduced considerably after 13 days. Similar results have been obtained in four other experiments,

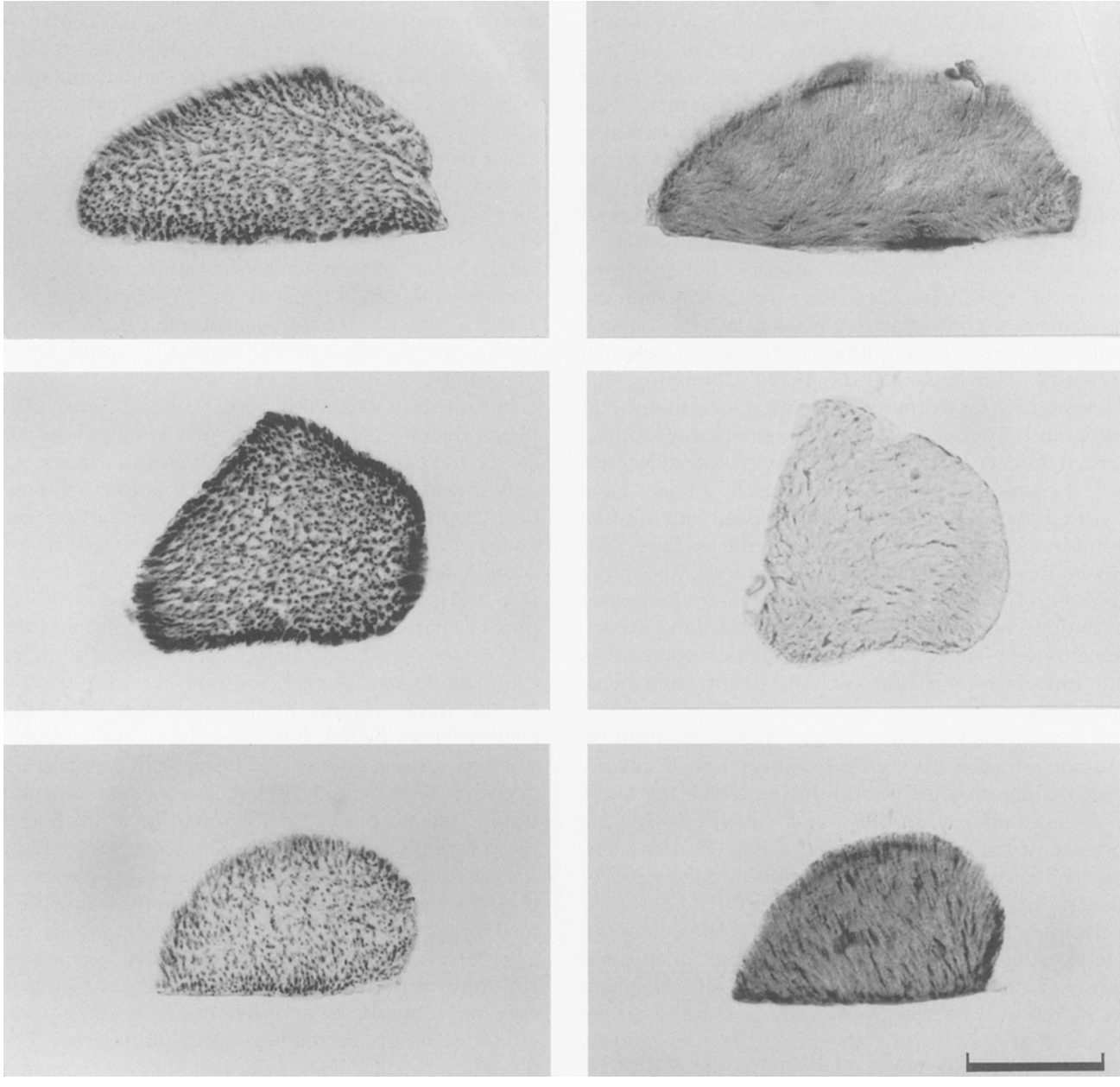

Fig. 6. Serial cross-sections of freeze-dried muscle fibres stained for succinate dehydrogenase (left) and glycogen (right). Photomicrographs of a control fibre (top), and a fibre after 9 (middle) and 13 (bottom) days in culture medium with 10 mM phosphate. Scale bar = 50 μm.

suggesting that loss of succinate dehydrogenase activity occurred after about 10 days, i.e. after the major metabolic changes have taken place.

Discussion

We demonstrated that it is possible to dissect single muscle fibres under aseptic conditions, and that these fibres can be kept in culture for about 2 weeks while their contractile properties can be monitored. In the simplest culture medium that we applied (60% L-15 medium with creatine and antibiotics) twitch tension was fairly constant for about 4 days and then started to decline. This is similar to the results of McDonagh (1983) and Ohira and colleagues (1989), who cultured whole anuran skeletal muscles. However, we observed that the stimulus threshold increased rather rapidly in this medium. The cause of this increase is not known. It has been shown that the membrane potential of anuran muscle fibres in cultured whole muscle is fairly constant for at least 2 weeks (Harris & Miledi, 1972; Szczupack *et al.*, 1989). Szczupack and colleagues (1989) showed that the input resistance does not change for about 3 weeks. After this time the input resistance does increase, the membrane depolarizes slightly (Harris & Miledi, 1972; Szczupack *et al.*, 1989), and the amplitude of the action potential decreases (Harris & Miledi, 1972). Combining the above results on cultured whole muscles suggest that fairly normal action potentials do not always result in normal force production. Glavinović and colleagues (1983), using isolated single muscle fibres, have reported that an action potential does not always result in a contraction of a cultured muscle fibre. This can be due to the fact that the contractile apparatus does not function any more and/or to failure of excitation–contraction coupling. Our caffeine experiments clearly show that the contractile apparatus is still functional and that excitation–contraction coupling fails.

We have tried to improve the culture medium by addition of FCS. The addition alters twitch characteristics, increases the period during which the fibres were excitable electrically and slows down the increase of the stimulus threshold (Fig. 2). However, the addition of FCS could not prevent a decrease of the CrP and ATP contents of the fibre. The observed metabolic changes either indicate that the sarcoplasmic phosphate concentration is very high in cultured fibres, or, alternatively, is due to loss of phosphate across the sarcolemma. The former possibility can be excluded at least during the first 5 days in culture. This is because an increased sarcoplasmic phosphate concentration reduces both Ca-sensitivity of the contractile apparatus and maximum tension (Godt & Nosek, 1989), and hence also reduces twitch tension. A reduction of twitch tension is not observed during the first 5 days in culture. After this time, when twitch tension starts to decline, we cannot exclude that the sarcoplasmic phosphate concentration increased. The phosphate concentration in the sarcoplasm (about 7 mM in fully rested frog muscle at 20° C (Kushmerick, 1983)) is not in electrochemical equilibrium with extracellular phosphate – about 2 mM in anuran plasma (Biology Data Book, 1974), and 0.9 mM in the culture medium without added phosphate – indicating the necessity for active phosphate transport into the fibres. An increase of the phosphate concentration of the culture medium proves to be beneficial in some respects: fibres remain excitable electrically for at least as long as those cultured in medium containing FCS, and the increase of the stimulus threshold is delayed relative to that in culture media containing FCS. The results obtained with 5 and 10 mM phosphate are the same, indicating that the beneficial effect of phosphate saturates between 0.9 and 5 mM. It has been shown recently that phosphate influx into L6 myoblasts depends mainly on the transmembrane Na^+ gradient, and is fairly independent of the extracellular phosphate concentration above 1 mM (Kemp *et al.*, 1992). Despite the beneficial effects of a fairly high phosphate concentration in the culture media, the decrease of CrP and ATP could not be prevented.

In culture media with both FCS and 5 mM phosphate, the effects of phosphate on twitch tension and on the increase of the stimulus threshold are lost and only the effect of FCS remains. The individual effects of phosphate and FCS on twitch contraction time and half relaxation time are additive, at least to some extent. Unfortunately, the individual effects of FCS and phosphate on the period during which the fibres remained electrically excitable are not additive.

We observe some minor, but statistically significant, effects of different additions to L-15 medium on the fibre volume: addition of phosphate (final concentration 5 mM) increases the volume after 1 week in culture, whereas addition of FCS results in a decrease. These volume changes can either be due to trophic factors present in FCS and/or to changes in the concentrations of osmotically active molecules in the sarcoplasm. The fact that the water content of the fibres depends on the composition of the culture medium (Fig. 4b) indicates that osmotic effects play a role. Figure 7 shows the relative volume of cultured fibres as a function of the amount of creatine compounds and free nucleotides: a weak, but significant correlation was found ($r = 0.51$, $n = 24$, $p < 0.05$). This indicates that only a small fraction ($r^2 = 0.26$) of the volume variation can be explained by these metabolite concentrations. The correlation did not improve when it was assumed that the phosphate content of the fibre did not

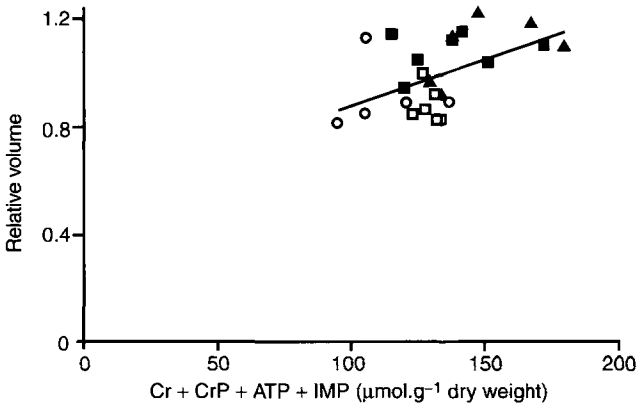

Fig. 7. The fibre volume during the second week in culture relative to the volume on the day of dissection as a function of the sum of measured contents of osmotically active molecules. Note that the volumes have been determined in Ringer's solution. The symbols are the same as in Fig. 2.

change in culture, i.e. by using 2 × [Cr] instead of [Cr] in the calculation ($r = 0.47$). The latter also suggests that the phosphate concentration does not increase to high levels in all fibres.

A fairly small amount of protein is lost in fibres cultured in the presence of 10 mM phosphate or FCS, whereas the protein content remained constant in culture medium with 5 mM phosphate, suggesting that protein synthesis occurs even in the absence of insulin (contrary to Ohira *et al.*, 1989).

Irrespective of culture conditions, the ATP content of the fibres eventually diminished. This can be due to a lack of substrates required for *de novo* ATP synthesis. Because glycogen was present in fibres which were unexcitable electrically, it can be excluded that substrates for glycolysis limit ATP formation. Cross-sections stained for succinate dehydrogenase suggest that mitochondria remain intact for at least one week; the enzyme activity decreases only after a substantial decrease of the CrP and ATP concentrations. The latter suggests that the decrease of the succinate dehydrogenase activity is due to a degenerative process rather than adaptation to reduced contractile activity.

To summarize, single *Xenopus* skeletal muscle fibres can be kept in culture under fully defined extracellular conditions for at least one week at 20° C. This is sufficient to study gene expression at the mRNA level, but, in view of the rather small changes in twitch characteristics, must be prolonged to study changes in contractile properties.

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